Region-selective effects of thiamine deficiency on cerebral metabolism in pyrithiamine-treated rats

Darren Navarro

Department of Medicine

Division of Experimental Medicine

McGill University

Montreal, Quebec, Canada

Submission: October 2008

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Ph.D.

Your file Votre référence
ISBN: 978-0-494-66696-8
Our file Notre référence
ISBN: 978-0-494-66696-8

This thesis is dedicated to my family and friends.

TABLE OF CONTENTS

CONTRIBUTIONS OF THE AUTHORS

In Article 1, the coauthor Claudia Zwingmann is credited for her multinuclear NMR spectroscopy studies performed at the University of Bremen, in Bremen, Germany. Alan S. Hazell is given authorship credit for his role in teaching the processes of formalin fixation of experimental rats and immunohistochemistry. Claudia Zwingmann and Roger F. Butterworth also took active roles in the editing process of this manuscript.

In Article 2, the coauthor Claudia Zwingmann is acknowledged for her aid in all experiments involving multinuclear NMR spectroscopy. Nicolas Chatauret is acknowledged as a coauthor for his teaching of the surgical techniques required for this study, namely femoral artery and jugular vein cannulation, and suturing of experimental rats. In addition, Claudia Zwingmann and Roger F. Butterworth were integral in the editing process of this document.

In Article 3, the coauthor Claudia Zwingmann is credited for her contributions in the multinuclear NMR spectroscopy studies performed at the University of Bremen, in Bremen, Germany. In addition, Claudia Zwingmann and Roger F. Butterworth took active roles in the editing process of this manuscript.

In Article 4, Claudia Zwingmann is acknowledged as a coauthor for her role in the NMR spectroscopy studies. Furthermore, Claudia Zwingmann and Roger F. Butterworth were actively involved in the editing process of this document.

ABSTRACT

Pyrithiamine-induced thiamine deficiency in rats is a well-established animal model of Wernicke's Encephalopathy (WE). This thesis project, submitted as four articles, presents an examination of metabolic events that contribute to the selective neuronal lesions observed in the medial thalamus (MT) of thiamine-deficient (TD) rat. In addition, the phenomenon of glucose-precipitated worsening of neurological status in WE patients (Wallis *et al.*, 1978; Watson *et al.*, 1981) is explored.

Lactate accumulation is known to occur selectively in regions of the TD brain, which ultimately express neuronal cell death (McCandless, 1982; Munujos *et al.*, 1996). In Article 1, the metabolic origin and cellular localization of region-selective lactate accumulation in the MT of TD rats was studied using combined ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Parallel studies were performed to examine the effects of glucose loading on regional brain lactate synthesis in TD animals. Thiamine deficiency caused focal increases in the *de novo* synthesis of lactate via elevated glycolytic flux in the MT, while contribution via pyruvate recycling and the periphery remained nominal. Lactate levels remained unaltered in the frontal cortex (FC), a brain region that is spared in thiamine deficiency. Administration of a glucose load intensified the selective increases in lactate *de novo* synthesis and accumulation in the MT of TD rats, positing a role for lactic acidosis in the glucose-precipitated worsening of neurological status in TD patients. Accordingly, Article 2 addresses the effect of glucose loading on local cerebral pH in the vulnerable MT, compared to the FC, of TD rats. Administration of a glucose load resulted in detrimental decreases in

regional pH selectively in the MT, implying that alterations of brain pH contribute to the pathogenesis of thalamic neuronal damage and consequent cerebral dysfunction in WE.

Region-specific alterations in the steady state levels of cerebral amino acid neurotransmitters have been well-documented in experimental animal models of thiamine deficiency (Butterworth *et al.*, 1979; Butterworth & Heroux, 1989; Gaitonde *et al.*, 1975; Plaitakis *et al.*, 1979); however, the dynamics of these changes have never been systematically explored. In Article 3, we examined the metabolic fluxes through thiamine-dependent pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (α-KGDH) using multinuclear NMR spectroscopy. Furthermore the cellular localization of the metabolic changes in relation to regional vulnerability to thiamine deficiency was addressed. Our studies clearly demonstrate that early decreases in metabolic flux through α-KGDH result in commensurate declines in aspartate concentrations in the MT of TD rats. Impairments to PDH flux manifest secondarily to the metabolic block at α-KGDH, likely due to depleted oxaloacetate pools. As a result of impaired pyruvate oxidation, declines in the *de novo* synthesis of glutamate and GABA ensue. The present findings also suggest that inhibition of flux through α-KGDH in TD brain occurs primarily in the neurons, while astrocytes possess compensatory mechanisms, i.e. the anaplerotic pathway, to replenish oxaloacetate concentrations via metabolic pathways that do not involve thiamine-dependent enzymes.

In Article 4, we investigated the regional effects of thiamine deficiency on the activity of thiamine-dependent branched-chain α-ketoacid dehydrogenase (BCKDH) and the resultant effects on regional

cerebral branched-chain amino acid (BCAA) oxidation. Thiamine deficiency resulted in significant impairments in BCKDH activity; while parallel studies on enzyme distribution confirmed a lower oxidative capacity for BCAAs in the MT compared with the FC.

The data presented in these four articles confirm and extend findings for the region-selective impairments in thiamine-dependent metabolic processes as the foundation of vulnerability of the MT to thiamine deficiency. In addition, glucose loading of TD rats exacerbates both lactic acidosis and impaired pyruvate oxidation in this vulnerable brain region, positing a role for these processes in the glucose-precipitated worsening of neurological status in TD patients. Impaired oxidative metabolism of glucose and BCAAs in the MT leads to the accumulation of potentially harmful metabolic intermediates, contributing to the mitochondrial dysfunction, cellular energy failure and ultimately neuronal cell death observed in thiamine deficiency.

ABRÉGÉ

La déficience en thiamine induite par la pyrithiamine chez le rat est un modèle établi de l'encéphalopathie de Wernicke (EW). Cette thèse, soumise sous forme de quatre articles, présente une étude des altérations métaboliques contribuant aux lésions touchant sélectivement les neurones du thalamus médian (TM) chez le rat déficient en thiamine (DT). De plus, le phénomène connu de l'aggravation de l'état neurologique causée par le glucose chez les patients atteints de EW (Wallis et al., 1978; Watson et al., 1981) est exploré.

Une accumulation de lactate survenant sélectivement dans les régions du cerveau éventuellement affectées par une mort neuronale lors de la DT a été documentée (McCandless, 1982; Munujos et al., 1996). Dans l'Article 1, l'origine métabolique et la localisation cellulaire de l'accumulation de lactate survenant sélectivement dans le TM de rats ayant une DT est étudiée grâce à une combinaison de spectroscopie par résonance magnétique nucléaire (RMN) du ^{1}H et du ^{13}C. Des études parallèles ont été menées afin de déterminer les effets d'une charge de glucose sur la synthèse de lactate en fonction de la région cérébrale dans les animaux déficients en thiamine. Ces études démontrent que la DT cause une augmentation focalisée dans le TM de la synthèse de novo de lactate via une activation du flux glycolytique, alors que le recyclage du pyruvate et l'apport de lactate de la périphérie restent minimes. Les niveaux de lactate sont par ailleurs inchangés dans le cortex frontal (CF), une région cérébrale non affectée par la déficience en thiamine. L'administration d'une charge de glucose intensifie l'augmentation de la synthèse de novo de lactate spécifique au TM chez les rats déficients en

thiamine, ce qui suggère une contribution de l'acidose lactique dans le phénomène de l'aggravation de l'état neurologique causée par le glucose chez les patients atteints de EW. Dans cette perspective, l'Article 2 propose une étude de l'effet d'une charge de glucose sur le pH cérébral local dans une région vulnérable à la DT (le TM) et dans une région non vulnérable (le CF) chez le rat avec une DT. L'administration d'une charge de glucose cause une baisse délétère du pH sélectivement dans le TM, suggérant que des modifications du pH cérébral contribuent aux mécanismes responsables de la mort neuronale dans le thalamus et aux dysfonctions neurologiques propres à l'EW en découlant.

Des modifications des niveaux d'acides aminés neurotransmetteurs survenant spécifiquement dans les régions affectées ont également été bien documentées dans les modèles expérimentaux de déficience en thiamine (Butterworth et al., 1979; Butterworth & Heroux, 1989; Gaitonde et al., 1975; Plaitakis et al., 1979), cependant, la dynamique de ces modifications n'a jamais été explorée de façon systématique. Dans l'Article 3, nous avons examiné les flux métaboliques attribuables à la pyruvate déshydrogénase (PDH) et à l'α-kétoglutarate déshydrogénase (α-KGDH), deux enzymes dépendants de la thiamine, grâce à la spectroscopie par RMN multinucléaire. De plus, la localisation cellulaire des changements métaboliques en fonction de la vulnérabilité régionale à la déficience en thiamine a été abordée. Cette étude démontre clairement qu'une baisse du flux métabolique attribuable à l' α-KGDH survenant tôt dans la progression de la pathologie résulte en une baisse proportionnelle des niveaux d'aspartate dans le TM de rats ayant une déficience en thiamine. D'autre part, des anomalies de l'activité de la PDH se manifestent secondairement au blocage métabolique au niveau de l'α-

KGDH, probablement en raison d'une baisse des niveaux d'oxaloacétate. Il en résulte une réduction de l'oxydation du pyruvate, et un déclin de la synthèse de novo du glutamate et du GABA. Nos données suggèrent également que l'inhibition de l'α-KGDH cérébrale lors de la DT se manifeste principalement dans les neurones, alors que les astrocytes sont pourvus de mécanismes compensatoires, par exemple la voie anaplérotique, leur permettant de maintenir leurs niveaux d'oxaloacétate via des voies métaboliques ne nécessitant pas la participation d'enzymes dépendants de la thiamine.

Dans l'article 4, nous avons investigué les effets de la déficience en thiamine sur l'activité de l'enzyme dépendant de la thiamine acide-cétone déshydrogénase à chaîne ramifiée (ACDCR) et l'effet résultant sur l'oxydation régionale d'acides aminés à chaîne ramifiée (AACR). La déficience en thiamine cause une baisse significative de l'activité de la ACDCR, et l'étude de la distribution de l'enzyme confirme une baisse de la capacité à oxyder les AACR dans le TM comparativement au CF.

Les données présentées dans ces quatre articles confirment et approfondissent le concept voulant que des anomalies de voies métaboliques dépendantes de la thiamine soient à la base de la vulnérabilité du TM à la déficience en thiamine. De plus, une charge de glucose chez le rat ayant une DT aggrave l'acidose lactique et les anomalies de l'oxydation du pyruvate dans le TM, ce qui suggère un rôle de ces voies métaboliques dans le phénomène de l'aggravation de l'état neurologique précipitée par le glucose chez les patients atteints de EW. Une anomalie du métabolisme du glucose et des AACR dans le TM conduit à une accumulation potentiellement délétère d'intermédiaires métaboliques, contribuant à la dysfonction mitochondriale, à la défaillance

du métabolisme énergétique et éventuellement à la mort neuronale observées lors de la DT.

INTRODUCTION

Thiamine is one of the B vitamins and plays an important role in energy metabolism and tissue building. Once inside the body, thiamine combines with phosphate to form the coenzyme thiamine diphosphate (TDP), which is essential in metabolic reactions that produce energy from glucose, as well as the oxidative metabolism of branched-chain amino acids. When there is not enough thiamine in the diet, these basic metabolic functions are disturbed, leading to problems throughout the body.

Special situations, such as an over-active metabolism, prolonged fever, pregnancy, and breast-feeding, can increase the body's thiamine requirements and lead to symptoms of deficiency. Extended periods of diarrhea or chronic liver disease can result in the body's inability to maintain normal levels of many nutrients, including thiamine. Other persons at risk are patients with kidney failure on dialysis and those with severe digestive problems who are unable to absorb nutrients. Alcoholics are susceptible because they may substitute alcohol for food and their frequent intake of alcohol decreases the body's ability to absorb thiamine. A deficiency of thiamine causes a condition known as beri-beri. The major symptoms of beri-beri or vitamin B_1 deficiency involve the nervous system causing sensory disturbances, muscle weakness, and impaired memory; and the heart causing shortness of breath, palpitations, and, eventually, heart failure.

Thiamine deficiency has marked effects on the brain and nerve function; and may result in irreversible damage. In particular, the Wernicke-Korsakoff syndrome is a neurological manifestation of thiamine

deficiency, and is a combination of two eponymous disorders: Wernicke's Encephalopathy (WE) and Korsakoff's Psychosis, named after Drs. Carl Wernicke and Sergei Korsakoff, respectively. The Wernicke-Korsakoff syndrome is discussed in more detail in Section 3.2.

At present, thiamine deficiency remains an important health care issue in several world populations, and its presence often goes unrecognized until the symptoms are more pronounced and often times irreversible. The causes of thiamine deficiency in developing populations include inadequate diets, prolonged cooking of foods and ingestion of certain foods containing significant quantities of thiaminases or anti-thiamine compounds. In more developed nations such as the U.S., thiamine deficiency is usually found in malnourished chronic alcoholics, though it is also found in patients who undergo prolonged intravenous (IV) therapy without vitamin B_1 supplementation, gastric stapling or intensive care unit stays, and in pregnancies accompanied by severe vomiting. Furthermore, in individuals with sub-clinical thiamine deficiency, a large dose of glucose (either as sweet food or glucose infusion), can precipitate the onset of overt encephalopathy (Wallis et al., 1978; Watson et al., 1981). Other populations at risk for the development of thiamine deficiency include patients with HIV-AIDS (Butterworth et al., 1991) as well as patients with severe gastrointestinal disorders.

1.0 THIAMINE

1.1 Discovery

Beri-beri puzzled medical experts for years as it ravaged people of all ages in Asia. Doctors thought it was caused by something in food. The first stage in discovering the cause of beri-beri was in the 1890s, when a Dutch doctor, Christiaan Eijkman, found that fowls fed only on polished rice developed similar symptoms to his patients who had beri-beri, and that they could be cured if they were also fed some of the husks from the rice grains. It was determined that the outer covering that was removed to create the polished white rice preferred by Asians, actually contained something that prevented the disease. In 1912, Casimir Funk isolated the anti-beri-beri factor from rice and called it *vitamine* - an amine essential for life. In the 1930s, the chemical formula of this vitamin B_1 was published by Robert R. Williams, and it was named *thiamine*. Thus, thiamine was the first vitamin identified, and its discovery marked the beginning of modern vitamin categorization.

1.2 Requirements and Sources

Thiamine is an essential nutrient for most mammals since they are unable to adequately synthesize it, and the body is incapable of storing the free vitamin. Research shows that dietary thiamine requirements are based on caloric intake. Those individuals who consume more calories, such as athletes, are likely to require a higher than average intake of thiamine in order to help process the extra carbohydrates into energy. During acute periods of stress, thiamine needs may also be increased. The recommended dietary allowance (RDA) of thiamine ranges from 1.1 mg/day for adults to 1.5 mg/day during pregnancy to accommodate

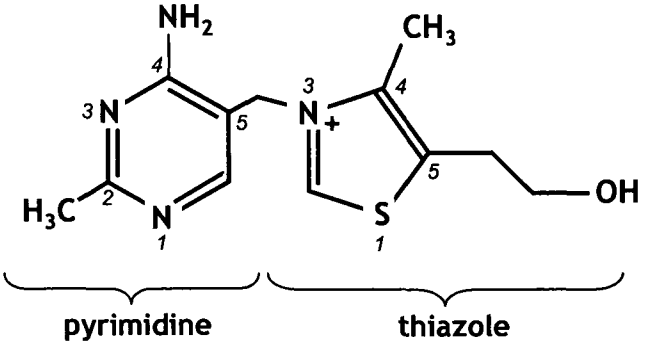
maternal and fetal growth as well as increased maternal caloric intake. A further increment of 0.5 mg/day is recommended during lactation. No adverse side effects are known with thiamine intakes at RDA levels or even at levels several times the RDA.

Dietary sources of thiamine include whole grain cereals, brewer's yeast, meat (pork, lamb and poultry), nuts, liver, spinach, green peas and some legumes. Widely used cereal products (i.e. bread and breakfasts cereals) are now enriched with thiamine and these sources probably provide as much as 30 to 40% of the daily intake. Thiamine content of these foods is sensitive to alkaline pH, high temperatures, oxygen and radiation, thus considerable amounts of this vitamin can be lost during food processing and/or cooking.

1.3 Chemical Structure

Thiamine, also known as vitamin B_1 or aneurine, is a colourless compound that is soluble in water and insoluble in alcohol. Thiamine is the currently accepted name for vitamin B_1 in the US. Aneurine is still widely used in Europe, especially in the United Kingdom. Its chemical formula is $C_{12}H_{17}N_4OS$, and the chemical name for this water-soluble vitamin is 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxy-ethyl)-4-methylthiazolium. Thiamine consists of a pyrimidine ring and a thiazole moiety connected by a methylene bridge as displayed in Figure 1.

Figure 1: Chemical structure of thiamine



The pyrimidine ring is substituted with a methyl radical and an amino group. The thiazole ring possesses a quaternary ammonium and a sulphur atom, and is substituted with a ß–hydroxyethyl group and a methyl radical. The nitrogen in the third position of the thiazole ring has a charge of +1, serving as an important electron sink in thiamine pyrophosphate mediated reactions. The reactive site of the molecule is the carbon in the second position of the thiazole ring. The methylene bridge is the fragile part of the thiamine molecule, explaining its thermolability. Because thiamine is positively charged, it requires a counterion (e.g. chloride). In the isolated state, thiamine usually exists as thiamine hydrochloride.

1.4 Metabolism

1.4.1 Synthesis

Thiamine can be synthesized in various species; particularly in the vegetable kingdom and perhaps in some lower animals. In mammals and particularly in humans, bacteria in the intestinal tract may synthesize some thiamine; however, since this synthesis occurs primarily at the level

of the caecum, which is downstream of the principal regions of thiamine absorption, namely the small intestines, it is considered to be a nominal source of thiamine. Accordingly, mammals are almost completely dependent on dietary thiamine (Stryer, 1997).

1.4.2 Intestinal absorption and transport

Thiamine is actively transported from the intestinal lumen to the blood (via the serosal compartment) by a mechanism that requires Na^+ ions and ATPase activity. In the rat, ingested thiamine is rapidly and actively absorbed in the proximal portion of the small intestine, namely the duodenum and the proximal jejunum (Hoyumpa, 1982; Sklan & Trostler, 1977). In humans, absorption occurs at the level of the jejunum and the ileum (Hoyumpa, 1982).

Transport of thiamine across the intestinal mucosa to the serosal compartment proceeds via two mechanisms (Hoyumpa et al., 1982) on both the luminal and contraluminal (basolateral) membranes of the enterocyte. At low concentrations of intraluminal thiamine (<2.0 μM), the process of active transport is saturable and occurs against a concentration gradient (Hoyumpa et al., 1975b; Komai et al., 1974; Ventura & Rindi, 1965). This process is carrier-mediated and is inhibited by metabolic inhibitors, anoxia, low temperature and structural analogues of thiamine such as pyrithiamine, oxythiamine and amprolium (Hoyumpa et al., 1975b; Komai et al., 1974; Rindi & Ventura, 1969). When concentrations of intraluminal thiamine are elevated (>2.0μM), the active transport carrier becomes saturated and the nonsaturable process of passive diffusion predominates (Hoyumpa et al., 1975b).

In both humans and rats, the intraluminal concentration of thiamine is estimated to be usually less than 2.0 μM (Hoyumpa, 1982; Sklan & Trostler, 1977; Thomson et al., 1970). Therefore, the intestinal transport of thiamine appears to be accounted for predominantly by the saturable mechanism at both the luminal (Casirola et al., 1988) and contraluminal sides of the enterocyte (Laforenza et al., 1993), whereas at higher pharmacologic concentrations thiamine transport is predominantly a passive process.

This energy-requiring transport system requires the extrusion of Na^+ from the basolateral membrane of the enterocyte by the Na^+-K^+ ATPase (Laforenza et al., 1993). However, the transport of thiamine into the enterocyte across both the luminal and basolateral membranes, respectively, are not influenced by Na^+ (Ferrari et al., 1971; Hayashi et al., 1981), but rather the net transport of thiamine from the intestinal lumen to the serosal compartment is strictly dependent on a functional Na^+-K^+ ATPase (Laforenza et al., 1993).

Once inside the enterocyte, thiamine is rapidly phosphorylated to TDP (and TMP to a lesser extent) which accumulates in the cells (Casirola et al., 1988). Ethanol adversely affects the active, but not the passive, component of thiamine transport. Moreover, ethanol appears to block thiamine exit from the enterocytes into the serosal compartment, but does not affect cellular uptake of thiamine (Hoyumpa et al., 1975a). The similarity of this inhibition to the action of ouabain administration suggests that ethanol may impair active thiamine transport by inhibiting Na^+-K^+ ATPase activity on the basolateral membrane.

Thiamine absorption is controlled and limited, and modest increases in the serum concentration are accompanied by active renal

clearance (Davis & Icke, 1983). In humans, an oral dose of 8.3 mg of thiamine is sufficient to saturate these absorption mechanisms; and an oral dose greater than 10 mg of will have no supplementary effect on the levels of thiamine in the blood (Thomson & Leevy, 1972). Given that the intestinal absorption of thiamine is a factor limiting its bioavailability, any conditions that interfere with intestinal absorption (e.g. prolonged diarrhea) may lead to thiamine deficiency in susceptible subjects.

1.4.3 Tissue distribution and storage

The average total amount of thiamine in a normal adult is approximately 0.11 mmol (30mg). High concentrations are found in skeletal muscle, heart, liver, kidneys and brain. About 50% of the total thiamine is present in muscles. The biologic half-life of ^{14}C-thiamine in the body is 9 to 18 days. Because thiamine is not stored in large amounts in any tissue, a continuous supply of thiamine is necessary (Gubler, 1968).

1.4.4 Phosphorylation and diffusion in the blood and tissue

Ingested thiamine must be phosphorylated in order to become metabolically active as a cofactor. Once in the bloodstream, thiamine is rapidly phosphorylated and distributed throughout the entire body. Phosphorylation is accomplished by a group of enzymes referred to as thiamine phosphokinases, which are most active in the liver, but also found in brain and other tissues. However, phosphorylated thiamine is less diffusible than free thiamine; thus, in order to facilitate diffusion within tissues the phosphate linkages must be split off. For this reason, pyrophosphatases of various types are also present in various tissues.

In the bloodstream, thiamine can be found in the following proportions: 75% phosphorylated forms and 25% free thiamine. TMP and free thiamine are the main thiamine compounds in blood and plasma serum; while TDP is essentially localized within erythrocytes (Herve et al., 1994). In species such as rat, mouse, guinea pig, dog, rabbit, chicken and pigeon, total thiamine concentrations in blood are 1 μM (Harata et al., 1993; Kimura et al., 1982; Kimura & Itokawa, 1985), whereas they are only 0.1 μM in humans (Brunnekreeft et al., 1989; Tallaksen et al., 1991; Wielders & Mink, 1983). Free thiamine concentrations in human plasma or serum are 10-15 nM in normal healthy individuals (Bettendorff et al., 1986; Tallaksen et al., 1991), compared with 200 nM in rats for instance (Bettendorff et al., 1990; Tallaksen et al., 1991; Weber & Kewitz, 1985). Thus, thiamine availability may become a limiting factor in several human disorders such as alcoholic liver cirrhosis (Tallaksen et al., 1992), Wernicke's Encephalopathy (Tallaksen et al., 1993) and alcoholic cardiomyopathy (Bettendorff et al., 1986), where blood and plasma thiamine concentrations are further decreased.

1.4.5 Transport into the brain

In order to enter the brain, thiamine must first be transferred from the blood into the cerebrospinal fluid (CSF) and brain (Dreyfus & Victor, 1961; McCandless et al., 1968). According to Spector (1982), the entry of thiamine into the brain is regulated at three levels: first, plasma levels are regulated by the kidneys and gut; secondly, mechanisms at the blood-CSF barrier (the choroid plexus) and/or the blood-brain barrier (BBB) regulate the concentration of total thiamine in the extracellular space of the brain and CSF; and finally, the cells of the brain are assumed to have saturable

uptake and release systems that further regulate the intracellular total thiamine concentrations (Bettendorff & Wins, 1994; Sharma & Quastel, 1965; Spector, 1976).

Thiamine enters the CSF and brain from blood by a saturable system in the choroid plexus (Spector, 1976). Thiamine transport across this blood-CSF barrier was found to be carrier-mediated (Greenwood et al., 1986; Spector, 1976), but the process does not appear to be energy-dependent (Greenwood et al., 1986). Once within the CSF, thiamine also enters the brain by a saturable process that depends in large part on thiamine phosphorylation to TDP (Sharma & Quastel, 1965; Spector, 1976). The continuous entry of thiamine from the blood to brain is necessary to replace the total thiamine that is constantly leaving the CNS, e.g. in the CSF.

1.4.6 Cellular uptake

Thiamine is transported across cell membranes by three mechanisms: two saturable components that are responsive to low and high physiological concentrations of thiamine (Bettendorff 1995; Bettendorff and Wims 1994) and one nonsaturable (diffusive) component (Casirola et al., 1988; Yamamoto et al., 1981). At low extracellular concentrations, thiamine is taken up through a saturable high affinity mechanism (K_m = 35 nM). This process can be blocked by low concentrations of the Na^+ channel activators veratridine and batrachotoxin. This high affinity thiamine uptake has been consistently found in most cell types studied so far (Casirola et al., 1988; Casirola et al., 1990; Yamamoto et al., 1981; Yoshioka, 1984).

At high extracellular concentrations, thiamine uptake proceeds essentially via a low affinity carrier (K_m = 0.8 mM), insensitive to veratridine, but blocked by divalent cations. In both cases, the uptake is independent on external sodium, partially inhibited by depolarization and sensitive to metabolic inhibitors. Thiamine uptake is also dependent on intracellular ATP concentrations; the apparent affinity for ATP is low and close to the affinity of thiamine pyrophosphokinase for ATP (Bettendorff & Wins, 1994). This suggests a secondary active transport of thiamine; the driving forces being phosphorylation to TDP rather than the sodium gradient.

1.4.7 Degradation and elimination

Excess amounts of free (physiologically inactive) thiamine in the bloodstream are rapidly eliminated by the kidneys and excreted in the urine (Rindi et al., 1968). Thiamine can also be degraded into various pyrimidine and thiazole derivatives within the tissue before excretion via the kidneys (Gubler, 1968).

1.5 Thiamine Homeostasis within the CNS

Adequate supply of thiamine is particularly important for the mammalian brain. Neuronal function is strongly dependent on oxidative energy metabolism, which absolutely requires the presence of the coenzyme TDP. In addition to TDP, TTP may play a specific role in membrane permeability (Bettendorff et al., 1993a; Bettendorff et al., 1993b; Cooper & Pincus, 1979).

In the mammalian brain, there is substantial evidence that the total thiamine content is maintained relatively constant. For example, in

thiamine-depleted rat brain, levels of thiamine were much better maintained than total thiamine levels in other organs (Lowry, 1952). On the other hand, following the parenteral injection of large doses of thiamine, there were relatively small changes of total thiamine levels in CSF and brain (Lowry, 1952), suggesting that total thiamine levels in brain are closely regulated.

1.5.1 Thiamine phosphate esters

The total thiamine content of mammalian tissues exists as free thiamine and as thiamine phosphates, which include thiamine monophosphate (TMP), thiamine diphosphate (TDP) and thiamine triphosphate (TTP) (Cooper & Pincus, 1979). Of the total thiamine in the body, about 80% is TDP, 10% TTP, and the remainder is TMP and thiamine. Significant concentrations of TDP exist within the liver, the brain, the kidneys and the heart while free thiamine and TMP are present in most tissue (Cooper & Pincus, 1979; Pincus & Grove, 1970; Spector, 1976).

1.5.1.1 Thiamine diphosphate

TDP is an important cofactor for enzymes involved in brain glucose metabolism such as transketolase, pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH) and branched-chain α-ketoacid dehydrogenase (BCKDH).

1.5.1.2 Thiamine triphosphate

The precise physiological role of TTP has not yet been elucidated, but it has been proposed that the triphosphate ester activates high

conductance chloride channels (Bettendorff et al., 1993a). TTP also has regulatory properties on proteins involved in the clustering of acetylcholine receptors (Gautam et al., 1995) suggestive of a direct role in the regulation of neurotransmission in the brain. Interestingly, rats with a high sensitivity to the CNS effects of ethanol express low levels of TTPase (Zimatkina et al., 2000).

1.5.1.3 Thiamine monophosphate

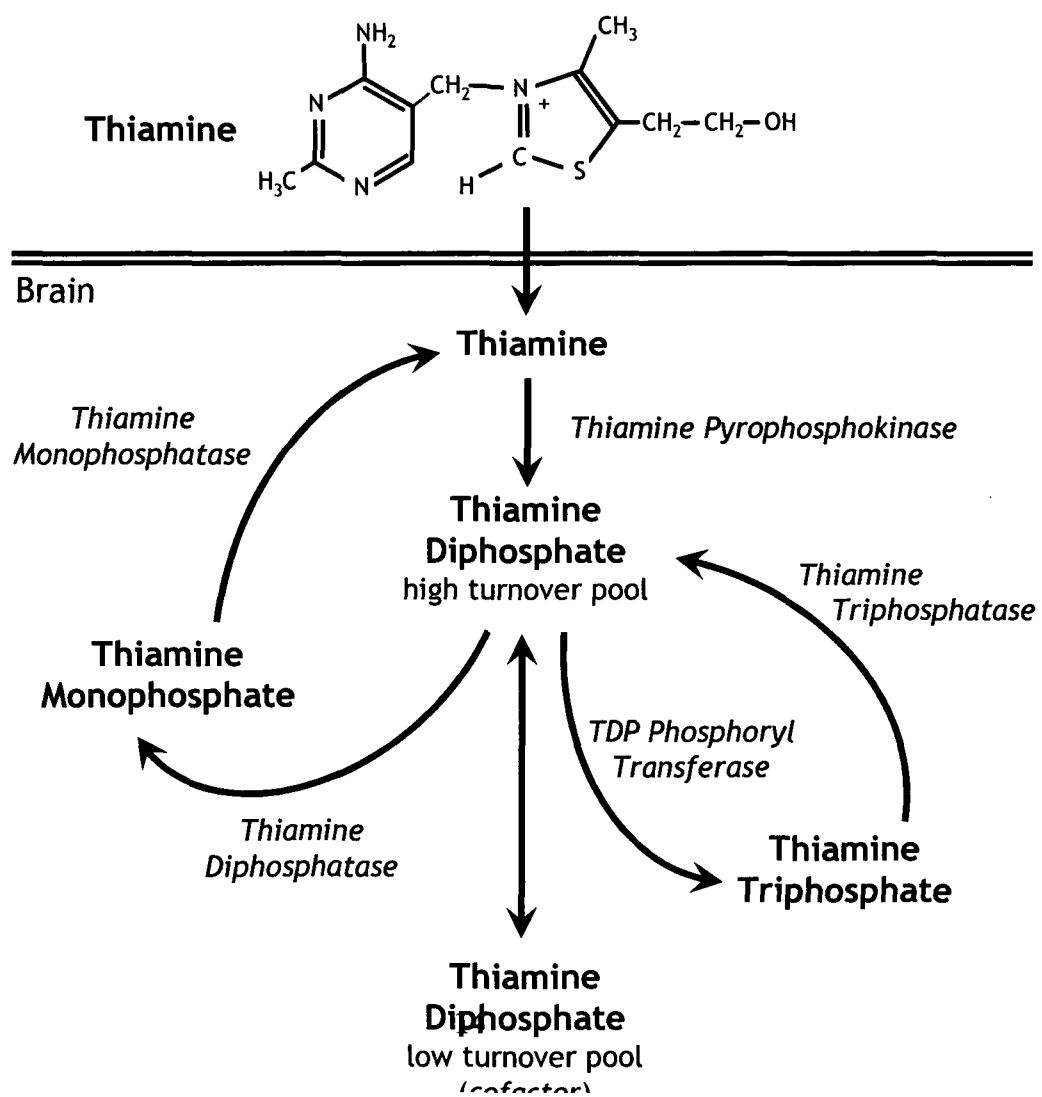
TMP and free thiamine are present in most tissues and are the main thiamine compounds in blood plasma and serum. In brain, TMP is prevalent in the cytosolic fraction. The role of TMP has not been fully elucidated.

1.5.2 Enzymes of thiamine metabolism in brain

Thiamine uptaken into the brain is phosphorylated to TDP by the enzyme thiamine pyrophosphokinase (see Figure 2). TDP can be further phosphorylated to TTP by TDP phosphoryl transferase, or dephosphorylated to TMP by thiamine diphosphatase. It is becoming clear that thiamine phosphorylation/dephosphorylation is a compartmented process in the brain. Evidence for this concept is derived from studies of the cellular localization of the thiamine phosphorylating and dephosphorylating enzymes as well as the phosphate esters themselves. Thiamine phosphate esters are significantly more concentrated in neurons compared to other brain cells (Laforenza *et al.*, 1998). Moreover, TDPase activities are 20-fold higher in neurons whereas TMPase is expressed primarily in glial cells. In nerve terminals, TTP is rapidly synthesized from TDP by the action of TDP

phosphoryltransferase, but the TTP ester does not accumulate to high concentrations; rather it is rapidly hydrolyzed to TDP by the action of TTPase, an enzyme which is also enriched in presynaptic terminals. Nerve stimulation results in release of thiamine which is mainly in the form of TMP (Cooper & Pincus, 1979). Taken together, these findings suggest that trafficking of thiamine and TMP occurs in brain as shown in a simplified schematic manner.

Figure 2: Brain thiamine homeostasis



Previous studies have demonstrated the existence of a low and a high-turnover pool of TDP in neuroblastoma cells and rat brain (Bettendorff, 1994a, 1994b). The low turnover pool, with a turnover time close to 20 h, represents bound TDP cofactor predominantly localized in the mitochondria; while the high turnover pool (1—2h) is free, cytosolic TDP. The latter is the precursor of TTP, which itself has a high turnover.

1.5.3 Regional distribution of thiamine in brain

Several studies report a rather uniform distribution of thiamine derivatives in rat (Dreyfus 1976). Dreyfus speculated that the parts of the central nervous system (CNS) having the highest content of TDP tend to be affected by thiamine deficiency earlier and to a greater extent than others by thiamine deficiency; however, the correlation was not strong. The region of the brain which had the highest amount of TDP in humans is the cerebellum; and this is likely due to the high cellular density in this brain region.

1.5.4 Turnover rates of thiamine in the CNS

In rats, depending upon the technique used and the region studied, approximately 2-10% of the total brain thiamine is turned over per hour (Rindi et al., 1980; Sen, 1976). However, due to the fact that there are multiple compartments of the brain with different turnover times, the concept of an overall turnover rate is marginally useful. Studies on the regional turnover rates of thiamine in the brain showed that the cerebellum was the brain area with the highest turnover rate, followed by the medulla and the pons, the spinal cord and hypothalamus, then the midbrain, thalamus and corpus striatum; while the cerebral cortex had the

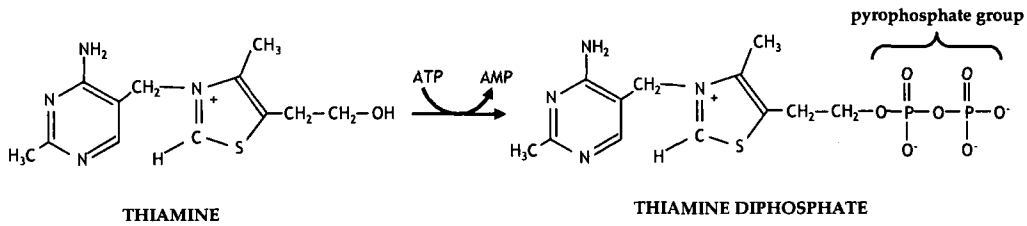
lowest thiamine turnover rate (Rindi et al., 1980). These results show a general (although not decisive) agreement between turnover rate values and brain regional sensitivity to thiamine deficiency, with some of the most vulnerable areas to thiamine depletion (e.g. cerebellum, pons) being those with higher turnover rates. Thiamine turnover rate alone, however, does not account for the degree of neuronal cell loss that is observed in regions such as the medial thalamus of TD rats.

2.0 THIAMINE-DEPENDENT ENZYMES

2.1 Thiamine Diphosphate: Coenzyme

Thiamine diphosphate (TDP), also known as thiamine pyrophosphate (TPP) or cocarboxylase, is the metabolically-active form of thiamine used as a coenzyme for oxidative decarboxylation reactions of α-ketoacids and transketolation. It is derived from thiamine by transfer of a pyrophosphate group from ATP to thiamine, yielding TDP and AMP (see Figure 3).

Figure 3: Pyrophosphorylation of thiamine



The enzymes that require TDP as a cofactor include pyruvate dehydrogenase (PDH, EC 1.2.4.1), α-ketoglutarate dehydrogenase (α-KGDH, EC 1.2.4.2), branched-chain α-ketoacid dehydrogenase (BCKDH, EC 1.2.4.4) and transketolase (EC 2.2.1.1). The first three enzyme complexes are located in mitochondria and catalyze the oxidative decarboxylation of α-ketoacids (see Figure 4). PDH and α-KGDH are important for mitochondrial oxidative metabolism which, especially in neurons, is crucial for cell survival. Transketolase is cytosolic and a key enzyme in the pentose phosphate pathway, a major source of NADPH for reductive biosynthesis of fatty acids and ribose sugars (Stryer, 1997).

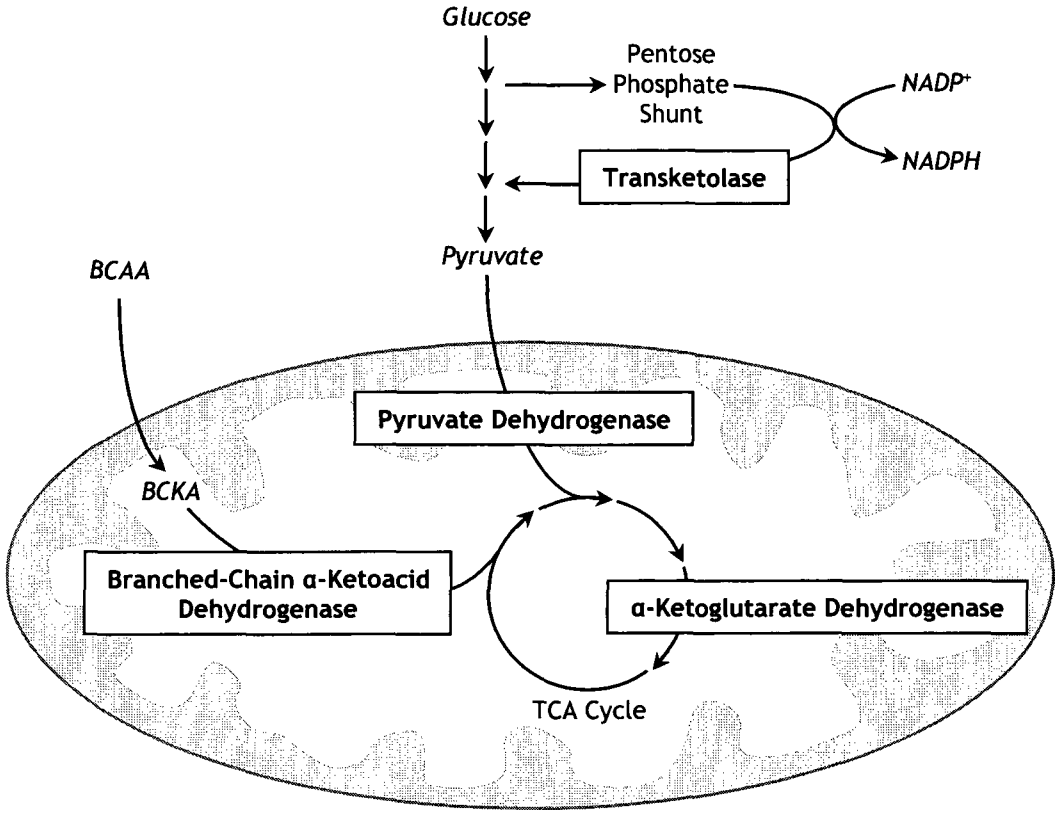


Figure 4: Thiamine-dependent enzymes in the brain

2.2 Oxidative Decarboxylation of α-Ketoacids

Pyruvate, α-ketoglutarate and branched-chain α-ketoacids undergo oxidative decarboxylation; and the α-ketoacid dehydrogenases are large enzyme complexes that serve this essential process in metabolism.

2.2.1 Pyruvate Dehydrogenase

The PDH complex sequentially catalyzes the conversion of pyruvate into acetyl-CoA, thereby providing the link between glycolysis and the tricarboxylic (TCA) cycle. The PDH complex is an organized assembly of multiple copies of three enzymes localized in the

mitochondria: pyruvate decarboxylase (E_1), dihydrolipoamide acyltransferase (E_2), and dihydrolipoamide dehydrogenase (E_3). The net reaction of the oxidative decarboxylation of pyruvate catalyzed by PDH is:

Pyruvate + CoA + NAD^+ → Acetyl-CoA + CO_2 + NADH + H^+

In addition to Coenzyme A (CoA-SH) and NAD^+, TDP, lipoic acid, and flavin adenine dinucleotide (FAD) also serve as coenzymes (Voet & Voet, 1990).

2.2.1.1 Regulation

PDH is a major regulatory point for the entry of materials into the TCA cycle as is accordingly regulated by end-product inhibition, NADH and the appropriate acetyl-CoA:

- E_2 – inhibited by acetyl-CoA, activated by CoA-SH
- E_3 – inhibited by NADH, activated by NAD^+

ATP is an allosteric inhibitor of the complex, and AMP is an activator. The activity of these key reactions is coordinated with the energy charge, the [NAD^+]/[NADH] ratio, and the ratio of acetylated to free CoA (Stryer, 1997).

In addition, the PDH complex is covalently regulated by phosphorylation-dephosphorylation state (James et al., 1995; Wieland, 1983). In particular, part of the PDH complex, pyruvate dehydrogenase kinase, phosphorylates three specific E_1 serine residues, resulting in a loss of activity of pyruvate dehydrogenase. NADH and acetyl-CoA both activate the kinase. The serines are dephosphorylated by a specific enzyme called pyruvate dehydrogenase phosphatase that hydrolyzes the phosphates from the E_1 subunit of PDH. This has the effect of activating

the complex. The phosphatase is activated by Ca^{2+} and Mg^{2+}. Because ATP and ADP differ in their affinities for Mg^{2+}, the concentration of Mg^{2+} reflects the ATP/ADP ratio within the mitochondrion. Thus, PDH responds to ATP levels by being turned off when ATP is abundant and further energy production is unneeded.

In mammalian tissues at rest, much less than half of the total pyruvate dehydrogenase is in the active, nonphosphorylated form (Voet & Voet, 1990). The complex can be turned on when low ATP levels signal a need to generate more ATP. The kinase protein is an integral part of the PDHC, whereas the phosphatase is loosely bound.

2.2.2 α-Ketoglutarate Dehydrogenase

α-KGDH is a rate-limiting enzyme in the TCA cycle; and similar to PDH, it exists as a complex with three analogous enzyme activities and the same five coenzymes — TDP, NAD^+, FAD, lipoic acid and CoA-SH. The net reaction in the oxidative decarboxylation of α-ketoglutarate catalyzed by the α-KGDH complex is:

α-Ketoglutarate + CoA + NAD^+ → Succinyl-CoA + CO_2 + NADH + H^+

2.2.2.1 Regulation

Notably, α-KGDH differs from PDH in that the regulatory activities associated with the PDH complex are not present in the α-KGDH complex.

2.2.3 Branched-Chain α-Ketoacid Dehydrogenase

The BCKDH complex catalyzes the irreversible oxidative decarboxylation of the three branched-chain α-ketoacids (BCKAs), obtained by the deamination of the branched-chain amino acids (leucine, isoleucine, and valine). Similar to PDH and α-KGDH, this enzyme complex consists of multiple copies of the BCKA decarboxylase (E_1), dihydrolipoamide acyltransferase (E_2), and dihydrolipoamide dehydrogenase (E_3) (Voet & Voet, 1990). The BCKA decarboxylation reactions are analogous to those for pyruvate and α-ketoglutarate:

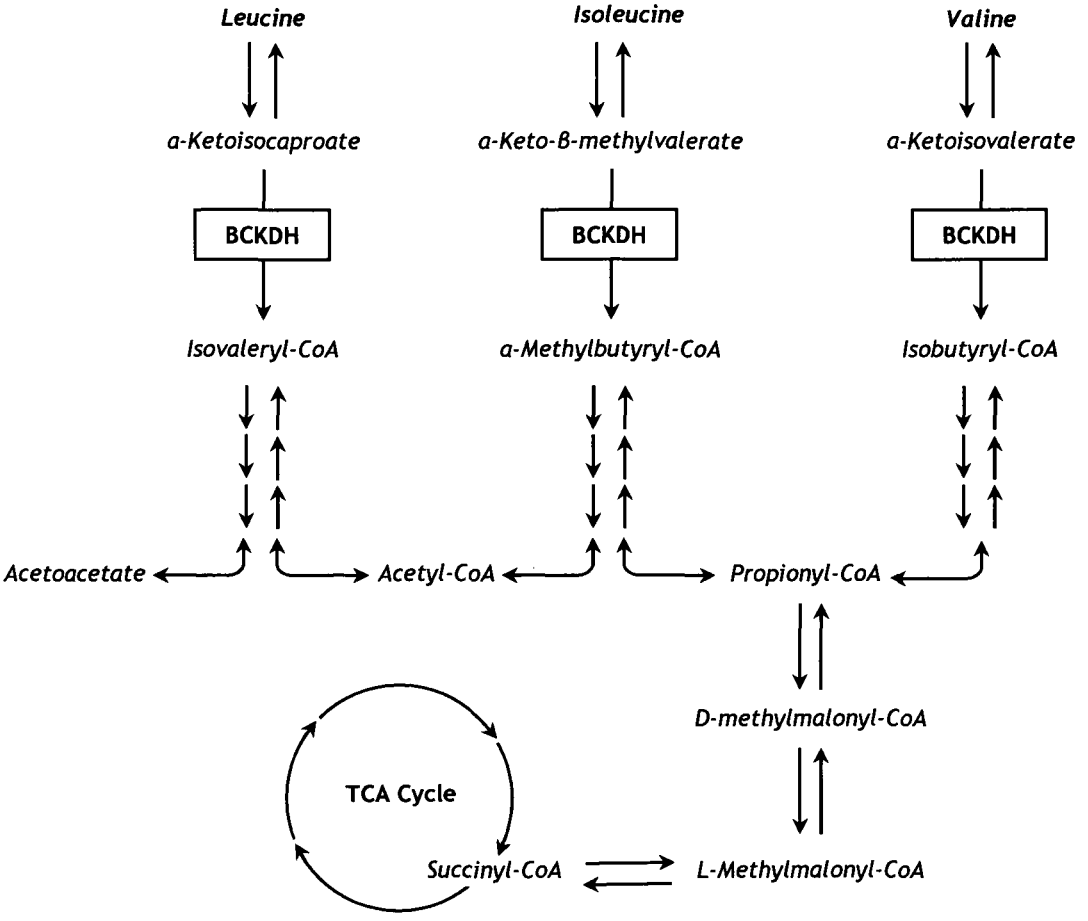
(i) α-Ketoisocaproate + CoA + NAD^+ → Isovaleryl-CoA + CO_2 + NADH + H^+

(ii) α-Keto-β-methylvalerate + CoA + NAD^+ → α-Methylbutyryl-CoA + CO_2 + NADH + H^+

(iii) α-Ketoisovalerate + CoA + NAD^+ → Isobutyryl-CoA + CO_2 + NADH + H^+

Aside from their involvement in synthetic processes, BCAAs can be oxidized after deamination to produce energy substrates (i.e. in times of starvation). A genetic defect in the BCKDH complex is responsible for maple syrup urine disease, emphasizing how the disposal of excess BCKAs is critical for the maintenance of good health (Stryer, 1997). Figure 5 illustrates how the carbon skeleton of BCAAs can be gluconeogenic (isoleucine, valine) or ketogenic (leucine, isoleucine).

Figure 5: Thiamine-dependent branched-chain α-ketoacid dehydrogenase (BCKDH) with respect to the tricarboxylic acid (TCA) cycle.



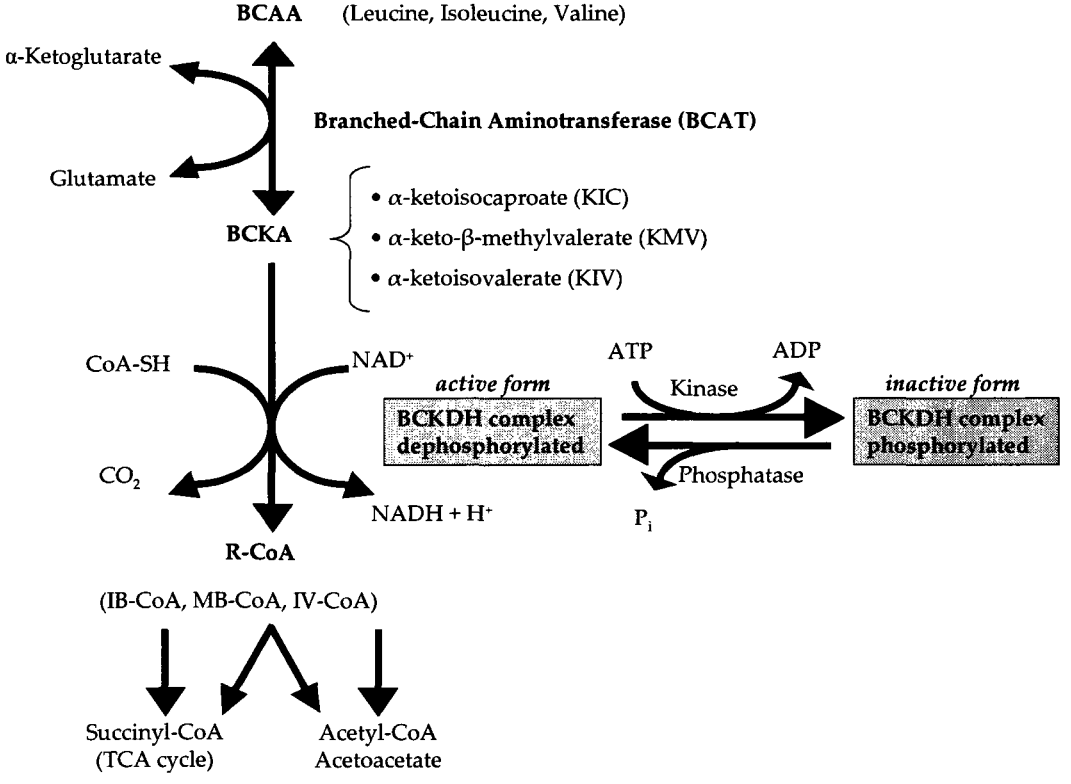
2.2.3.1 Regulation

BCKDH regulates the tightly controlled enzymatic system for BCAA degradation (see

Figure 6). Similar to PDH, BCKDH is regulated by both covalent and allosteric mechanisms. Phosphorylation of E_1 (on its a subunit) by the BCKDH kinase inhibits its activity; this may be reversed by dephosphorylation via a phosphatase. The BCKDH kinase has been

isolated, characterized, and cloned (Harris *et al.*, 2004) and is considered to be the key regulator of BCKDH activity. The kinase is regulated in two different ways. It is allosterically inhibited by α-ketoisocaproate (and by the other BCKAs, although they are less effective), which provides an elegant means of enhancing BCAA disposal when they are present in excess and of conserving these essential amino acids when they are less available. This effect of a-ketoisocaproate also explains the finding that the provision of excess leucine results in decreased circulatory levels of valine and isoleucine. The active dephosphorylated BCKDH is also susceptible to allosteric inhibition, in particular by NADH and by the CoA esters that arise during BCAA catabolism (Shimomura *et al.*, 2001).

Figure 6: The BCAA catabolic pathway.



TCA, tricarboxylic acid; KIV, α-ketoisovalerate; KMV, α-keto-β-methylvalerate; CoA-SH, reduced coenzyme A; IB-CoA, isobutyryl-CoA; MB-CoA, α-methylbutyryl-CoA; IV-CoA, isovaleryl-CoA; R-CoA, acyl-CoA.

2.3 Transketolase

A TDP-dependent transketolase found in the cytoplasm catalyzes the reversible transfer of a glycoaldehyde moiety from the first two carbons of a donor ketose phosphate to the aldehyde carbon of an aldose phosphate in the pentose phosphate pathway. These reversible reactions are:

(i) Xylulose-5-phosphate + Ribose-5-Phosphate ←→

Glyceraldehyde-3-Phosphate + Sedoheptulose-7-Phosphate

(ii) Xylulose-5-Phosphate + Erythrose-4-Phosphate ←→

Glyceraldehyde-3-Phosphate + α-D-Fructose-6-Phosphate

3.0 THIAMINE DEFICIENCY DISORDERS

3.1 Beri-Beri

Beri-beri is a peripheral manifestation of thiamine deficiency with symptoms including weight loss, weakness and pain in the limbs, and periods of irregular heart rate. There are two distinct forms of the disease: wet beriberi and dry beriberi. Wet beriberi affects the heart; it is sometimes fatal, as it causes a combination of heart failure and weakening of the capillary walls, which leads to peripheral edema. Dry beriberi, on the other hand, causes peripheral nerve damage, leading to muscle wasting and partial paralysis; and is also referred to as endemic neuritis.

3.2 Wernicke Korsakoff's Syndrome

In developed countries, thiamine deficiency results in Wernicke's Encephalopathy (WE), which, especially in chronic alcoholics, may lead to irreversible sequelae known as Korsakoff's Psychosis (Butterworth, 1993). WE is a serious complication of alcoholism and thiamine deficiency that may manifest as a combination of ataxia, nystagmus, opthalmoplegia, and marked confusion. Administration of thiamine at an early stage of deficiency will allow a complete patient recovery, with no morphological brain lesions observed before or after thiamine treatment; thereby reinforcing the concept of a "biochemical lesion" initially introduced by Rudolph Peters (Peters, 1936). Korsakoff's Psychosis is characterized by confusion, anterograde and retrograde amnesia, and confabulation. It is generally agreed that WE results from severe acute deficiency of thiamine, while Korsakoff's Psychosis is a more chronic neurologic sequela presenting after prolonged WE. Unlike WE, once amnesia and psychosis have occurred, complete recovery is unlikely. Subclinical thiamine

deficiency, especially in elderly people, may be more common than previously thought (Smidt et al., 1991) and often goes unnoticed.

3.3 Thiaminase

In certain fish (e.g. carp) there exists a heat-labile enzyme, thiaminase, which destroys thiamine by cleaving the molecule into its respective pyrimidine and thiazole components (Deolalkar & Sohonie, 1954, 1957; Sealock & White, 1949). This had led to signs of thiamine deficiency in certain animals (e.g. foxes) to which the raw fish has been fed. In those countries where large amounts of fish are eaten raw, human thiamine deficiency may also occur (Boros, 2000; Vimokesant et al., 1975). Thiaminase is also known to be found in certain fern species (e.g. bracken) amongst other plants. Some bacteria (e.g. *Bacillus thiaminolyticus*) are also capable of destroying thiamine (Douthit & Airth, 1966).

4.0 NEURONAL CELL DEATH DUE TO THIAMINE DEFICIENCY

Thiamine deficiency in humans (WE) is characterized by a selective loss of neurons in the midbrain, thalamus and cerebellum. Magnetic resonance imaging (MRI) of patients with WE reveals bilateral ventricular enlargement, mammillary body atrophy and cerebellar degeneration indicative of neuronal loss that is characteristic of WE. Several mechanisms have been proposed to explain this selective loss of neurons including a cerebral energy deficit resulting from reduction in activity of TDP-dependent enzymes, oxidative stress and N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity.

4.1 Impaired Cerebral Energy Metabolism

Both WE and experimental thiamine deficiency are characterized by decreases in brain concentrations of TDP and a reduction in activities of TDP-dependent enzymes (Butterworth, 1993; Butterworth & Heroux, 1989). A great deal of attention has been focused particularly on the role of decreased α-KGDH activity in the pathogenesis of neuronal cell death due to thiamine deficiency, since it is well-established that α-KGDH is a rate-limiting enzyme in the tricarboxylic acid cycle, which is responsible for the maintenance of cellular energy metabolism. Prolonged reductions in α-KGDH activity due to thiamine deficiency result in decreased glucose (pyruvate) oxidation; and direct measurement of high-energy phosphates in brain of TD rats revealed decreased levels of ATP in the brainstem (Aikawa *et al.*, 1984).

Reductions in TCA cycle flux due to thiamine deficiency results in decreased synthesis of relevant neurotransmitter amino acids glutamate and GABA(Butterworth & Heroux, 1989). In addition, impaired pyruvate

oxidation in the TD rat brain leads to the focal accumulation of lactate, which has been shown to cause a reduction in tissue pH (Hakim, 1984).

4.2 Oxidative and Nitrosative Stress

The effect of free radicals appears to link thiamine deficiency to the observed neuropathology; and both microglia and perivascular endothelial cells are sources of nitric oxide and oxidative stress in thiamine deficiency. Some markers of oxidative/nitrosative stress that have been shown to occur in TD animal brains include elevated:

- ICAM-1 (Calingasan *et al.*, 2000);
- heme oxygenase-1 (Langlais *et al.*, 1997) ;
- endothelial nitric oxide synthase (eNOS) (Gibson & Zhang, 2002; Kruse *et al.*, 2004);
- inducible nitric oxide synthase (iNOS) (Calingasan & Gibson, 2000b);
- redox active iron (Langlais et al., 1997) ; and
- microglial activation (K. Todd & Butterworth, 1999).

Induction of iNOS results in increased nitrotyrosine immunoreactivity in regions of the brain shown ultimately to manifest neuronal cell death. Nitrotyrosine is a specific nitration product of peroxynitrite, a highly potent oxidant generated by the reaction of superoxide with nitric oxide. Thiamine-dependent enzymes are particularly sensitive to oxidative stressors as both nitric oxide and peroxynitrite have been shown to inactivate α-KGDH (Park *et al.*, 2000).

Vascular factors also contribute to oxidative damage to neurons in thiamine deficiency. For example, eNOS is increased in thiamine

deficiency; while knockout of the eNOS gene significantly attenuates the neuronal cell death in TD mice. Furthermore, other studies found neuronal cell loss in TD rats to be attenuated by the free radical scavenger L-deprenyl (K. G. Todd & Butterworth, 1998b).

4.3 NMDA Receptor-Mediated Excitotoxicity

It has been proposed that the nature of the neuropathologic damage due to thiamine deficiency resembles that encountered in excitotoxic brain injury (i.e. brain injury resulting from excessive stimulation of NMDA receptors by glutamate). Excitotoxicity is a process shown to result in excessive accumulation of intracellular calcium leading to the activation of apoptotic cell death mechanisms. Evidence consistent with a role for excitoxicity in the neuronal cell death observed in thiamine deficiency includes the consistent finding of increased extracellular concentrations of glutamate in brain regions known to be selectively lesioned by thiamine deficiency(Hazell *et al.*, 1993). Pre-treatment of TD rats with the competitive NMDA receptor antagonist MK801 was shown to be neuroprotective (Langlais & Mair, 1990); however, this effect was largely due to the anticonvulsant properties of the drug (K. G. Todd & Butterworth, 1998a). A more plausible explanation for the increased extracellular brain glutamate levels in thiamine deficiency relates to the finding of selective down-regulation of astrocytic glutamate transporters in vulnerable brain structures in the TD brain (Hazell *et al.*, 2001).

5.0 EXPERIMENTAL WERNICKE'S ENCEPHALOPATHY

Further insights into the nature and cause of the neuropathologic features of WE have been (and continue to be) provided by studies in experimental animal models of WE; the most popular and well-characterized of which is the rat treated for 12-14 days with the central thiamine antagonist, pyrithiamine.

5.1 Pyrithiamine-Induced Thiamine Deficiency

Pyrithiamine is a competitive thiamine antagonist that inhibits thiamine pyrophosphokinase, the enzyme responsible for converting free thiamine to the diphosphate ester (TDP). Consequently, pyrithiamine administration to experimental animals leads to the decline in levels of TDP.

The pyrithiamine-treated rat recapitulates much of the neurological syndrome and neuropathology of WE in humans, being characterized by the presence of focal lesions with symmetrical distribution(Troncoso *et al.*, 1981). Both pyrithiamine and oxythiamine, another thiamine analogue, have been used to produce thiamine deficiency in rats. While pyrithiamine administration produces neurological symptoms such as ataxia, opisthotonus, nystagmus, loss of righting reflex, convulsions, and finally coma, treatment with oxythiamine does not lead to any of these deficits. It is clear that oxythiamine crosses the blood-brain barrier very poorly, while pyrithiamine readily accumulates in cerebral structures (Rindi *et al.*, 1963; Rindi & Perri, 1961). This observation is probably also the basis of the finding that pyrithiamine rapidly depletes brain thiamine stores (McCandless et al., 1968; Murdock & Gubler, 1973). Because pyrithiamine treatment fairly accurately reproduces the distribution of

lesions seen in WE, this analogue is most often chosen for investigating the pathogenesis of thiamine-deficiency induced brain damage.

SUMMARY

Substantial evidence to date clearly indicates that thiamine deficiency has deleterious effects in selectively vulnerable brain regions which are predisposed to manifest neuronal cell death and mitochondrial dysfunction. The following four articles will provide an examination of some of the region-selective effects of thiamine deficiency in various aspects of thiamine-dependent metabolic processes within the rat brain. In addition, the phenomenon of glucose-precipitated worsening of neurological symptoms in TD patients will be explored.

ARTICLE 1

BRAIN LACTATE SYNTHESIS IN THIAMINE DEFICIENCY: A RE-EVALUATION USING $^{1}H/^{13}C$ NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Darren Navarro, Claudia Zwingmann, Alan S. Hazell and

Roger F. Butterworth

Neuroscience Research Unit, Hôpital Saint-Luc,

Montreal, Quebec, Canada.

Running Title: Brain lactate in thiamine deficiency

Keywords: Thiamine deficiency, Glucose metabolism, Pyruvate recycling, Wernicke's Encephalopathy, Lactate dehydrogenase

Address for reprints and correspondence:

Roger. F. Butterworth, Ph.D, D.Sc.

Neuroscience Research Unit

CHUM (Campus Saint-Luc)

1058 Saint-Denis Street

Montreal, Quebec, Canada H2X 3J4

Phone: (514) 890-8310 ext. 35759

FAX: (514) 412-7314

Email: roger.butterworth@umontreal.ca

ABSTRACT

Region-selective accumulation of brain lactate occurs in thiamine deficiency. However, the mechanisms responsible have not been fully elucidated. ^{1}H and ^{13}C Nuclear Magnetic Resonance spectroscopy were therefore used to investigate *de novo* lactate synthesis from [1-^{13}C]glucose in vulnerable (medial thalamus) and non-vulnerable (frontal cortex) brain regions of rats made thiamine-deficient by administration of the central thiamine antagonist pyrithiamine. *De novo* synthesis of lactate was increased in the medial thalamus to 148% and 226% of pair-fed control values at presymptomatic and symptomatic stages of thiamine deficiency, whereas no such changes were observed in the frontal cortex. Administration of a glucose load selectively worsened the changes in medial thalamus. Pyruvate recycling and peripherally-derived lactate did not contribute significantly to the lactate increase within the thiamine-deficient brain. Increases in immunolabeling of the lactate dehydrogenase isoenzymes (LDH1 and LDH5) were observed in the medial thalamus of thiamine-deficient animals. Thus, metabolic impairment due to thiamine deficiency results in increased glycolysis, increased LDH immunolabeling of neurons and astrocytes and increased *de novo* synthesis of lactate in brain regions vulnerable to thiamine deficiency. These results are consistent with the notion that focal lactate accumulation participates in the worsening of neurological symptoms in thiamine-deficient patients.

INTRODUCTION

The accumulation of lactate in the brains of thiamine-deprived animals is a phenomenon that has been recognized for several decades (Kinnersley and Peters, 1930; Holowach et al., 1968; McCandless et al., 1968). Lactate accumulation (McCandless, 1982; Munujos et al., 1993) and lactic acidosis (Hakim, 1984) have been shown to be localized to brain regions that develop histological lesions and selective accumulation of lactate and associated pH changes have been proposed as a possible cause of neuronal cell death in thiamine deficiency (Hakim et al., 1983; Parker et al., 1984). However, since plasma levels of lactate are also elevated in thiamine deficiency (Park and Gubler, 1969), it is still unknown whether the increased lactate accumulation results from de novo synthesis within the brain or from the periphery.

Administration of glucose precipitates neurological symptoms (Wernicke's Encephalopathy) in thiamine-deficient (TD) patients (Watson et al., 1981). Although the exact mechanism underlying this phenomenon remains unclear, it has been proposed that the accumulation of glucose-derived intermediates is a causal factor in its development. For example, previous studies using ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy in TD rats have shown a substantial increase of lactate in the brain after a glucose load (Rose et al., 1993).

In order to elucidate the metabolic origin and cellular localization of brain lactate accumulation in thiamine deficiency, a series of experiments were performed. Firstly, *de novo* synthesis of lactate together with the magnitude of pyruvate recycling via the tricarboxylic acid cycle were studied in vulnerable versus non-vulnerable brain regions of TD rats using combined ^{1}H and ^{13}C NMR spectroscopy. Parallel studies were

performed to examine the effects of glucose loading on regional brain lactate synthesis in TD animals. Furthermore, in order to shed light on the cellular selectivity of increased brain lactate, the effects of thiamine deficiency on lactate dehydrogenase (LDH1 and LDH5) expression was investigated in vulnerable versus non-vulnerable brain regions of TD animals.

MATERIALS AND METHODS

Animal model

Adult male Sprague Dawley rats weighing 200-225g obtained from Charles River (St. Constant, Quebec, Canada) were used in all experiments. Rats were housed individually under constant conditions of temperature, humidity and 12 hour light/dark cycles and had free access to water at all times. Rats were allowed to adapt to their environment for 3 days prior to the initiation of treatments. Animals were assigned to either thiamine-deficient (TD) or pair-fed control (PFC) groups. TD rats were further divided into presymptomatic (PS) and symptomatic (SYM) groups (see below). All animal treatment procedures were approved by the Animal Ethics Committee of Saint-Luc Hospital (CHUM) and the University of Montreal.

Thiamine deficiency protocol

Rats in the TD group were fed a diet deficient in thiamine (ICN Nutritional Biochemicals, Cleveland, OH, USA) and administered daily pyrithiamine hydrobromide (0.5 mg per kg body wt) intraperitoneally (i.p.). Control rats were pair-fed to equal food consumption with the TD rats using the same TD diet but with supplemental daily i.p. injections of thiamine (0.1 mg per kg body wt) (Troncoso et al., 1981; Héroux and Butterworth, 1992). Rats in the PS group were sacrificed on day 11 of treatment, a time-point characterized by the onset of ataxia, at which no cell death had occurred in the vulnerable brain regions. After 12 days of treatment, rats were assessed twice daily for neurological abnormalities. When rats displayed loss of righting reflex (usually between days 13-15) they were considered to be at the symptomatic stage. Any rats exhibiting

spontaneous seizures were eliminated from the protocols. Animals were sacrificed by decapitation and the brains were promptly removed and flash frozen in isopentane on dry ice and stored at – 80 °C. Frontal cortex and medial thalamus were dissected on ice according to the coordinates described in the rat brain atlas of Paxinos and Watson (1982).

Sample preparation for Nuclear Magnetic Resonance (NMR)

For NMR investigations, [1-^{13}C]glucose (Cambridge Isotopes, Andover, MA, USA) was administered either as a minimal labeling dose or a glucose load (200 or 500 mg/kg, i.p., respectively) 60 min prior to sacrifice. The rats were killed by decapitation, the brains were removed from the skull, immediately frozen in isopentane over dry ice, and dissected on ice to obtain medial thalamus (MT, vulnerable brain region) and the frontal cortex (FC, non-vulnerable brain region). Tissue samples were ground over liquid nitrogen and homogenized in 12% perchloric acid (PCA) at 0°C using a motor-driven polished glass tube-Teflon homogenizer. The homogenate was centrifuged at 4000 g for 15 min and this was repeated once. The supernatants were combined and neutralized on ice with KOH. The precipitated $KClO_4$ was removed by centrifugation (4000 g, 15 min). Arterial blood was immediately mixed with 20% PCA, centrifuged (4000 g, 40 min), neutralized with KOH, and centrifuged again (4000 g, 15 min) to precipitate $KClO_4$. The supernatants from each sample were lyophilized.

NMR spectroscopy

The lyophilized PCA extracts of the blood and the brain tissue were dissolved in 0.6 mL deuterium oxide (D_2O; Merck, Darmstadt, Germany) and centrifuged. Prior to NMR analysis, the pH was adjusted to 7.0 with DCl and NaOD. ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker DRX 600 or AVANCE NB/WB 360 spectrometers, operating at frequencies of 600 MHz or 360 MHz for ^{1}H-measurements, and 150.9 MHz or 90.5 MHz for ^{13}C-measurements.

^{1}H-NMR spectra were recorded with a 5-mm H,C,N inverse triple resonance probe (5-mm HX probe), 400 accumulations, repetition time 15 s, spectral width 7183 Hz (DRX 600) or 3623 Hz (NB/WB 360). Chemical shifts were referenced to lactate at 1.33 ppm. ^{13}C-NMR spectra were recorded with a 5-mm $^{1}H/^{13}C$ dual probe, 20,000 accumulations, repetition time 2 s, composite pulse decoupling with WALTZ-16, spectral width 47,619 Hz (DRX 600) or 20,833 Hz (NB/WB 360). Chemical shifts were referenced to the C-3 signal of lactate at 21.3 ppm.

Measurement of metabolite concentrations

Lactate concentrations were determined enzymatically (adapted for measurement in 96-well microtiter ELISA plates) using a commercially available kit (Roche Molecular Biochemicals, Mannheim, Germany). Concentrations of unlabeled lactate and its pool size ($[^{13}C] + [^{12}C]$) were determined from fully relaxed ^{1}H-NMR spectra of brain extracts, obtained after injection of [1-^{13}C]glucose, using known lactate concentrations as internal standard.

Calculation of fractional ^{13}C-enrichment and amount of ^{13}C-labeled lactate

The ^{13}C-enrichments in C-3 of lactate were determined from ^{1}H-NMR spectra by integration of peak areas of the [^{1}H-^{12}C] signal and both [^{1}H-^{13}C] satellite signals of the respective methyl groups (Fig. 1):

$$^{13}\text{C-enrichment} = \frac{\text{area } [^{1}\text{H-}^{13}\text{C}]}{\text{area } [^{1}\text{H-}^{12}\text{C}] + \text{area } [^{1}\text{H-}^{13}\text{C}]}$$

The sum (area [^{1}H-^{12}C] + area [^{1}H-^{13}C]) is equivalent to the pool size of the metabolite, measured enzymatically. All values are corrected for 1.1 % natural abundance ^{13}C. ^{13}C-enrichments in [1-^{13}C]glucose were calculated accordingly using the H1α glucose resonances in ^{1}H-NMR spectra. Since the protons at C-2 of lactate overlap with other compounds in ^{1}H-NMR spectra, the fractional ^{13}C-enrichment and the absolute amount of ^{13}C in the C-2 carbon of lactate were derived from ^{13}C-NMR spectra by the peak area ratio of the ^{13}C-labeled carbon/natural abundance carbon and using the known ^{13}C-enrichment in [3-^{13}C]lactate as internal standard as described previously (Zwingmann et al., 2003). The ^{13}C signal intensities were corrected for NOE (Nuclear Overhauser Enhancement) and naturally abundant ^{13}C (1.1%) in [2-^{13}C]lactate.

Labeling of metabolites from [1-^{13}C]glucose

[1-^{13}C]glucose metabolites are labeled in different carbon positions, depending on the relative contribution of the enzymatic pathways (Figure 1). Through glycolysis, [1-^{13}C]glucose is converted to [3-^{13}C]pyruvate,

then to [3-^{13}C]lactate via lactate dehydrogenase (LDH). [3-^{13}C]pyruvate may also enter the TCA cycle via the anaplerotic pathways (pyruvate carboxylase, PC or malic enzyme, ME) or the oxidative pathway (pyruvate dehydrogenase, PDH). After entry of labeled pyruvate into the TCA cycle and scrambling of the label at the symmetrical succinate step, generation of [2-^{13}C]pyruvate, and hence [2-^{13}C]lactate, may occur only after the exit of a TCA cycle intermediate via a pyruvate recycling pathway (i.e. malate, via ME; or oxaloacetate, via phosphoenolpyruvate carboxykinase, PEPCK and pyruvate kinase, PK).

Immunohistochemistry

Rats were anaesthetised with pentobarbital (80mg/kg) and perfused transcardially with 150 mL of saline followed by 150 mL of neutral-buffered formalin containing 4% formaldehyde, 0.5% sodium phosphate buffer, 1.5% methanol and 0.02% glutaraldehyde, pH 7.0 (Fisher Scientific, Fair Lawn NJ, U.S.A). Brains were then removed and post-fixed overnight in the same fixative. Paraffin-embedded 6μm coronal sections were cut at the level of the frontal cortex and medial thalamus, and mounted on slides coated with Vectabond Reagent (Vector Laboratories Inc., Burlingame, CA, U.S.A.).

For studies of LDH1 and LDH5 immunohistochemistry, paraffin-embedded slides were deparaffinized by heating in an oven at 60°C for 30 min, immersed in xylene, and sequentially rehydrated using ethanol 100%, 95%, 85%, 70%, followed by distilled water. Endogenous peroxidase was blocked by means of 1% H_2O_2 in methanol for 30 min at room temperature and then washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 5.37 mM Na_2HPO_4, 1.76 mM KH_2PO_4). For

LDH1 antigen retrieval, the slides were transferred into microwaveable-glass baths containing 5% urea/Tris-HCl buffer, pH 9.5, as described by Shi et al., 1996. The baths were heated twice for 5 min in a microwave oven (Sanyo Model EM-802TW, operating at 2450 mHz power setting 600W). When necessary, more AR buffer was added after the first 5 min to compensate for loss due to boiling and to avoid drying of tissue sections. After heating, slides remained in the baths for an additional 30 min before washing in double-distilled H_2O, followed by PBS. Excess PBS was removed; and the sections were circled with a waterproof pen. The non-specific binding sites were blocked using 10% normal horse serum in 0.5% Triton X-100/PBS for 30 min. The slides were incubated overnight with mouse-derived primary antibodies for LDH1 or LDH5 (1:500, Sigma, St. Louis MO, U.S.A.) in a humid chamber at 4ºC. After washing in PBS, the sections were incubated with biotinylated horse anti-mouse IgG secondary antibody (1:1000, Vector Laboratories, Inc. Burlingame, CA, U.S.A.), followed by incubation with ABC reagent (Vector Laboratories Inc. Burlingame, CA, U.S.A.). LDH1/LDH5 immunoreactivity was subsequently detected by incubation with 3-3'-diaminobenzidine (DAB) containing urea hydrogen peroxide (Sigma-Aldrich Co., St. Louis MO, U.S.A.). Consecutive sections were immunostained for GFAP (DakoCytomation Inc., Mississauga ON, Canada) for astrocytic identification. Negative control slides were treated identically except that the primary antibodies were omitted.

Statistical analysis

The NMR studies were carried out on four to five animals per group. Data are expressed as mean ± SD values. Data from all

experimental groups were analyzed using two-way analysis of variance (ANOVA) and post-hoc Tukey`s test. Differences were considered significant when $P < 0.05$.

RESULTS

Concentration and *de novo* synthesis of lactate

[1-^{13}C]glucose is converted to [3-^{13}C]pyruvate via the glycolytic pathway, and subsequently to [3-^{13}C]lactate via LDH (Fig. 1). ^{1}H-^{13}C-NMR spectroscopy, (the heteronuclear spin pattern of lactate in the high-field region of ^{1}H-NMR spectra), allows for distinction between lactate synthesized after glycolytic transformation of [1-^{13}C]glucose ([3-^{13}C]lactate) which is evident in the spectra as an additional doublet splitting due to ^{1}H-^{13}C-coupling (J = 128 Hz), and the unlabeled isotopomer ([^{12}C]lactate) synthesized from unlabeled pyruvate. Figure 2 shows representative segments of ^{1}H-NMR spectra of extracts obtained from the medial thalamus of pair-fed control and TD rats at symptomatic stages, which were sacrificed 60 min after injection of either 200 mg/kg (minimal labeling dose) or 500 mg/kg (glucose load) [1-^{13}C]glucose, respectively.

The effect of thiamine deficiency on the *de novo* synthesis of lactate from glucose was measured in the non-vulnerable frontal cortex (FC) compared to the vulnerable medial thalamus (MT) in the presence or absence of a glucose load (Fig. 3). After injection of TD rats with the minimal labeling dose of [1-^{13}C]glucose (200 mg/kg, i.p.), ^{13}C-labeled lactate levels increased concomitantly with the stage of encephalopathy (Fig. 3b). Significant alterations were observed in the MT (148% and 226% of pair-fed controls, at presymptomatic and symptomatic stages, respectively; $p < 0.01$), whereas changes were significantly less in the FC (120% and 128% of pair-fed controls; $p < 0.05$). Changes in the total pool of lactate ([^{13}C] + [^{12}C]) in the MT were also observed (Fig. 3a,b) resulting in increased fractional enrichments in ^{13}C-lactate (indicating *de novo*

synthesis from glucose via the glycolytic pathway) ($p < 0.05$). No alterations in the fractional enrichment of ^{13}C-lactate were observed in FC (Fig. 3c).

Glucose loading (500 mg/kg) of PFC rats had no observable effect on levels and fractional enrichment of [3-^{13}C]lactate synthesized from [1-^{13}C]glucose in both FC and MT. However, after administration of a glucose load to TD rats at the presymptomatic stage, significant increases in concentration of [3-^{13}C]lactate were already observed in both the FC and MT (150% and 149% respectively, $p<0.05$) compared to presymptomatic TD rats without a glucose load (Fig. 3b). When TD rats at the symptomatic stage were administered a glucose load, levels of [3-^{13}C]lactate were significantly increased in FC (165%, $p<0.01$), and to a much larger extent in MT (275%, $p<0.001$) compared to symptomatic TD rats without a glucose load. Following a glucose load, an increase in the fractional enrichment in [3-^{13}C]lactate, thus *de novo* synthesis from glucose, was observed in FC only at the symptomatic stage (166% compared to symptomatic TD rats without a glucose load, $p<0.05$) (Fig.3c); however in MT, significant increases in the fractional enrichment in [3-^{13}C]lactate were already observed at the presymptomatic stage of thiamine deficiency (130% compared to presymptomatic TD rats without a glucose load, $p<0.01$). In the symptomatic MT the effect of glucose loading was largely augmented as the fractional enrichment in [3-^{13}C]lactate was increased approximately 212% ($p<0.001$) compared to symptomatic rats without a glucose load.

The ^{13}C-enrichment in blood lactate was very low (< 1 %) compared to brain tissue. While the amount of blood [3-^{13}C]lactate was unchanged, the concentration of unlabeled lactate increased to 124.71 ± 9.44% of

control at presymptomatic stages resulting in decreased fractional enrichments in [3-^{13}C]lactate (data not shown).

Pyruvate recycling

The appearance of [2-13C]lactate is indicative of lactate formation through a pyruvate recycling pathway (Fig. 4). In the non-vulnerable FC, [2-13C]lactate formation via pyruvate recycling remained unchanged in both the presence and absence of a glucose load. However, in the MT, significant increases in [2-13C]lactate enrichment were observed at symptomatic stages of thiamine deficiency (from 0.95 ± 0.11% in PFC to 1.96 ± 0.16% at symptomatic stage); and this increase was augmented after glucose loading (up to 2.4 ± 0.18%).

Immunohistochemistry of LDH1/LDH5 isoenzymes in the medial thalamus

Sections of the medial thalamus were taken from either PFC or presymptomatic TD rat brains (Day 11). LDH1-immunostaining was observed in both neurons and glia in the MT of the PFC rats (Fig. 5). Immunostaining was more intense in the MT of the TD rat compared to PFC, and was particularly evident in neurons. Similarly, LDH5-immunostaining increased in the TD MT compared to PFC and was preferentially localized within astrocyte cell bodies. The intensity of staining for the astrocyte-marker GFAP was similar between pair-fed control and TD MT, confirming the lack of significant astrogliosis.

DISCUSSION

Accumulation of lactate in the thiamine-deficient brain has been observed since the pioneering studies of Peters and colleagues (Kinnersley and Peters, 1930; Peters, 1936). Since that time, increased brain lactate levels have been described in a wide range of experimental thiamine deficiency paradigms involving rodents treated with the central thiamine antagonist pyrithiamine (Holowach et al., 1968; McCandless et al., 1968) as well as in studies of the effects of thiamine antagonists on cultured neural cells (Bettendorf et al., 1995; Pannunzio et al., 2000). Results of the present study demonstrate that pyrithiamine-induced thiamine deficiency results in lactate accumulation within brain structures that are destined to manifest neuronal cell damage and loss. Furthermore, brain lactate accumulation is shown to parallel the progression of thiamine deficiency and to result primarily from increased *de novo* synthesis of lactate from glucose within the brain. Although plasma levels of lactate are increased, its ^{13}C-fractional enrichment remains unchanged. Therefore, peripheral lactate does not contribute significantly to the [3-^{13}C]lactate increase within the TD brain.

There is a general consensus of opinion that the major cause of reduced glucose (pyruvate) oxidation in the TD brain resides is an initial impairment of α-ketoglutarate dehydrogenase activity, resulting in a reduction of tricarboxylic acid cycle flux (Héroux and Butterworth, 1995) in brain regions that are vulnerable to thiamine deficiency (Parker et al., 1984; Butterworth and Héroux, 1989; Munujos et al., 1996). Results of the present study confirm and extend these findings.

Recent studies demonstrate that lactate is produced not only from pyruvate but also from precursors derived from the TCA cycle

(Sonnewald et al., 1993; Bachelard et al., 1994). This "recycling" of pyruvate, a process that occurs when pyruvate is incorporated into the TCA cycle and then subsequently regenerated from TCA cycle constituents, was first demonstrated in the brain by Cerdan et al. (1990). Pyruvate recycling preferentially occurs within the astrocytic, rather than the neuronal compartment (Hassel and Sonnewald, 1995). In the present study, [2-^{13}C]lactate (which can only arise from [1-^{13}C]glucose via pyruvate recycling) was increased in TD MT at the symptomatic stage. The metabolic advantage of such recycling is unknown. However, it has been suggested that astrocyte-derived lactate formed via glycolysis may serve as an energy substrate for neurons, particularly in situations of impaired glucose metabolism (Pellerin and Magistretti, 1994; Pellerin et al., 1998). Although 2-fold increases in [2-^{13}C]lactate enrichment, indicative of pyruvate recycling, were observed in MT at the symptomatic stage of thiamine deficiency, results of the present study show that the contribution of this pathway to total lactate synthesis remains relatively minor. However, it is conceivable that increased pyruvate recycling may occur in an additional attempt to supply more lactate to energy-impaired neurons in thiamine deficiency.

Previous investigations have shown that lactate dehydrogenase (LDH) exists in two isoforms within the brain, namely LDH1 and LDH5 (Cahn et al., 1962). These two isoenzymes are under the control of separate genes (Nance et al., 1963) and isoenzyme variation has been shown to occur in several disease states (Lample et al., 1990; Subhash et al., 1993). In the present study, increased neuronal and astrocytic LDH1-immunolabeling coincided with the increased *de novo* synthesis of lactate in this brain structure. Increases in LDH5-immunolabeling appeared

predominantly in astrocytes of MT; and the increased staining intensities of both LDH1 and LDH5 were not caused by astrogliosis, as shown by comparable levels of staining for the astrocytic-marker GFAP. Increased astrocytic immunolabeling of LDH isoenzymes is consistent with increased lactate production in astrocytes. Whether this occurs in order to provide an energy substrate to the metabolically-impaired neurons remains a possibility worthy of further evaluation.

It is well established in both experimental and clinical studies that glucose loading causes worsening of the neurological symptoms of thiamine deficiency and can lead to the precipitation of Wernicke's Encephalopathy (Watson et al., 1981; Wallis et al., 1978; Harper, 1980; Miyajima et al., 1993)in TD patients. Studies using magnetic resonance imaging in rats reveal that T_2-weighted signal hyperintensities (indicative of neuronal cell damage and death) appear within 30 min of administration of a glucose load to TD rats. These signal hyperintensities are confined to vulnerable thalamic and brain stem structures in these animals (Jordan et al., 1998). Rapid appearance of neurological and spectroscopic changes have also been reported following glucose loading of TD rats (Zimitat et al., 1999; Rose et al., 1993). The mechanisms responsible for glucose precipitation of Wernicke's Encephalopathy in thiamine deficiency have not been elucidated. However, results of the present study demonstrate that the *de novo* synthesis of lactate is further increased following glucose loading of TD rats and that this increase is most evident in the vulnerable MT. Furthermore, the effect of glucose loading on *de novo* lactate synthesis increases with progression of neurological impairment in TD rats. These findings suggest that focal lactate accumulation may be implicated in the worsening of neurological

symptoms of thiamine deficiency following glucose loading. In support of this possibility, a previous report described reductions in pH consistent with focal lactic acidosis in vulnerable brain regions in pyrithiamine-induced thiamine deficiency (Hakim et al., 1984). Altered pH is known to adversely affect vascular tone (Kim et al., 2004) and calcium homeostasis (Hriciga et al., 1983). Whether or not glucose-loading causes more intense acidosis in vulnerable brain structures in thiamine deficiency requires further study.

In conclusion, results of the present study demonstrate that the regional lactate accumulation observed by ^{1}H and ^{13}C-NMR spectroscopy in the selectively vulnerable regions of the TD brain is primarily due to increased *de novo* synthesis from glucose. This suggests that brain lactate accumulation in thiamine deficiency is a direct consequence of the metabolic impairment of the TCA cycle. The increased immunolabelling of both LDH isoforms in vulnerable brain structures suggests that increased lactate production by both neurons and astrocytes contributes to the accumulation of brain lactate in thiamine deficiency. Lactate formation via pyruvate recycling in astrocytes makes a significant but minor contribution to the total lactate accumulation at late stages of thiamine deficiency. Administration of a glucose load exacerbates the region-selective increases of *de novo* lactate synthesis and pyruvate recycling. These findings suggest that lactate accumulation is a likely mechanism contributing to the glucose-precipitation of Wernicke's Encephalopathy in TD patients.

ACKNOWLEDGMENTS

The studies from the authors' research unit were funded by the Canadian Institutes for Health Research (CIHR). Claudia Zwingmann is a recipient of research awards from the Quebec Ministry of Education and Deutsche Forschungsgemeinschaft, Germany.

REFERENCES

Bachelard, H., Badar-Goffer, R., Morris, P., & Thatcher, N. (1994). Magnetic resonance spectroscopy studies on Ca^{2+}, Zn^{2+} and energy metabolism in superfused brain slices. *Biochem Soc Trans*, 22, 988-991.

Bettendorff, L., Sluse, F., Goessens, G., Wins, P., & Grisar, T. (1995). Thiamine deficiency-induced partial necrosis and mitochondrial uncoupling in neuroblastoma cells are rapidly reversed by addition of thiamine. *J Neurochem*, 65(5), 2178-2184.

Butterworth, R.F., & Héroux, M. (1989). Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acids and thiamine-dependent enzymes. *J Neurochem*, 52(4), 1079-1084.

Cahn, R.D., Kaplan, N.O., Levine, L., & Zwilling, E. (1962). Nature and development of lactic dehydrogenases. *Science*, 136, 962-969.

Cerdan, S., Kunnecke, B., & Seelig, J. (1990). Cerebral metabolism of [1,2-^{13}C2]acetate as detected by in vivo and in vitro ^{13}C NMR. *J Biol Chem*, 265, 12916-12926.

Hakim, A.M., Carpenter, S., & Pappius, H.M. (1983). Metabolic and histological reversibility of thiamine deficiency. *J Cereb Blood Flow Metab*, 3(4), 468-477.

Hakim, A.M. (1984). The induction and reversibility of cerebral acidosis in thiamine deficiency. *Ann Neurol*, 16(6), 673-679.

Harper, C.G. (1980). Sudden, unexpected death and Wernicke's Encephalopathy: a complication of prolonged intravenous feeding. *Aust N Z J Med*, 10, 230-235.

Hassel, B., & Sonnewald, U. (1995). Glial formation of pyruvate and lactate from TCA cycle intermediates: implications for the inactivation of transmitter amino acids. *J Neurochem*, 65, 2227-34.

Héroux, M., & Butterworth, R.F. (1992). Animal Models of the Wernicke-Korsakoff Syndrome. *Neuromethods*, 22, 95-131.

Héroux, M., & Butterworth, R.F. (1995). Regional alterations of thiamine phosphate esters and of thiamine diphosphate-dependent enzymes in relation to function in experimental Wernicke's Encephalopathy. *Neurochem Res*, 20, 87-93.

Holowach, J., Kauffman, F., Ikossi, M.G., Thomas, C., & McDougal, D.B. Jr. (1968). The effects of a thiamine antagonist, pyrithiamine, on levels of selected metabolic intermediates and on activities of thiamine-dependent enzymes in brain and liver. *J Neurochem*, 15, 621-631.

Hriciga, A., & Lehn, J.M. (1983). pH regulation of divalent/monovalent Ca/K cation transport selectivity by a macrocyclic carrier molecule. *Proc Natl Acad Sci USA*, 80, 6426-6428.

Jordan, L.R., Zelaya, F.O., Rose, S.E., Bower, A.J., Galloway, G., Wholohan, T., & Nixon, P.F. (1998). Changes in the hippocampus induced by glucose in thiamin deficient rats detected by MRI. *Brain Res*, 791, 347-351.

Kim, Y.C., Lee, S.J., & Kim, K.W. (2004). Effects of pH on Vascular Tone in Rabbit Basilar Arteries. *J Korean Med Sci*, 19, 42-50.

Kinnersley, H.W., & Peters, R.A. (1930). Brain localization of lactic acidosis in avitaminosis B1 and its relation to the origin of symptoms. *Biochem J*, 24, 711-722.

Lample, Y., Paniviz, Y., & Eshel, Y. (1990). LDH isoenzymes in CSF in various brain tumors. *Neurol Neurosurg Psychiat,* 53, 697-699.

McCandless, D.W., Schenker, S., & Cook, M. (1968). Encephalopathy of thiamine deficiency: studies of intracerebral mechanisms. *J Clin Invest,* 47(10), 2268-2280.

McCandless, D.W. (1982). Energy metabolism in the lateral vestibular nucleus in pyrithiamin-induced thiamin deficiency. *Ann N Y Acad Sci,* 378, 355-364.

Miyajima, Y., Fukuda, M., Kojima, S., Matsuyama, T., Shylaja, N., & Aso, K. (1993). Wernicke's Encephalopathy in a child with acute lymphoblastic leukemia. *Am J Pediatr Hematol Oncol,* 15, 331-334.

Munujos, P., Vendrell, M., & Ferrer, I. (1993). Proto-oncogene c-fos induction in thiamine-deficient encephalopathy. Protective effects of nicardipine on pyrithiamine-induced lesions. *J Neurol Sci,* 118(2), 175-180.

Munujos, P., Coll-Canti, J., Beleta, J., Gonzalez-Sastre, F., & Gella, F.J. (1996). Brain pyruvate oxidation in experimental thiamin-deficiency encephalopathy. *Clin Chim Acta,* 255(1), 13-25.

Nance, W.E., Claflin, A., & Smithies, O. (1963). Lactic dehydrogenase: genetic control in man. *Science,* 142, 1075-1077.

Pannunzio, P., Hazell, A.S., Pannunzio, M., Rao, K.V., & Butterworth, R.F. (2000). Thiamine deficiency results in metabolic acidosis and energy failure in cerebellar granule cells: an in vitro model for the study of cell death mechanisms in Wernicke's Encephalopathy. *J Neurosci Res,* 62(2), 286-292.

Park, D.H., & Gubler, C.J. (1969). Studies on the physiological functions of thiamine. V. Effects of thiamine deprivation and thiamine

antagonists on blood pyruvate and lactate levels and activity of lactate dehydrogenase and its isozymes in blood and tissues. *Biochim Biophys Acta,* 177, 537-543.

Parker, W.D. Jr, Haas, R., Stumpf, D.A., Parks, J., Eguren, L.A., & Jackson, C. (1984). Brain mitochondrial metabolism in experimental thiamine deficiency. *Neurology,* 34(11), 1477-1481.

Paxinos, G., & Watson, C. (1982). The Rat Brain in Stereotaxic Coordinates. New York : Academic Press Inc.

Pellerin, L., & Magistretti, P.J. (1994). Glutamate uptake by astrocytes stimulates aerobic glycolysis : A mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci USA,* 91, 10625-10629.

Pellerin, L., Pellegri, G., Bittar, P.G., Charnay, Y., Bouras, C., Martin, J.L., Stella, N., & Magistretti, P.J. (1998). Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. *Dev Neurosci,* 20, 291-299.

Peters, R.A. (1936). The biochemical lesion in vitamin B1 deficiency. *Lancet,* 1, 1161-1165.

Rose, S.E., Nixon, P.F., Zelaya, F.O., Wholohan, B.T., Zimitat, C., Moxon, L.N., Crozier, S., Brereton, I.M., Doddrell, D.M. (1993). Application of high field localised in vivo ^{1}H MRS to study the biochemical changes in the thiamine deficient rat brain under glucose load. *NMR Biomed,* 6(5), 324-328.

Shi, S.R., Cote, R.J., Young, L., Imam, S.A., & Taylor, C.R. (1996). Use of pH 9.5 Tris-HCl buffer containing 5% urea for antigen retrieval immunohistochemistry. *Biotech Histochem,* 71, 190-196.

Sonnewald, U., Westergaard, N., Petersen, S.B., Unsgard, G., & Schousboe, A. (1993). Metabolism of [U-^{13}C]glutamate in astrocytes studied by ^{13}C-NMR spectroscopy: incorporation of more label into lactate than into glutamine demonstrates the importance of the tricarboxylic acid cycle. *J Neurochem,* 61, 1179-1182.

Subhash, M.N., Rao, B.S.S., & Shankar, S.K. (1993). Changes in lactate dehydrogenase isoenzyme pattern in patients with tumors of the central nervous sytem. *Neurochem Int,* 22, 121-124.

Troncoso, J.C., Johnston, N.V., Hess, K.M., Griffin, J.W., & Price, D.L. (1981). Model of Wernicke's Encephalopathy. *Archives of Neurology,* 38(6), 350-354.

Wallis, W.E., Willoughby, E., & Baker, P. (1978). Coma in the Wernicke-Korsakoff syndrome. *Lancet,* 2, 400-401.

Watson, A.J., Walker, J.F., Tomkin, G.H., Finn, M.M., & Keogh, J.A. (1981). Acute Wernicke's Encephalopathy precipitated by glucose loading. *Ir J Med Sci,* 150, 301-303.

Zimitat, C., & Nixon, P.F. (1999). Glucose loading precipitates acute encephalopathy in thiamin-deficient rats. *Metab Brain Dis,* 14, 1-20.

Zwingmann, C., Leibfritz, D., & Hazell, A.S. (2003). Energy metabolism in astrocytes and neurons treated with manganese: Relation among cell-specific energy failure, glucose metabolism, and intercellular trafficking using multinuclear NMR-spectroscopic analysis. *J Cereb Blood Flow Metab,* 23, 756-771.

LEGENDS

Figure 1: Metabolic fate of ^{13}C-label from [1-^{13}C]glucose

Label distribution in glycolytic and TCA cycle intermediates following metabolism of [1-^{13}C]glucose. The fate of the ^{13}C-label from [1-^{13}C]glucose is indicated by filled circles: ^{13}C-label position in pyruvate and lactate synthesized via lactate dehydrogenase (LDH) without prior entry of the label into the TCA cycle; ^{13}C-label position due to pyruvate (and lactate) generated from labeled TCA cycle intermediates via pyruvate recycling. Open circles indicate unlabeled carbons. PDH: pyruvate dehydrogenase; PC: pyruvate carboxylase; ME: malic enzyme; PEPCK: phosphoenolpyruvate carboxykinase; PK: pyruvate kinase.

Figure 2: Lactate portion of ^{1}H-NMR spectra of extracts from medial thalamus

Segments of ^{1}H-NMR spectra of extracts obtained from medial thalamus of PFC rats and TD rats at symptomatic stage administered either a labeling dose (200mg/kg) or a [1-^{13}C]glucose load (500mg/kg). In these expanded spectra, the methyl resonance of unlabeled lactate ([^{1}H-^{12}C]) can be distinguished from the ^{13}C-labelled isotopomer ([^{1}H-^{13}C]) by the additional doublet splitting arising from ^{1}H-^{13}C-coupling (J = 128 Hz). This allows for the calculation of the percentage ^{13}C-enrichment in lactate.

Figure 3: Effect of thiamine deficiency on brain lactate synthesis

The amounts of *a)* unlabeled (^{12}C) lactate, of *b)* ^{13}C-labeled lactate (labeled 60 min after administration of [1-^{13}C]glucose (200 or 500 mg/kg)), and *c)* the fractional ^{13}C-enrichments in lactate were calculated from ^{13}C-NMR

spectra of extracts from frontal cortex and medial thalamus of pair-fed control (white bars) rats, rats at presymptomatic (grey bars) and symptomatic (black bars) stages of thiamine deficiency. Values represent means ± SD for n = 5 (no glucose load) or n = 4 (glucose load). Data was analyzed using ANOVA and post-hoc Tukey's test († significantly different from controls; ‡ significantly different at symptomatic versus presymptomatic stage; § significantly different change after administration of 500 mg/kg versus 200 mg/kg glucose; *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$).

Figure 4: Effect of thiamine deficiency on pyruvate recycling

Percentage ^{13}C-enrichment of [2-^{13}C]lactate (labeled 60 min after administration of [1-^{13}C]glucose (200 or 500 mg/kg)) calculated from the ^{13}C-NMR spectra of frontal cortex and medial thalamus of pair-fed control rats (white bars) and rats at presymptomatic (grey bars) and symptomatic (black bars) stages. Values represent means ± SD for n = 5 (no glucose load) or n = 4 (glucose load). Data was analyzed using ANOVA and post-hoc Tukey's test († significantly different from controls; ‡ significantly different at symptomatic versus presymptomatic stage; § significantly different change after administration of 500 mg/kg versus 200 mg/kg glucose; *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$).

Figure 5: LDH1/LDH5 and GFAP Immunohistochemistry

Paraffin-embedded sections of rat MT were probed with respective antibodies (see Materials and Methods section). Coronal sections from TD animals were taken at a presymptomatic time-point (Day 11). LDH1 immunolabeling can be seen in neurons (arrowheads) and astrocytes

(arrows). LDH5 was preferentially located in astrocytic cell bodies, showing clear increases in MT sections from TD rats. Similar levels of staining are present for the astrocytic-marker GFAP in control and TD sections. Negative control sections were incubated with normal sera instead of primary antibody; absence of immunostaining was noted in all cases (data not shown). Images are magnified at 200X.

Figure 1 (Navarro et al., 2005)

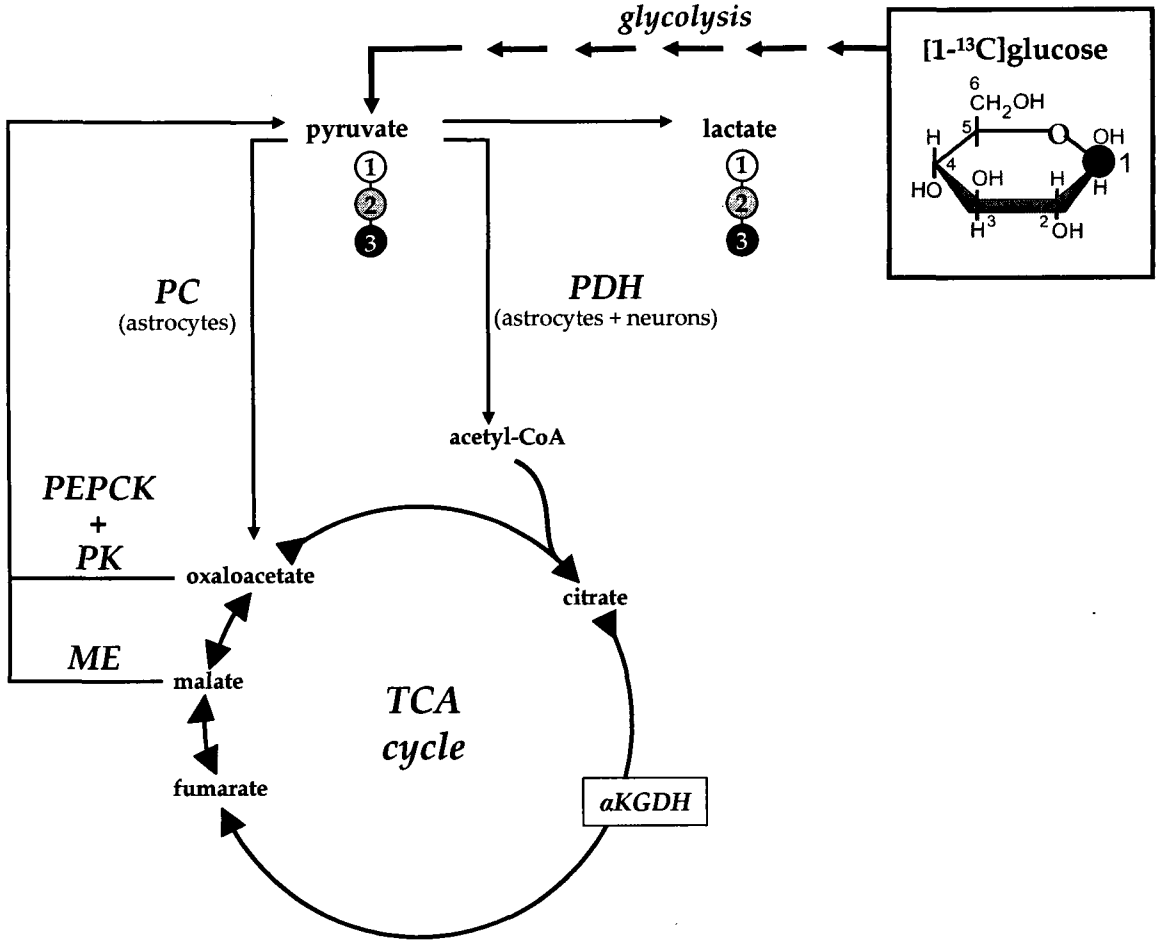


Figure 2 (Navarro et al., 2005)

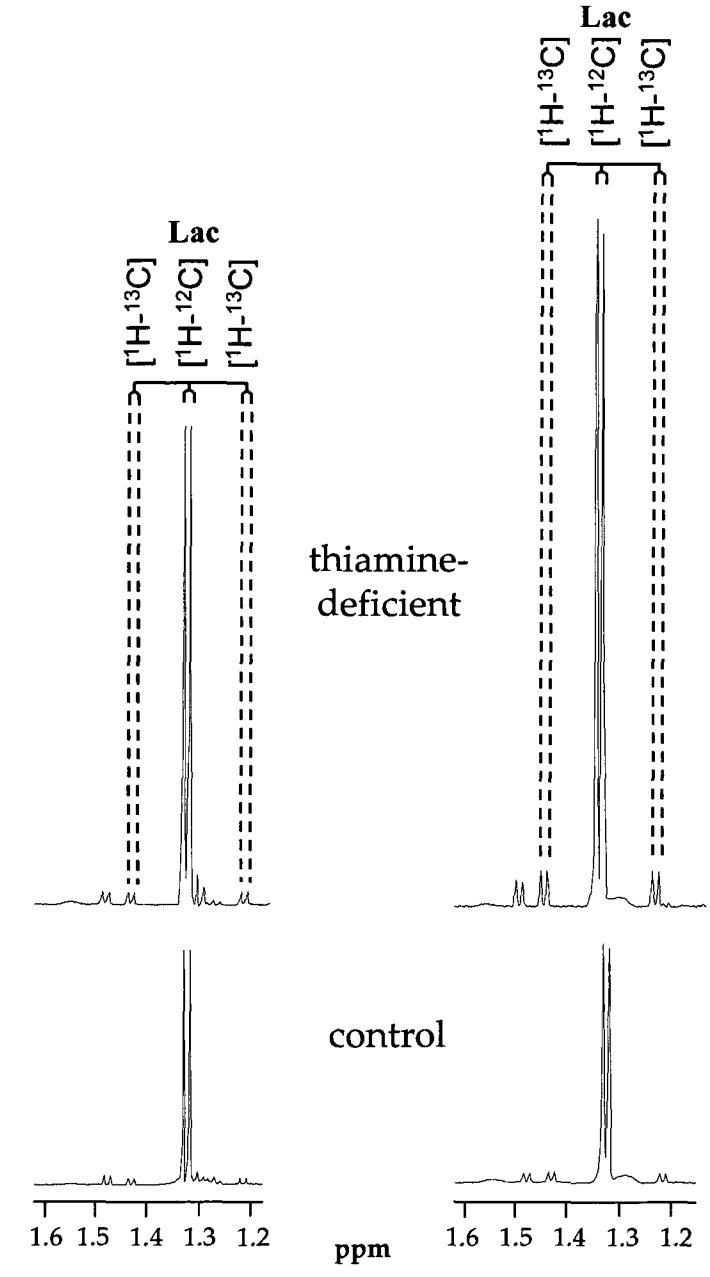


Figure 3 (Navarro et al., 2005)

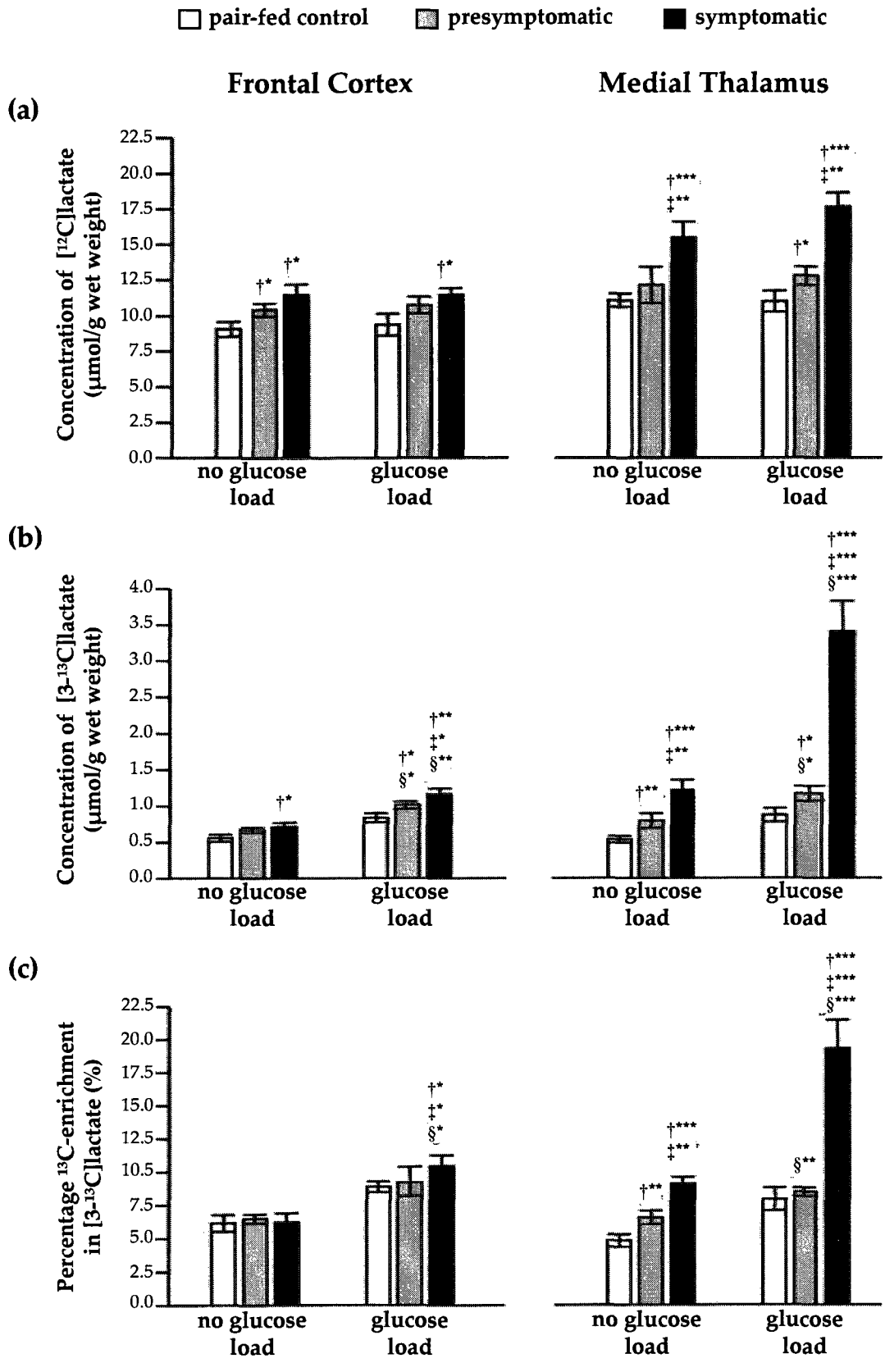


Figure 4 (Navarro et al., 2005)

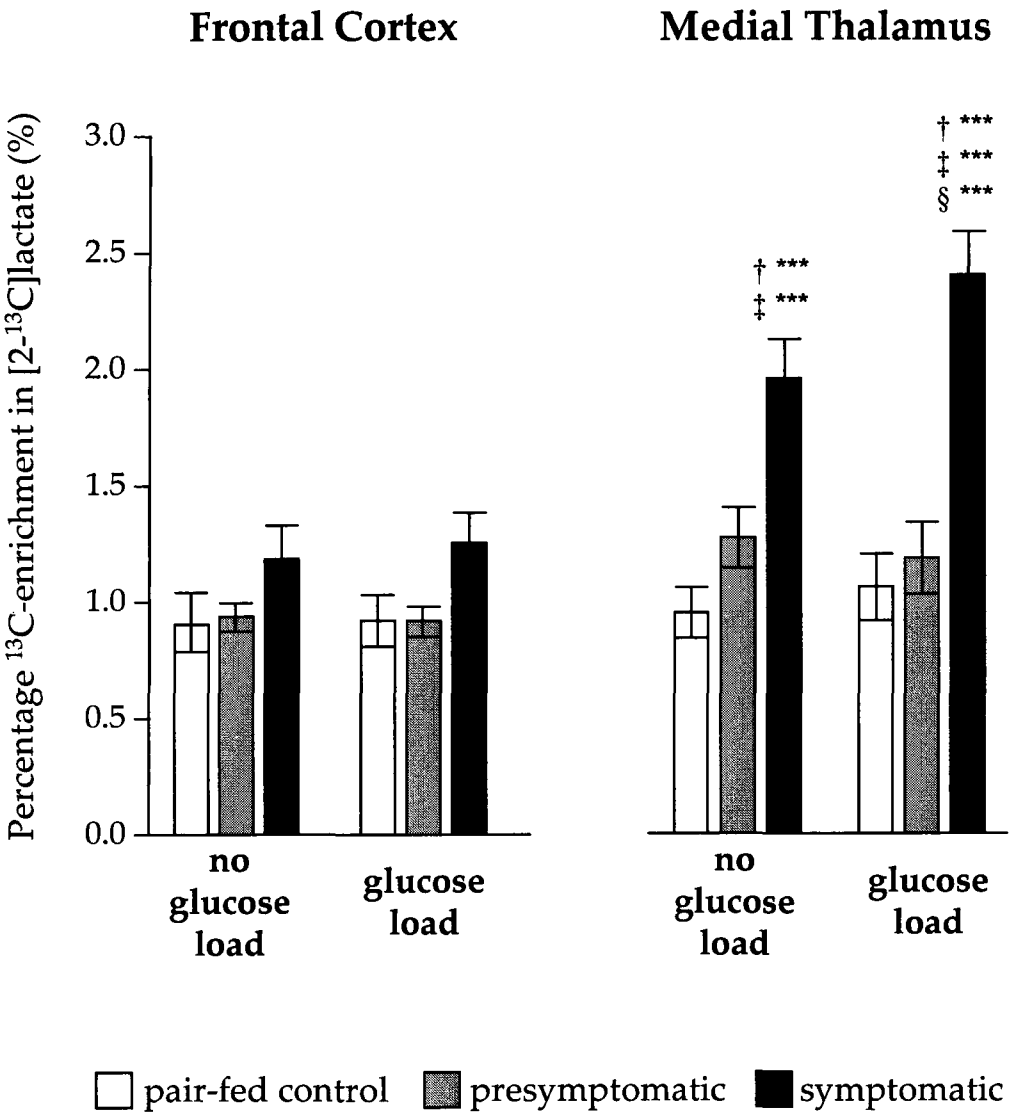
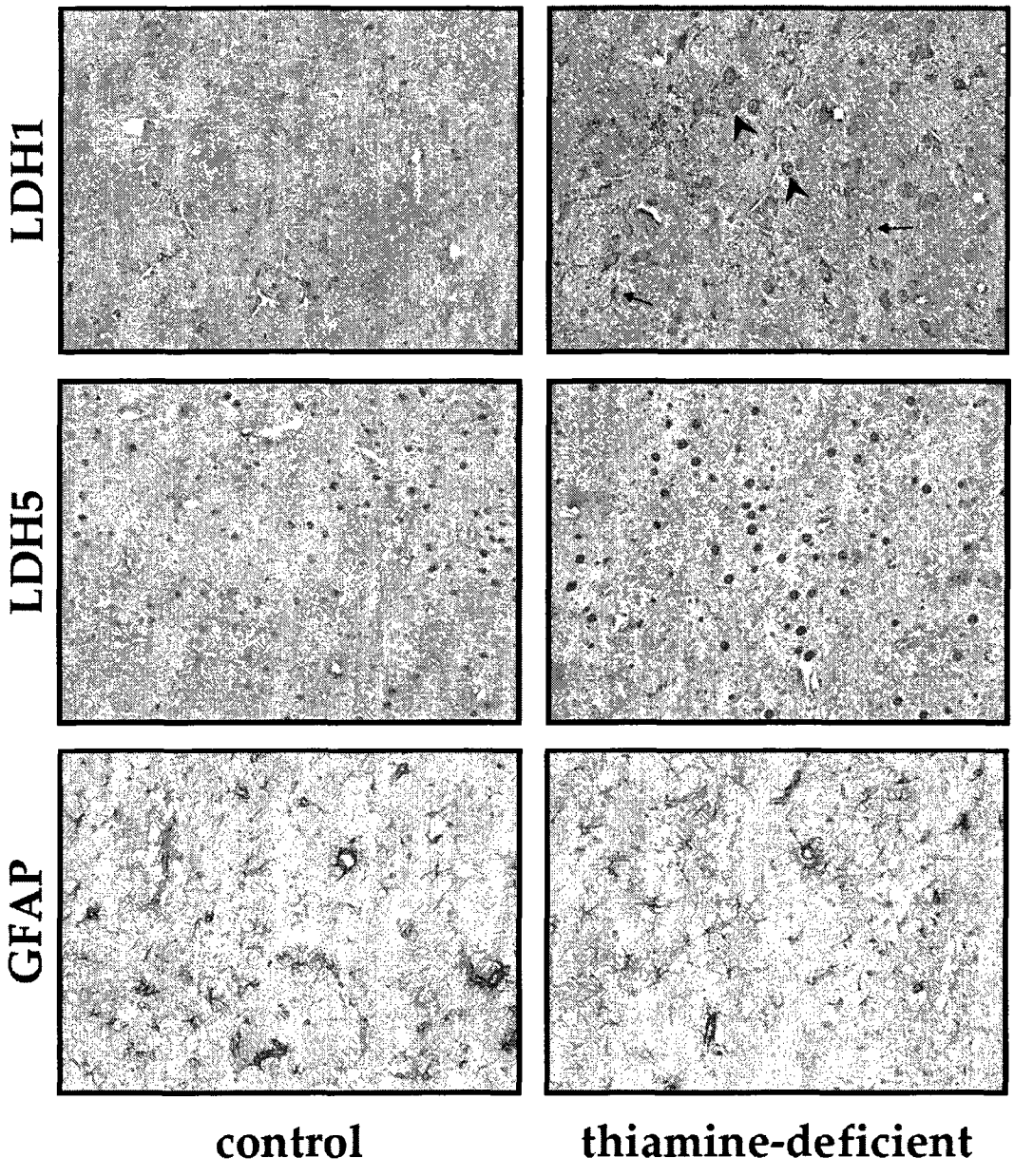


Figure 5 (Navarro et al., 2005)



ARTICLE 2

The results of Article 1 clearly demonstrate that increased lactate *de novo* synthesis predicts regional vulnerability in the TD rat brain, while the increased accumulation of brain lactate parallels the worsening of neurological status and the appearance of neuronal cell loss in vulnerable brain structures. Furthermore, glucose loading of TD rats causes even further augmentations of brain lactate production and accumulation in vulnerable brain regions. The following article addresses whether or not this increased lactate production and accumulation in brain precipitates lactic acidosis, which may be involved in the worsening of neurological symptoms in patients with Wernicke's Encephalopathy.

GLUCOSE LOADING PRECIPITATES FOCAL LACTIC ACIDOSIS IN THE VULNERABLE MEDIAL THALAMUS OF THIAMINE-DEFICIENT RATS

Darren Navarro, Claudia Zwingmann, Nicolas Chatauret,
Roger F. Butterworth

Neuroscience Research Unit, Hôpital Saint-Luc,
Montreal, Quebec, Canada.

Running Title: Glucose loading precipitates focal acidosis in thiamine-deficient rat brain

Keywords: Thiamine deficiency, Lactate, Glucose loading, Cerebral acidosis, Wernicke's Encephalopathy

Address for reprints and correspondence:
Roger. F. Butterworth, Ph.D, D.Sc.
Neuroscience Research Unit
CHUM (Campus Saint-Luc)
1058 Saint-Denis Street

Montreal, Quebec, Canada H2X 3J4

Phone: (514) 890-8310 ext. 35759

FAX: (514) 412-7314

Email: roger.butterworth@umontreal.ca

ABSTRACT

Glucose loading in thiamine-deficient patients is known to precipitate Wernicke's Encephalopathy; however, the mechanisms responsible have not been fully elucidated. Lactate accumulation occurs in brains of thiamine-deficient rats. In order to determine whether glucose loading in thiamine-deficient rats causes selective lactic acidosis in vulnerable brain structures, cerebral pH was measured autoradiographically using ^{14}C-labeled 5,5-dimethyloxazolidine-2, 4-dione ([^{14}C]DMO) in the medial thalamus, a vulnerable brain region, versus cerebral cortex, a brain region that is spared in thiamine deficiency. Following administration of a glucose load, regional lactate levels and *de novo* synthesis measured by ^{1}H-^{13}C-NMR spectroscopy, increased significantly to 21.86 ± 3.04 μmol/g (wet weight) in the medial thalamus ($p<0.001$) and pH in this brain region was decreased significantly from 7.08 ± 0.04 to 6.87 ± 0.05 ($p<0.001$). No such changes were observed in cerebral cortex following a glucose load. These results demonstrate that the increased production and accumulation of brain lactate result in acidosis following glucose loading in thiamine deficiency. Alterations of brain pH could contribute to the pathogenesis of thalamic neuronal damage and consequent cerebral dysfunction in Wernicke's Encephalopathy.

INTRODUCTION

Thiamine deficiency continues to be a problem in some world communities leading to infantile beriberi (Luxemburger et al., 2003; McGready et al., 2001); and Wernicke's Encephalopathy (WE) is a serious complication of chronic alcoholism and other disorders associated with grossly impaired nutritional status (Butterworth, 1989; Butterworth et al., 1991). The precise cause of the selective lesions in WE is still unknown.

Lactate accumulation and its increased *de novo* synthesis have been shown to occur in brains of rats made thiamine-deficient (TD) by the thiamine antagonist, pyrithiamine (Butterworth & Heroux, 1989; McCandless, 1982; Navarro *et al.*, 2005). However, the mechanisms by which lactate may bring about neuronal injury are not fully understood. Although it is well established that glucose loading of TD patients precipitates Wernicke's Encephalopathy (Wallis et al., 1978; Watson et al., 1981), the mechanisms implicated have not been established. Lactic acidosis is one such mechanism.

The present study was undertaken to determine the extent to which glucose loading leads to brain lactate accumulation and using ^{13}C-NMR spectroscopy, its *de novo* synthesis within the TD medial thalamus (a brain region vulnerable to thiamine deficiency), and whether this increase of lactate results in acidosis. Local cerebral pH (LCpH) was determined using an autoradiographic technique and carbon 14-labelled 5,5-dimethyloxazolidine-2, 4-dione ([^{14}C]DMO) as a pH marker.

MATERIALS AND METHODS

Thiamine Deficiency Protocol

Adult male Sprague Dawley rats weighing 200-225g obtained from Charles River (St. Constant, Quebec, Canada) were used in all experiments. Rats were housed individually under constant conditions of temperature, humidity and 12 hour light/dark cycles and had free access to water at all times. Rats were allowed to adapt to their environment for 3 days prior to the initiation of TD protocol, which consisted of feeding a diet deficient in thiamine (MP Biochemicals, Solon, OH, USA) and daily administration of pyrithiamine hydrobromide (0.5 mg per kg body wt) intraperitoneally (i.p.) (Troncoso et al., 1981) until onset of anorexia and ataxia (12 days), but prior to appearance of opisthotonus. This time-point is not associated with significant neuronal cell loss in this model of thiamine deficiency (K. Todd & Butterworth, 1999). All animal treatment procedures were approved by the Animal Ethics Committee of Saint-Luc Hospital (CHUM) and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

Sample Preparation for Nuclear Magnetic Resonance (NMR) Spectroscopic Studies

For NMR investigations, [1-^{13}C]glucose (Cambridge Isotopes, Andover, MA, USA) was administered either as a labeling dose (200 mg/kg, i.p.) or a glucose load (500 mg/kg, i.p.) 60 min prior to sacrifice. Rats were killed by decapitation, brains were removed from the skull, immediately frozen in isopentane over dry ice, and dissected on ice to

obtain medial thalamus (MT, vulnerable brain region) and the frontal cortex (FC, non-vulnerable brain region). Tissue samples were ground over liquid nitrogen and homogenized in 12% perchloric acid (PCA) at 0°C using a motor-driven polished glass tube-Teflon homogenizer. The homogenate was centrifuged at 4000 g for 15 min and this was repeated once. The supernatants were combined and neutralized on ice with KOH. The precipitated $KClO_4$ was removed by centrifugation (4000 g, 15 min). Arterial blood was immediately mixed with 20% PCA, centrifuged (4000 g, 40 min), neutralized with KOH, and centrifuged again (4000 g, 15 min) to precipitate $KClO_4$. The supernatants from each sample were lyophilized.

NMR Spectroscopy

The lyophilized PCA extracts of the blood and the brain tissue were dissolved in 0.6 mL deuterium oxide (D_2O; Merck, Darmstadt, Germany) and centrifuged. Prior to NMR analysis, the pH was adjusted to 7.0 with DCl and NaOD. ^1H- and ^{13}C-NMR spectra were recorded on Bruker DRX 600 or AVANCE NB/WB 360 spectrometers, operating at frequencies of 600 MHz or 360 MHz for ^1H-measurements, and 150.9 MHz or 90.5 MHz for ^{13}C-measurements.

^1H-NMR spectra were recorded with a 5-mm H,C,N inverse triple resonance probe (5-mm HX probe), 400 accumulations, repetition time 15 s, spectral width 7183 Hz (DRX 600) or 3623 Hz (NB/WB 360). Chemical shifts were referenced to lactate at 1.33 ppm. ^{13}C-NMR spectra were recorded with a 5-mm $^1H/^{13}C$ dual probe, 20,000 accumulations, repetition time 2 s, composite pulse decoupling with WALTZ-16, spectral width

47,619 Hz (DRX 600) or 20,833 Hz (NB/WB 360). Chemical shifts were referenced to the C-3 signal of lactate at 21.3 ppm.

Measurement of Regional Brain Lactate Concentrations

Lactate concentrations were determined enzymatically (adapted for measurement in 96-well microtiter ELISA plates) using a commercially available kit (Roche Molecular Biochemicals, Mannheim, Germany). Concentrations of unlabeled lactate and its pool size ($[^{13}C]$ + $[^{12}C]$) were determined from fully relaxed ^{1}H-NMR spectra of brain extracts, obtained after injection of [1-^{13}C]glucose, using known lactate concentrations as internal standards.

Determination of Local Cerebral pH (LCpH)

The methods used to determine local cerebral pH are based on previous studies using [^{14}C]DMO (Hakim, 1984; Kobatake et al., 1984), with minor modifications. After 12 days of thiamine deficiency, rats were anesthetized with isofluorane and the femoral artery and jugular vein cannulated, after which they were allowed to recover for at least 4 hours. Once the serum pH was stable and within the range established for normal rats, 60μCi of [^{14}C]DMO (specific activity, 55 mCi/mmol American Radiolabeled Chemicals, Inc., St. Louis, USA) dissolved in saline solution at a concentration of 100μCi/ml was injected into the jugular vein. In order to examine the effects of glucose loading on acidosis, animals were administered either a glucose load (500 mg/kg in saline, i.p.) or a saline (i.p.) 45 min before the [^{14}C]DMO injection (2 hours before decapitation). This 2 hour timeline for the glucose/vehicle injection was employed in order to allow ample time for acidosis to occur while not interfering with

the administration and of [^{14}C]DMO. Arterial samples were taken at 30, 45, 60 and 75 minutes after [^{14}C]DMO injection for determination of plasma [^{14}C]DMO radioactivity and at 30 and 60 min for determination of plasma pH.

Seventy-five minutes following [^{14}C]DMO injection, animals were sacrificed, the brain rapidly removed, frozen in isopentane on dry ice and stored at –80 °C. Brain sections, 20 μm thick, were then cut in a microtome cryostat (International Equipment Company, USA) at –22°C. Sections were collected on gelatin-coated slides and dried at room temperature within 60 min of slicing since [^{14}C]DMO is somewhat unstable at room temperature and could be lost from tissue slices exposed for long periods to room temperature. Sections were then exposed to x-ray film (Hyperfilm MP, Amersham Pharmacia Biotech, Buckinghamshire, UK) for 8 days at 4°C. Local tissue concentrations of ^{14}C were determined from the optical densities of the medial thalamus, and autoradiographic [^{14}C] micro-scales (Amersham Biosciences, UK) were included in every audioradiograph. Films were developed and tissue concentrations of [^{14}C]DMO were determined by quantitative densitometry analysis using an MCID computer-based densitometer and image-analysis system (Imaging Research Inc., St. Catharines, Canada). The amount of ligand bound to various regions was calculated from the specific activity of the ligand.

Calculation of LCpH

Autoradiographic studies in which [^{14}C]DMO concentrations are used to determine regional pH consider the brain to be composed of two functional compartments, one plasma and the other tissue. The LCpH was calculated from the following formulation:

$$\frac{[DMO^-]_t}{[DMO^-]_p} = \frac{10^{pH_t}-6.13+1}{10^{pH_p}-6.13+1}$$

Comprehensive derivations of this equation are well-documented in previous studies using [^{14}C]DMO as a pH marker in brain (Hakim, 1984; Kobatake et al., 1984). This equation was used to calculate the regional pH in this study where pH_p and pH_t are plasma and tissue pH respectively, and [DMO^-] is the ionized species concentration in plasma or tissue. The pK of DMO is 6.13. From the above equation, the concentration of DMO in brain is proportional to the pH of the tissue. In all animals the water contents of plasma and brain gray matter and white matter were assumed to be 93, 81 and 69% respectively (Katzman & H.M., 1973). $[DMO]_t$ was obtained from the densitometric readings; pH_p was the average of arterial pHs determined at 30 and 60 min following [^{14}C]DMO injection; and $[DMO]_p$ was the average of the plasma concentration in the last 45 minutes of the experiment (30, 45, 60, 75 min). This average was used to avoid reliance on one determination and was made feasible by equilibrium studies showing that after the initial 30 minutes, [^{14}C]DMO concentrations in plasma declined by only 4% per hour (Kobatake et al., 1984). The calculated LCpH values (pH_t) are a composite of intracellular and extracellular pH, but are weighted more heavily by pH in the intracellular space due to its larger size.

Statistical Methods

Studies were carried out on four rats per group. Data are expressed as mean ± SD values. Regional lactate concentrations and the LCpH values were compared using one-way analysis of variance (ANOVA) to detect overall variation. Differences were considered significant when $p < 0.05$.

RESULTS

Regional Brain Lactate Concentrations

[1-^{13}C]glucose is converted to [3-^{13}C]pyruvate via the glycolytic pathway, and subsequently to [3-^{13}C]lactate via lactate dehydrogenase. ^{1}H-^{13}C-NMR spectroscopy, (the heteronuclear spin pattern of lactate in the high-field region of ^{1}H-NMR spectra), allows for distinction between lactate synthesized after glycolytic transformation of [1-^{13}C]glucose ([3-^{13}C]lactate) which is evident in the spectra as an additional doublet splitting due to ^{1}H-^{13}C-coupling (J = 128 Hz), and the unlabeled isotopomer ([^{12}C]lactate) synthesized from unlabeled pyruvate. Table 1 shows the total concentrations of lactate ([^{12}C] + [^{13}C]) calculated from the high-field region of ^{1}H-NMR spectra in both cerebral cortex and medial thalamus, with or without a glucose load. Following administration of a glucose load, the concentration of lactate increased significantly from 17.80 ± 2.07 to 21.86 ± 2.26 μmol/g (wet weight) in the vulnerable medial thalamus ($p<0.05$), while levels remained relatively unchanged in the non-vulnerable cerebral cortex.

Autoradiographic Determination of LCpH in TD Rat Brain

Immediately prior to administration of [^{14}C]DMO to TD rats arterial pH was 7.37 ± 0.08, and rectal temperature was 35.6 ± 0.5 ºC. These parameters were not significantly different in TD rats administered a glucose load.

Figure 1 shows representative autoradiographic sections at the level of the medial thalamus made 75 min after [^{14}C]DMO administration in TD rats with or without a glucose load. Acidosis, evidenced by hypodensity, can be clearly seen in the medial thalamus of the glucose-loaded TD rat.

Table 1 also shows the calculated values for LCpH in the cerebral cortex versus the medial thalamus, in the presence or absence of a glucose load. LCpH (pH_i values) were decreased significantly from 7.05 ± 0.04 to 6.87 ± 0.05 ($p<0.001$) in the medial thalamus following a glucose load, while no significant differences were observed in the cerebral cortex.

DISCUSSION

Lactate accumulation in brains of thiamine-deficient animals has been well-documented since the pioneering experiments of Peters in the early 1930s. More recently, the selective accumulation of lactate has been shown to occur within cerebral structures which are susceptible to histological damage with continued thiamine deficiency. This pattern of vulnerability closely resembles that encountered in WE in humans.

Thiamine deficiency does not result in early compromise of brain activities of the thiamine-dependent enzyme PDH (Butterworth et al., 1985), but activity of a second thiamine-dependent dehydrogenase, α-KGDH, on the other hand has been found to be substantially decreased, leading to impairments in TCA cycle flux (Butterworth et al., 1986; Gibson et al., 1984). Impaired pyruvate oxidation resulting from decreased entry of pyruvate into the TCA cycle is accompanied by increased *de novo* synthesis of lactate, which has been found to be elevated in circulation and brain tissue of both experimental animals and patients with WE (Holowach et al., 1968; McCandless, 1982; McCandless et al., 1968; Munujos et al., 1996).

Cerebral acidosis has been shown to occur in the medial thalamus of TD rats following the onset of opisthotonus (Hakim, 1984). Medial thalamus is known ultimately to manifest neuronal cell loss in thiamine deficiency. Results of the present study reveal that the concentration of lactate in the TD medial thalamus prior to administration of a glucose load is already elevated relative to that of the TD cerebral cortex, however this produces no observable differences in tissue pH between the two brain regions. Thalamic pH values in the present study are comparable to those

previously published using this and other techniques (Hakim, 1984; Kobatake et al., 1984; Kogure et al., 1980; Roos, 1971).

Administration of a glucose load has been shown to precipitate WE in TD patients exhibiting no prior neurological symptoms (Wallis et al., 1978; Watson et al., 1981); however, the mechanisms involved in this phenomenon remain unclear. In the present study, brain lactate concentrations increased to 21.86 ± 2.26 μmol/g (wet weight) in the TD medial thalamus following the administration of a glucose load. Such a concentration has the potential to adversely affect cellular function. For example, in periods of hypoxia and ischemia, recovery varies with the nutritional state of the animals, and results of previous studies suggest that tissue lactate concentrations exceeding 20 μmol/g (wet weight) are detrimental for recovery (Myers, 1979; Rehncrona et al., 1980, 1981). This focal accumulation of lactate was accompanied by a significant decrease in local cerebral pH; a characteristic of later stages of thiamine deficiency (Hakim, 1984). In contrast, both lactate concentrations and tissue pH values within the non-vulnerable cerebral cortex remained unaffected following administration of a glucose load.

Previous studies reveal that an acidic environment may have detrimental effects on cellular metabolism (Rehncrona et al., 1980; Van Nimmen et al., 1986); and pH changes can affect vascular tone and may lead to deleterious hemodynamic changes (Kuschinsky et al., 1972). Acidosis is also known to enhance the production of reactive oxygen species, and oxidative stress is a feature of TD neuropathology, as evidenced by the accumulation of heme oxygenase-1, ferritin, reactive iron and superoxide dismutase in microglia, nitrotyrosine and 4-hydroxynonenal in neurons, as well as induction of endothelial nitric

oxide synthase within vulnerable brain regions such as MT (Calingasan et al., 2000; Kruse et al., 2004). Endothelial cells are believed to be targets of free radicals (Kontos, 1985, 1989); and impairment of blood-brain barrier function has been demonstrated in TD rats (Zelaya et al., 1995) and in WE patients (Schroth et al., 1991). Thus the microvasculature may be a potential primary target of acidosis-mediated damage, suggesting that a compromise in the blood-brain barrier, as observed in thiamine deficiency, may occur secondarily to focal cerebral acidosis in brain regions vulnerable to thiamine deficiency.

ACKNOWLEDGEMENTS

Research funded by the Canadian Institutes for Health Research (CIHR). We thank Professor Dieter Leibfritz, University of Bremen, for the generous availability of the NMR laboratory.

REFERENCES

Butterworth, R. F. (1989). Effects of thiamine deficiency on brain metabolism: Implications for the pathogenesis of the wernicke-korsakoff syndrome. *Alcohol Alcohol, 24*(4), 271-279.

Butterworth, R. F., Gaudreau, C., Vincelette, J., Bourgault, A. M., Lamothe, F., & Nutini, A. M. (1991). Thiamine deficiency and Wernicke's Encephalopathy in aids. *Metab Brain Dis, 6*(4), 207-212.

Butterworth, R. F., Giguere, J. F., & Besnard, A. M. (1985). Activities of thiamine-dependent enzymes in two experimental models of thiamine-deficiency encephalopathy: 1. The pyruvate dehydrogenase complex. *Neurochem Res, 10*(10), 1417-1428.

Butterworth, R. F., Giguere, J. F., & Besnard, A. M. (1986). Activities of thiamine-dependent enzymes in two experimental models of thiamine-deficiency encephalopathy. 2. Alpha-ketoglutarate dehydrogenase. *Neurochem Res, 11*(4), 567-577.

Butterworth, R. F., & Héroux, M. (1989). Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acids and thiamine-dependent enzymes. *J Neurochem, 52*(4), 1079-1084.

Calingasan, N. Y., Huang, P. L., Chun, H. S., Fabian, A., & Gibson, G. E. (2000). Vascular factors are critical in selective neuronal loss in an animal model of impaired oxidative metabolism. *J Neuropathol Exp Neurol, 59*(3), 207-217.

Gibson, G. E., Ksiezak-Reding, H., Sheu, K. F., Mykytyn, V., & Blass, J. P. (1984). Correlation of enzymatic, metabolic, and behavioral deficits in thiamin deficiency and its reversal. *Neurochem Res, 9*(6), 803-814.

Hakim, A. M. (1984). The induction and reversibility of cerebral acidosis in thiamine deficiency. *Ann Neurol, 16*(6), 673-679.

Holowach, J., Kauffman, F., Ikossi, M. G., Thomas, C., & McDougal, D. B., Jr. (1968). The effects of a thiamine antagonist, pyrithiamine, on levels of selected metabolic intermediates and on activities of thiamine-dependent enzymes in brain and liver. *J Neurochem, 15*(7), 621-631.

Katzman, R., & H.M., P. (1973). *Brain electrolytes and fluid metabolism.* Baltimore: Williams & Wilkins.

Kobatake, K., Sako, K., Izawa, M., Yamamoto, Y. L., & Hakim, A. M. (1984). Autoradiographic determination of brain ph following middle cerebral artery occlusion in the rat. *Stroke, 15*(3), 540-547.

Kogure, K., Alonso, O. F., & Martinez, E. (1980). A topographic measurement of brain pH. *Brain Res, 195*(1), 95-109.

Kontos, H. A. (1985). George E. Brown Memorial Lecture. Oxygen radicals in cerebral vascular injury. *Circ Res, 57*(4), 508-516.

Kontos, H. A. (1989). Oxygen radicals in CNS damage. *Chem Biol Interact, 72*(3), 229-255.

Kruse, M., Navarro, D., Desjardins, P., & Butterworth, R. F. (2004). Increased brain endothelial nitric oxide synthase expression in thiamine deficiency: Relationship to selective vulnerability. *Neurochem Int, 45*(1), 49-56.

Kuschinsky, W., Wahl, M., Bosse, O., & Thurau, K. (1972). Perivascular potassium and ph as determinants of local pial arterial diameter in cats. A microapplication study. *Circ Res, 31*(2), 240-247.

Luxemburger, C., White, N. J., ter Kuile, F., Singh, H. M., Allier-Frachon, I., Ohn, M., et al. (2003). Beri-beri: The major cause of infant mortality in karen refugees. *Trans R Soc Trop Med Hyg, 97*(2), 251-255.

McCandless, D. W. (1982). Energy metabolism in the lateral vestibular nucleus in pyrithiamin-induced thiamin deficiency. *Ann N Y Acad Sci, 378*, 355-364.

McCandless, D. W., Schenker, S., & Cook, M. (1968). Encephalopathy of thiamine deficieny: Studies of intracerebral mechanisms. *J Clin Invest, 47*(10), 2268-2280.

McGready, R., Simpson, J. A., Cho, T., Dubowitz, L., Changbumrung, S., Bohm, V., et al. (2001). Postpartum thiamine deficiency in a Karen displaced population. *Am J Clin Nutr, 74*(6), 808-813.

Munujos, P., Coll-Canti, J., Beleta, J., Gonzalez-Sastre, F., & Gella, F. J. (1996). Brain pyruvate oxidation in experimental thiamin-deficiency encephalopathy. *Clin Chim Acta, 255*(1), 13-25.

Myers, R. E. (1979). A unitary theory of causation of anoxic and hypoxic brain pathology. *Adv Neurol, 26*, 195-213.

Navarro, D., Zwingmann, C., Hazell, A. S., & Butterworth, R. F. (2005). Brain lactate synthesis in thiamine deficiency: A re-evaluation using 1h-13c nuclear magnetic resonance spectroscopy. *J Neurosci Res, 79*(1-2), 33-41.

Rehncrona, S., Rosen, I., & Siesjö, B. K. (1980). Excessive cellular acidosis: An important mechanism of neuronal damage in the brain? *Acta Physiol Scand, 110*(4), 435-437.

Rehncrona, S., Rosen, I., & Siesjö, B. K. (1981). Brain lactic acidosis and ischemic cell damage: 1. Biochemistry and neurophysiology. *J Cereb Blood Flow Metab, 1*(3), 297-311.

Roos, A. (1971). Intracellular pH and buffering power of rat brain. *Am J Physiol, 221*(1), 176-181.

Schroth, G., Wichmann, W., & Valavanis, A. (1991). Blood-brain-barrier disruption in acute Wernicke Encephalopathy: Mr findings. *J Comput Assist Tomogr, 15*(6), 1059-1061.

Todd, K., & Butterworth, R. F. (1999). Mechanisms of selective neuronal cell death due to thiamine deficiency. *Ann N Y Acad Sci, 893*, 404-411.

Troncoso, J. C., Johnston, M. V., Hess, K. M., Griffin, J. W., & Price, D. L. (1981). Model of Wernicke's Encephalopathy. *Arch Neurol, 38*(6), 350-354.

Van Nimmen, D., Weyne, J., Demeester, G., & Leusen, I. (1986). Local cerebral glucose utilization during intracerebral pH changes. *J Cereb Blood Flow Metab, 6*(5), 584-589.

Wallis, W. E., Willoughby, E., & Baker, P. (1978). Coma in the Wernicke-Korsakoff syndrome. *Lancet, 2*(8086), 400-401.

Watson, A. J., Walker, J. F., Tomkin, G. H., Finn, M. M., & Keogh, J. A. (1981). Acute Wernickes Encephalopathy precipitated by glucose loading. *Ir J Med Sci, 150*(10), 301-303.

Zelaya, F. O., Rose, S. E., Nixon, P. F., Wholohan, B. T., Bower, A. J., Zimitat, C., et al. (1995). MRI demonstration of impairment of the

blood-CSF barrier by glucose administration to the thiamin-deficient rat brain. *Magn Reson Imaging, 13*(4), 555-561.

LEGENDS

Table 1: Effect of glucose loading on regional brain lactate concentrations and local cerebral pH in TD rats

Concentrations of lactate determined by ^{1}H-NMR spectroscopy of brain extracts obtained after administration of either 200 mg/kg (labeling dose) or 500 mg/kg (glucose load) [1-^{13}C]glucose. pH values were measured autoradiographically using [^{14}C]DMO administered 75 min prior to sacrifice. Glucose load (or vehicle) was administered 2 h before sacrifice. Values represent means ± SD for n = 4 animals per group. Data between all experimental groups were analyzed using ANOVA, where * $p < 0.05$, ** $p < 0.001$.

Figure 1: Acidosis due to glucose loading in TD rat brain

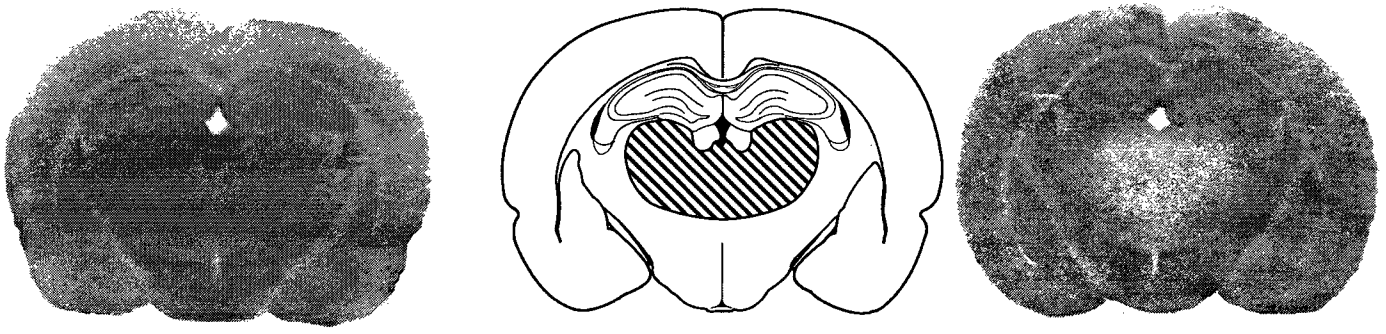
Representative coronal sections of medial thalamus from TD rats administered [^{14}C]DMO following 11 days of thiamine deficiency, with or without administration of a glucose load (500 mg/kg). Note the appearance of thalamic hypodensity, implying acidosis following a glucose load.

Table 1: Effect of glucose loading on regional brain lactate concentrations and local cerebral pH in TD rats

Regional Lactate Concentration (μmol/g)		
	No glucose load	Glucose load
Cerebral Cortex	11.92 ± 1.04	12.79 ± 1.50
Medial Thalamus	17.80 ± 2.07	21.86 ± 2.26 *

Local Cerebral pH		
	No glucose load	Glucose load
Cerebral Cortex	7.09 ± 0.04	7.10 ± 0.05
Medial Thalamus	7.05 ± 0.04	6.87 ± 0.05 **

Figure 1: Acidosis due to glucose loading in TD rat brain (Navarro et al., 2007)



-3.3 mm

No glucose load **Glucose load**

ARTICLE 3

Previous studies have demonstrated focal decreases in amino acid concentrations resulting from impaired pyruvate metabolism in brain regions that are known to be selectively vulnerable to thiamine deficiency. The following article provides further investigation into the effects of thiamine deficiency on the *de novo* synthesis of metabolically relevant neurotransmitter amino acids, and the cellular localization of these metabolic changes. The effects of glucose loading on metabolic flux through thiamine-dependent pathways will also be examined.

REGION-SELECTIVE ALTERATIONS OF GLUCOSE OXIDATION AND AMINO ACID SYNTHESIS IN THE THIAMINE-DEFICIENT RAT BRAIN: A RE-EVALUATION USING $^{1}H/^{13}C$ NMR SPECTROSCOPY

Darren Navarro, Claudia Zwingmann, Roger F. Butterworth

Neuroscience Research Unit, Hôpital Saint-Luc,
Montreal, Quebec, Canada.

Running Title: Brain amino acids in thiamine deficiency

Keywords: Thiamine deficiency, Glucose metabolism, Pyruvate dehydrogenase, Wernicke's Encephalopathy

Address for reprints and correspondence:
Roger. F. Butterworth, Ph.D, D.Sc.
Neuroscience Research Unit
CHUM (Campus Saint-Luc)

1058 Saint-Denis Street

Montreal, Quebec, Canada H2X 3J4

Phone: (514) 890-8310 ext. 35759

FAX: (514) 412-7314

Email: roger.butterworth@umontreal.ca

ABSTRACT

Impaired metabolism occurs in a region-selective manner in the thiamine-deficient (TD) brain; however, the cellular localization of these metabolic changes has not been fully elucidated. ^{1}H and ^{13}C-NMR were therefore used to investigate effects of thiamine deficiency on de novo synthesis of metabolically relevant neurotransmitter amino acids derived from [1-^{13}C]glucose in vulnerable (medial thalamus; MT) and non-vulnerable (frontal cortex; FC) brain regions of rats made TD by administration of the central thiamine antagonist pyrithiamine. A time-point of 11 days thiamine deficiency was chosen, in which minimal cell death had occurred in MT. Inhibition of α-ketoglutarate dehydrogenase (α-KGDH) was accompanied by decreased pyruvate oxidation and reduced de novo synthesis of glutamate, aspartate and GABA in TD brain; decreased aspartate synthesis being the earliest predictor of regional vulnerability. Analysis of isotopomer labeling-patterns in aspartate, glutamate and GABA helped to elucidate the cellular localization of metabolic changes. De novo synthesis of glutamate and GABA were unaffected at 11 days of thiamine deficiency, while synthesis of [2-^{13}C]aspartate was impaired in the MT. Detailed isotopomer analysis suggests impaired flux through α-KGDH and decreased aspartate synthesis occurred principally in neurons. Glucose loading accentuated decreases in TCA cycle flux and impairments in pyruvate dehydrogenase flux, leading to significant selective reductions in de novo synthesis of aspartate and glutamate in the MT.

INTRODUCTION

Previous biochemical studies suggest that selectively impaired activities of α-ketoglutarate dehydrogenase (α-KGDH; EC 1.2.4.2.) in brain are responsible for the early cerebral metabolic changes characteristic of thiamine deficiency which include decreases in concentrations of the amino acids aspartate, glutamate and GABA (Butterworth and Héroux, 1989). These metabolic changes are more severe in brain regions such as medial thalamus which are destined to manifest neuronal cell loss in thiamine deficiency (Butterworth et al., 1979; Hamel et al., 1979; Plaitakis et al., 1979). Pyruvate dehydrogenase (PDH; EC 1.2.4.1.) is a key mitochondrial enzyme regulating oxidative (pyruvate) metabolism and, like α-KGDH, is thiamine-dependent. However, biochemical studies reveal that, unlike α-KGDH, activities of PDH are unchanged in brain in thiamine deficiency (Gaitonde et al., 1975; Butterworth et al., 1985, 1986). Although thiamine-dependent enzymes have been studied extensively, few previous studies have examined the effects of thiamine deficiency on metabolic fluxes through PDH and α-KGDH. Furthermore the cellular localization of the metabolic changes in relation to regional vulnerability to thiamine deficiency has not been studied. The first aim of the present study was to address these issues by the application of *ex vivo* high-resolution ^{1}H- and ^{13}C-NMR spectroscopy.

It is well established that the administration of glucose precipitates neurological symptoms of Wernicke's Encephalopathy in thiamine-deficient (TD) patients (Wallis et al., 1978; Watson et al., 1981). In the TD rat, hyperglycemia worsens neurological status (Zimitat and Nixon, 1999, 2001) and leads to decreased concentrations of tricarboxylic acid (TCA) cycle-derived amino acids in the brains of symptomatic TD rats (Rose et

al., 1993). In order to study changes in flux through glucose metabolic pathways and the cellular localization of these changes, high-resolution *ex vivo* ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, following the *in vivo* administration of [1-^{13}C]glucose, was employed. This approach facilitates the measurement of *de novo* amino acid synthesis as a function of TCA cycle turnover and the relative flux of glucose via PDH compared to the pathway through the astrocyte-specific enzyme pyruvate carboxylase (PC; EC 4.1.1.1.). The effects of glucose loading on regional brain amino acid synthesis and TCA cycle flux were also studied using the *ex vivo* [^{1}H-^{13}C]NMR approach.

MATERIALS AND METHODS

Animal model

Adult male Sprague Dawley rats weighing 200-225g obtained from Charles River (St. Constant, Quebec, Canada) were used for all experiments. Rats were housed individually under constant conditions of temperature, humidity and 12 hr light/dark cycles with free access to water. Rats were allowed to adapt to their environment for 3 days prior to the initiation of treatments. Animals were assigned to either thiamine-deficient (TD) or control groups. All animal treatment procedures were approved by the Animal Ethics Committee of Saint-Luc Hospital (CHUM) and the University of Montreal.

Thiamine deficiency protocol

Rats in the TD group were fed a diet deficient in thiamine (ICN Nutritional Biochemicals, Cleveland, OH, USA) and administered daily injections of pyrithiamine hydrobromide (0.5 mg per kg body wt) intraperitoneally (i.p.). Control rats were pair-fed to equal food consumption with the TD rats (previous day) using the same TD food, but were supplemented with daily i.p. injections of thiamine (0.1 mg/kg body wt). Rats in the TD group were sacrificed by decapitation on day 11 of treatment (along with the corresponding PFC animals), a time-point characterized by the onset of ataxia and anorexia. Previous studies have shown that, at this time-point, no significant neuronal cell death had occurred in the vulnerable brain regions (Todd and Butterworth, 1999). All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of

Health [NIH publication 6-23 (revised), 1985]. Brains were promptly removed and flash frozen in isopentane on dry ice and stored at –80°C. The brain regions (described below) were dissected on ice according to the rat brain atlas of Paxinos and Watson (1986).

Sample preparation for Nuclear Magnetic Resonance (NMR) spectroscopic studies

[1-^{13}C]glucose (Cambridge Isotopes, Andover, MA, USA) was administered 60 min prior to decapitation. The rats in each group (PFC and TD) were administered 200 mg/kg [1-^{13}C]glucose (the minimal dose needed to label amino acids in the brain), while other rats from the same groups were injected with 500 mg/kg [1-^{13}C]glucose (glucose loading study).

Brains were rapidly frozen in isopentane over dry ice, and dissected on ice to provide samples of medial thalamus (MT), a brain region that is vulnerable to thiamine deficiency and frontal cortex (FC), a spared brain region. Tissue samples were immediately snap-frozen by immersion in liquid nitrogen and kept at –80 °C. Samples were ground over liquid nitrogen and homogenized in 12% PCA at 0°C using a motor-driven polished glass tube-Teflon homogenizer. Homogenates were centrifuged at 40,000 g for 15 min and this procedure was repeated once. The supernatants were combined and neutralized on ice with KOH. The precipitated $KClO_4$ was sedimented by centrifugation (40,000 g, 15 min). Arterial blood was immediately mixed with 20% perchloric acid (PCA), centrifuged (40,000 g, 40 min), neutralized with KOH, and centrifuged

again (40,000 g, 15 min) to precipitate $KClO_4$. The supernatant of each sample was lyophilized.

NMR spectroscopy

Lyophilized PCA extracts of the blood and the brain tissues were redissolved in 0.6 ml deuterium oxide (D_2O; Merck, Darmstadt, Germany) and centrifuged. Prior to NMR analysis, the pH was adjusted to 7.0 with DCl and NaOD. ^1H- and ^{13}C-NMR spectra were recorded on a Bruker DRX 600 spectrometer, operating at a frequency of 600 MHz for ^1H-, and of 150.9 MHz for ^{13}C-measurements.

^1H-NMR spectra were recorded with a 5-mm H,C,N inverse triple resonance probe (5-mm HX probe), 400 accumulations, repetition time 15 s, spectral width 7183 Hz. Chemical shifts were measured with reference to lactate at 1.33 ppm. ^{13}C-NMR spectra were recorded with a 5-mm $^1H/^{13}C$ dual probe, 20,000 accumulations, repetition time 1 s, composite pulse decoupling with WALTZ-16, spectral width 47,619 Hz. Chemical shifts were measured with reference to the C-3 signal of lactate at 21.3 ppm.

Measurement of metabolite concentrations

The concentrations (μmol/g tissue) of amino acids were determined from fully relaxed ^1H-NMR spectra of brain extracts using lactate as internal standard (lactate was determined enzymatically) or using (trimethylsilyl)propionic-2,2,3,3,d_4-acid (TSP) as external standard (Zwingmann et al., 2003).

Calculation of fractional 13C-enrichment and concentrations of 13C-labeled amino acids

In order to assess alterations in *de novo* amino acid synthesis, ^{13}C-NMR was employed to analyze changes in the percentage ^{13}C-enrichment and total amounts of ^{13}C-labeled glutamate, GABA and aspartate following (60 min) administration of [1-^{13}C]glucose. The fractional ^{13}C-enrichment of each carbon was derived from peak area ratios of the ^{13}C-labeled carbon/natural abundance carbon and using the known ^{13}C-enrichment lactate as internal standard as described in detail previously (Zwingmann et al., 2003) according to the formula:

$$E_{Met}\,(\%) = \frac{A_{Met} - A_{n.a.}\,(Met)}{A_{n.a.}\,(Met)} \times 100$$

A_{Met} represents ^{13}C carbon peak area of the amino acid, and $A_{n.a.}$ its natural abundance signal intensity. The signal intensities were corrected for 1.1% naturally abundant ^{13}C as well as for NOE (Nuclear Overhauser Enhancement) and saturation effects.

Labeling of amino acids from [1-^{13}C]glucose

[1-^{13}C]glucose metabolites are labeled in different carbon positions, depending on the relative contribution of enzymatic pathways (Figure 1). [1-^{13}C]glucose is converted to [3-^{13}C]pyruvate (via the glycolytic pathway) which may enter the TCA cycle via the anaplerotic pathway (pyruvate carboxylase, PC (or malic enzyme (ME)) or via pyruvate dehydrogenase (PDH). Via PC, glutamate is labeled at position C-2 (and at position C-3 after equilibration of oxaloacetate with fumarate). Via PDH, pyruvate enters the cycle as [2-^{13}C]acetyl-CoA, and glutamate is mono-labeled at

position C-4. During the second TCA cycle turn, glutamate is mono-labeled at positions C-2 or C-4 after condensation of ^{13}C-labeled oxaloacetate with unlabeled acetyl-CoA, but double-labeled ([3,4-^{13}C]- and [2,4-^{13}C]) after condensation with [2-^{13}C]acetyl-CoA. The order of labeled carbons is reversed in GABA (i.e. the isotopomer [2(4)-^{13}C]glutamate ends up as [4(2)-^{13}C]GABA). In ^{13}C-NMR spectra, mono-labeled and double-labeled isotopomers are observed as singlets and doublets, respectively.

TCA cycle flux

Metabolism of [3-^{13}C]pyruvate via PDH leads to labeling of glutamate at the C-4 position ([4-^{13}C]glutamate) and double-labeled [3,4-^{13}C]glutamate during the first and second TCA cycle turn, respectively. The labeling ratios of double/mono C-4 in glutamate reflect the number of turns that the ^{13}C-label from [2-^{13}C]acetyl-CoA has cycled before exiting as glutamate.

PDH flux: Enrichment in acetyl-CoA

If [4-^{13}C]α-ketoglutarate remains in the TCA cycle up to [2-^{13}C]oxaloacetate and condenses with unlabeled acetyl-CoA, mono-labeled glutamate C-3 will be formed during the second TCA cycle turn. Since double-labeled C-3 glutamate will result only if [2-^{13}C]acetyl-CoA and [2-^{13}C]oxaloacetate are converted to glutamate, its appearance is indirectly correlated with the entry of labeled acetyl-CoA into the TCA cycle. Hence, the ratio of double/mono-labeled glutamate C-3 reflects the ^{13}C-enrichment of the acetyl-CoA pool entering the TCA cycle.

Statistical analysis

The NMR studies were carried out on 4-6 independent experiments. Data are expressed as mean ± SD values. Comparison of data from experimental groups was analyzed using ANOVA and *post-hoc* Tukey`s test. Differences were considered significant when $P < 0.05$.

RESULTS

Concentrations of amino acids in rat brain

Brain amino acid concentrations were calculated from the 1.8 – 3.0 ppm region of ^{1}H-NMR spectra of brain extracts. Values in FC and MT of TD rats were compared to those of control animals (Table 1; Figure 2 shows typical ^{1}H-NMR spectra of extracts obtained from the MT). In both groups, plasma glucose levels remained within the normal range of 5.5 – 6.5 mM after injection of [1-^{13}C]glucose (data not shown). Levels of brain amino acids in control animals did not differ between the two glucose-administered groups (200 mg/kg or 500 mg/kg [1-^{13}C]glucose administration).

After 11 days of TD treatment, concentrations of the neuronal marker N-acetylaspartate (NAA) were unchanged in both brain regions whether a minimal labeling (200 mg/kg) or a loading (500 mg/kg) dose of [1-^{13}C]glucose was administered.

Following injection of the minimal labeling dose (200 mg/kg) of glucose to TD rats, aspartate levels decreased to 80.2% of control values in MT ($p < 0.05$). Aspartate concentrations remained unchanged in the non-vulnerable FC. Glutamate, glutamine and GABA concentrations were unchanged in both FC and MT of TD rats.

Glucose loading of TD rats led to a selective effect on MT amino acid levels. In particular, it led to significant decreases of aspartate levels to 61.4% of control values ($p < 0.01$). Furthermore, concentrations of glutamate in the MT of TD rats decreased to 71.5% of control values ($p < 0.01$) after glucose loading; while concentrations of glutamine increased to 131.5% of control values. In contrast to MT, administration of a glucose

load did not significantly alter the effects of thiamine deficiency on brain amino acid content of FC.

***De novo* synthesis of brain amino acids from [1-^{13}C]glucose in TD rats**

Figure 3 shows a representative section of a ^{13}C-NMR spectrum obtained from an extract of the MT of a control rat obtained 60 min after administration of [1-^{13}C]glucose (500 mg/kg). 60 min following [1-^{13}C]glucose injection, appreciable amounts of label were found in discrete carbon positions of glutamate, GABA and aspartate. Table 2 indicates the fractional ^{13}C-enrichments in the C-4 and C-2 carbon positions in glutamate and GABA, and in the C-3 and C-2 of aspartate, in MT compared to FC brain regions of control rats, exposed to either 200 or 500 mg/kg [1-^{13}C]glucose. In contrast to the total brain concentrations of these amino acids, the fractional ^{13}C-enrichments in amino acids differed between the 200 or 500 mg/kg [1-^{13}C]glucose-treated control groups. In all groups, the ^{13}C-label was predominantly present in C-4 of glutamate and in C-2 of GABA (formed via PDH), whereas C-2 of glutamate and C-4 or GABA (formed via PC) were more moderately labeled. In aspartate, the ^{13}C-label was evenly distributed between the C-3 and C-2 isotopomers. These ^{13}C-enrichments in control rats were used as baseline values for comparison with their respective TD groups.

The effects of thiamine deficiency on the fractional ^{13}C-enrichments in the aspartate, glutamate and GABA isotopomers in MT and FC are shown in Figure 4 (a-c) with the values expressed as percentages of the respective control values (see Table 2).

Together with the selective decreases in the total levels of aspartate (Table 1) in the MT, a significant reduction was observed for the fractional

enrichment in the C-2 aspartate isotopomer in TD rats (Fig. 4a), while C-3 aspartate remained the same as that observed in the control rats. The fractional ^{13}C-enrichments in both C-2 and C-3 of aspartate remained unchanged in the FC.

Following 11 days of thiamine deficiency, there were no significant changes in the fractional ^{13}C-enrichments in glutamate or GABA isotopomers in either the FC or MT (Fig. 4 b,c).

In the MT, glucose loading led to a significant deterioration of thiamine deficiency-induced reduction of ^{13}C-label incorporation into both the C-3 and C-2 aspartate isotopomers to 60.8% and 18.2% of control values ($p < 0.001$), respectively, while no such effect was observed in the FC (Fig. 4a). Following administration of a glucose load, the fractional ^{13}C-enrichments in C-4 and C-2 of glutamate in MT, however, declined to 52.1% and 70.2 % of control values ($p < 0.01$), respectively. *De novo* synthesis of these amino acids remained unchanged in the FC (Fig. 4 b,c). The fractional ^{13}C-enrichment in C-4 of GABA was reduced to 55.8% of control values ($p < 0.001$) in the MT of TD rats following glucose loading, while the synthesis of GABA remained unchanged in the FC of these animals (Fig. 4b).

TCA cycling ratio and ^{13}C-enrichment in acetyl-CoA

If the ^{13}C-label from [1-^{13}C]glucose remains in the TCA cycle for a second TCA cycle turn, it can result in the formation of double-labeled glutamate isotopomers, as shown in Figure 1. Figure 5 shows expanded plots of ^{13}C-NMR spectra obtained from MT of control and TD rats, showing the doublets for the C-4 and C-3 carbon positions of glutamate. The ratio of double/mono-labeled [4-^{13}C]glutamate is an index for the

relative number of turns that the ^{13}C-label derived from [2-^{13}C]acetyl-CoA has passed through the TCA cycle before exiting and labeling glutamate. For example: a ratio of 0.2 means that 1/5 of the label stays in the TCA cycle for more than one turn. Figure 6 shows that the ratio of double/mono-labeled [4-^{13}C]glutamate decreased to a greater extent (to 55 ± 9.2% of control values ($p<0.01$)) in the MT compared to a reduction to 75 ± 10.6% of controls ($p<0.05$) in FC of TD rats.

Administration of a glucose load caused further decreases of the TCA cycling ratio to 38 ± 8.5% of control values in MT ($p<0.001$) of TD rats, but did not significantly alter the effect of TD on this ratio in the FC.

Double-labeled [3-^{13}C]glutamate is formed after condensation of [2-^{13}C]acetyl-CoA with [2-^{13}C]oxaloacetate in the second TCA cycle turn. A decrease in the ratio of double/mono-labeled [3-^{13}C]glutamate therefore would reflect a decreased fractional ^{13}C-enrichment in acetyl-CoA (formed through PDH) entering the TCA cycle. As shown in Figure 6, the fractional ^{13}C-enrichment in acetyl-CoA remained unchanged in both regions of the TD brain. Glucose loading, however, decreased the double/mono-labeled [3-^{13}C]glutamate ratio to 34 ± 8.5% of control values in the TD MT ($p < 0.001$), but not in the FC.

DISCUSSION

Region-specific alterations in the steady state levels of cerebral amino acid neurotransmitters have been well-documented in experimental animal models of thiamine deficiency (Gaitonde et al., 1975; Butterworth et al., 1979; Plaitakis et al., 1979; Hamel et al., 1979; Butterworth and Héroux, 1989); however, the dynamics of these changes have never been systematically explored. Application of $^{1}H/^{13}C$-NMR enables the examination of these regional changes in amino acid concentrations in relation to the relative contribution of the various competing pathways of glucose metabolism. Moreover, to some extent the cellular localization of these metabolic changes can be inferred through the analysis of complex isotopomer labeling patterns. In the present study, an early end-point of 11 days TD treatment was chosen so that any observed metabolic changes in the vulnerable brain regions could not be attributed to the neuronal cell death that occurs at late stages of thiamine deficiency (Todd and Butterworth, 1999). The absence of neuronal cell death and mitochondrial dysfunction at this time-point of thiamine deficiency, with or without glucose loading, was confirmed by the absence of changes in the neuronal marker molecule N-acetylaspartate in both vulnerable and non-vulnerable brain structures of TD rats compared to control animals (Table 1).

^{1}H-NMR analysis showed no changes in concentrations of glutamate, glutamine and GABA by day 11 in the MT of TD rats (Table 1). Aspartate levels were significantly reduced in MT while remaining unaffected in the non-vulnerable FC. This is likely the consequence of impaired condensation of acetyl-CoA with a reduced pool of oxaloacetate, as aspartate synthesis depends upon the availability of this TCA cycle

intermediate. It has been consistently reported that cerebral activities of α-KGDH are more sensitive to thiamine deficiency than PDH in vulnerable brain regions (Gaitonde et al., 1975; Butterworth et al., 1985, 1986). The present finding of thiamine deficiency-related decreases of aspartate concentrations in the MT in the absence of changes in glutamate is consistent with a decrease in α-KGDH activity, leading to decreases of the oxaloacetate pool. Results of the present study also provide unequivocal evidence for decreased *de novo* synthesis of aspartate, again consistent with the limited availability of the transaminase substrate oxaloacetate. Administration of a glucose load caused further reductions in aspartate levels and synthesis. These metabolic consequences of TD occur selectively in MT, a brain structure shown previously to manifest decreased glucose utilization, mitochondrial dysfunction and ultimately neuronal cell death.

The classical concept of the compartmentation of brain metabolism is based on the presence of at least two glutamate pools, one small and one large, associated with two kinetically distinct TCA cycles localized in astrocytes and neurons, respectively (Cruz and Cerdan, 1999). According to this concept, the astrocytic and neuronal pools account for approximately 10% and 90% of total glutamate, respectively. This implies that brain glutamate is preferentially synthesized in neurons. Since neurons lack significant capacity for anaplerosis, neuronal glutamate synthesis relies primarily upon the activity of PDH and the provision of anaplerotic substrates from the astrocytes. Astrocytes selectively express pyruvate carboxylase (PC) (Shank et al., 1985, 1993). The synthesis of aspartate can occur in both astrocytes and neurons. The C-2 and C-3 isotopomers of aspartate may be synthesized in astrocytes via PC or in

both astrocytes and neurons via PDH. Via PC, [1-^{13}C]glucose is converted to [3-^{13}C]oxaloacetate and subsequently to [3-^{13}C]aspartate. If the ^{13}C-label enters the TCA cycle via PDH and remains within the cycle for two turns, it will be distributed equally between the C-2 and C-3 positions of oxaloacetate due to the scrambling of the label in the symmetrical fumarate step. However, impaired flux through this pathway would lead to similar decreases of both aspartate isotopomers. Consequently, the decrease of only the C-2 isotopomer observed in the MT of TD rats may result from impaired equilibration of PC-derived oxaloacetate with fumarate and/or a concomitant stimulation of astrocytic PC-mediated [3-^{13}C]aspartate synthesis. This data suggests that the early decrease in total levels of aspartate observed in the thiamine-deficient MT (Table 1) is primarily due to decreased PDH-mediated synthesis of [2-^{13}C]aspartate caused by a diminished TCA cycle flux at the level of α-KGDH (Fig. 4a), while the synthesis of [3-^{13}C]aspartate via the more direct astrocytic anaplerotic pathway is relatively maintained. Following glucose loading, synthesis of [2-^{13}]aspartate in brain via PDH decreased below 20% of control values.

Using [1-^{13}C]glucose, the enrichment in the C-4 position of glutamate ([4-^{13}C]glutamate) relative to the enrichment of [2-^{13}C]glutamate reflects the flux of pyruvate through PDH relative to PC (Figure 1). However, it should be borne in mind that the label at the C-2 position of glutamate may also arise if the ^{13}C-label enters the TCA cycle via PDH and remains within the cycle until the second turn before exiting and labeling glutamate. The isotopomer patterns for glutamate in the FC and MT of TD rats (Fig. 4b) indicate no significant decreases in the synthesis of glutamate via either the oxidative (PDH) or the anaplerotic (PC) pathway

in thiamine deficiency. Similarly, the isotopomer patterns for GABA show no effect of thiamine deficiency on synthesis of GABA in both brain regions at this time point. Glucose loading, on the other hand, resulted in selectively decreased synthesis of both glutamate and GABA in MT of TD rats via both pathways. Together, these results combined with those of aspartate suggest that the administration of a glucose load to TD rats exacerbates the "biochemical lesion" by causing an inhibition of pyruvate flux through PDH, thus resulting in further impairments in the TCA cycle.

Estimation of the relative number of turns that the ^{13}C-label has passed through the TCA cycle by the ratio of double/mono-labeling of [4-^{13}C]glutamate showed that the number of TCA cycle turns decreased to approximately 55% of control values in the MT of TD rats. Previous studies have demonstrated that activities of α-KGDH are decreased by up to 50% at this time-point; and MT showed the most marked reductions which occurred prior to the onset of neurological symptoms (Butterworth, 1986). Results of the present study extend these findings and demonstrate that the initial metabolic lesion in TD brain is a decreased *in vivo* flux through α-KGDH resulting in a reduction in TCA cycle turnover. This is confirmed by (i) the isotopomer pattern in aspartate and (ii) a decreased ratio of double/mono-labeling of [4-^{13}C]glutamate (Figure 6a). Hence, the selective decrease in TCA cycle flux predicts brain regional vulnerability to thiamine deficiency.

Glucose loading, shown previously to worsen neurological status in TD patients (Wallis et al., 1978; Watson et al., 1981) and experimental animals (Zimitat and Nixon, 1999), exacerbated the decreases in TCA cycle flux in the MT (Figure 6b) with a concomitant decrease in the flux through PDH (as estimated by the ratio of double/mono-labeled glutamate C-3;

Fig. 6); no such effects were observed in FC. Impaired pyruvate oxidation has been shown to occur in brain regions vulnerable to TD at later stages of thiamine deficiency (Dreyfus and Hauser, 1965; McCandless and Schenker, 1968), so these results demonstrate a possible mechanism for the worsening of neurological status observed in both TD patients and experimental animals following parenteral administration of glucose. It has recently been demonstrated that administration of a glucose load to TD rats at this time-point leads to increased *de novo* synthesis and accumulation of lactate in MT (Navarro et al., 2004). A key consequence of elevated lactate levels is to make the cytosolic redox potential more reducing, which would act to inhibit PDH activity. This in turn would limit the production of acetyl-CoA, resulting in the stimulation of PC, as suggested in this study by the ^{13}C-isotopomer pattern of aspartate following administration of a glucose load. Thus under conditions of glucose loading, the cytosolic NADH/NAD^+ ratio may increase enough to inhibit PDH. It is also possible that the focal accumulation of lactate in MT causes a focal acidosis, which is known to have detrimental effects on cellular metabolism by causing a depression in local cerebral glucose utilization (Van Nimmen et al., 1986) and by enhancing iron-catalysed production of reactive oxygen species.

Results of the present study using ^{13}C isotopomer analysis provide the first direct evidence that an early impairment of α-KGDH is responsible for the decreased glucose (or pyruvate) oxidation in TD brain; and administration of a glucose load caused further decreases in TCA cycle turnover and impairments in PDH flux. The present findings also suggest that inhibition of flux through α-KGDH in TD brain occurs primarily in the neurons, while astrocytes possess compensatory

mechanisms, i.e. the anaplerotic pathway, to replenish oxaloacetate concentrations via metabolic pathways that do not involve thiamine-dependent enzymes.

ACKNOWLEDGMENTS

The studies from the authors' research unit were funded by the Canadian Institutes for Health Research (CIHR). Claudia Zwingmann is a recipient of research awards from the Quebec Ministry of Education and Deutsche Forschungsgemeinschaft, Germany. We thank Professor Dieter Leibfritz, University of Bremen, for the generous availability of the NMR laboratory.

REFERENCES

Butterworth, R.F., (1986). Cerebral thiamine-dependent enzyme changes in experimental Wernicke's Encephalopathy. *Metab Brain Dis,* 1(3), 165-175.

Butterworth, R.F., & Héroux, M., (1989). Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acid and thiamine-dependent enzymes. *J Neurochem,* 52(4), 1079-1084.

Butterworth, R.F., Hamel, E., Landreville, F., & Barbeau, A., (1979). Amino acid changes in thiamine-deficient encephalopathy: Some implications for the pathogenesis of Friedrich's Ataxia. *Can J Neurol Sci,* 6(2), 217-222.

Butterworth, R.F., Giguere, J.F., & Besnard, A.M., (1985). Activities of thiamine-dependent enzymes in two experimental models of thiamine-deficiency encephalopathy: 1. The pyruvate dehydrogenase complex. *Neurochem Res,* 10(10), 1417-1428.

Butterworth, R.F., Giguere, J.F., & Besnard, A.M., 1986. Activities of thiamine-dependent enzymes in two experimental models of thiamine deficiency encephalopathy: 2. α–ketoglutarate dehydrogenase. *Neurochem Res,* 11(4), 567-577.

Cruz, F., & Cerdan, S., (1999). Quantitative ^{13}C NMR studies of metabolic compartmentation in the adult mammalian brain. *NMR in Biomed,* 12, 451-462.

Dreyfus, P.M., & Hauser G., (1965). The effect of thiamine deficiency on the pyruvate decarboxylase system of the central nervous system. *Biochim Biophys Acta,* 104, 78-84.

Elnageh, K.M., & Gaitonde, M.K., (1988). Effect of a deficiency of thiamine on brain pyruvate dehydrogenase: Enzyme assay by three different methods. *J Neurochem,* 51, 1482-1489.

Gaitonde, M.K., Fayein, N.A., & Johnson, A.L., (1975). Decreased metabolism in vivo of glucose into amino acids of the brain of thiamine-deficient rats after treatment with pyrithiamine. *J Neurochem,* 22, 53-61.

Hamel E., Butterworth R.F., & Barbeau, A., (1979). Effect of thiamine deficiency on levels of putative amino acid transmitters in affected regions of the rat brain. *J Neurochem,* 33(2), 575-577.

McCandless, D.W., & Schenker, S., (1968). Encephalopathy of thiamine deficiency: Studies of intracerebral mechanisms. *J Clin Invest,* 47(10), 2268-2280.

Navarro, D., Zwingmann, C., Hazell, A.S., & Butterworth, R.F., (2005). Brain lactate synthesis in thiamine deficiency: A re-evaluation using $^{1}H/^{13}C$ nuclear magnetic resonance spectroscopy. *J Neurosci Res,* 79(1-2), 33-41.

Paxinos, G., & Watson, C., (1982). The Rat Brain in Stereotaxic Coordinates. New York : Academic Press Inc.

Plaitakis, A., Nicklas, W.J., & Berl, S., (1979). Alterations in uptake and metabolism of aspartate and glutamate in brain of thiamine-deficient animals. *Brain Res,* 171(3), 489-502.

Rose, S.E., Nixon, P.F., Zelaya, F.O., Wholohan, B.T., Zimitat, C., Moxon, L.N., Crozier, S., Brereton, I.M., & Doddrell, D.M., (1993). Application of high field localised in vivo ^{1}H MRS to study biochemical changes in the thiamin deficient rat brain under glucose load. *NMR Biomed*, 6(5), 324-328.

Shank, R.P., Bennett, G.S., Freytag, S.O., & Cambell, G.L., 1985. Pyruvate carboxylase: an astrocyte-specific enzyme implicated in the replenishment of amino acid neurotransmitter pools. *Brain Res*, 329, 364-367.

Shank, R.P., Leo, G.C., & Zielke, H.R., 1993. Cerebral metabolic compartmentation as revealed by nuclear magnetic resonance analysis of D-[1-^{13}C]glucose metabolism. *J Neurochem*, 61, 315-323.

Todd, K., & Butterworth, R.F., (1999). Mechanisms of selective neuronal cell death due to thiamine deficiency. *Ann N Y Acad Sci*, 893, 404-411.

Van Nimmen, D., Weyne, J., Demeester, G., & Leusen, I., (1986). Local cerebral glucose utilization during intracerebral pH changes. *J Cereb Blood Flow Metab*, 6, 584-589.

Wallis, W.E., Willoughby, E., & Baker, P., (1978). Coma in the Wernicke-Korsakoff syndrome. *Lancet*, 2(8086), 400-401.

Watson, A.J., Walker, J.F., Tomkin, G.H., Finn, M.M., & Keogh, J.A., (1981). Acute Wernicke's Encephalopathy precipitated by glucose loading. *Ir J of Med Sci*, 150(10), 301-303.

Zimitat, C., & Nixon, P.F., (1999). Glucose loading precipitates acute encephalopathy in thiamin-deficient rats. *Metab Brain Dis*, 14, 1-20.

Zimitat, C., & Nixon, P.F., (2001). Glucose induced IEG expression in thiamin-deficient rat brain. *Brain Res*, 892, 218-227.

Zwingmann, C., Leibfritz, D., & Hazell, A.S., (2003). Energy metabolism in astrocytes and neurons treated with manganese: Relation among cell-specific energy failure, glucose metabolism, and intercellular trafficking using multinuclear NMR-spectroscopic analysis. *J Cereb Blood Flow Metab*, 23, 756-771.

LEGENDS

Figure 1: Metabolic fate of ^{13}C-label from [1-^{13}C]glucose

Label distribution in glycolytic and TCA cycle intermediates following administration of [1-^{13}C]glucose. For label distribution in TCA cycle intermediates and aspartate only one TCA cycle turn from pyruvate to malate is considered; for glutamine, glutamate, and GABA the label distribution after two TCA cycle turns are also presented. The fate of the ^{13}C-label from [1-^{13}C]glucose is indicated by filled circles: Black circles represent the ^{13}C-label position in lactate and alanine (synthesized from pyruvate via lactate dehydrogenase (LDH) and alanine aminotransferase (ALAT), respectively, and in TCA cycle-related metabolites synthesized through pyruvate carboxylase (PC) or pyruvate dehydrogenase (PDH) during the 1st TCA cycle turn. ^{13}C-label position due to equilibration of PC-derived oxaloacetate with fumarate and ^{13}C-label positions within the 2nd TCA cycle turn via PDH are indicated by grey circles. AAT: aspartate aminotransferase; GDH: glutamate dehydrogenase; GABA: γ-aminobutyric acid.

Figure 2: ^{1}H-NMR spectra of extracts from medial thalamus

Segments of typical ^{1}H-NMR spectra of extracts obtained from medial thalamus of a control and a thiamine-deficient rat. Peak assignments: Ac: acetate; Asp: aspartate; Cho: choline-containing compounds; Cr: creatine; GABA: γ-aminobutyric acid; Gln: glutamine; Glu: glutamate; Glx: Gln + Glu; myo-Ins: myo-inositol; NAA: N-acetylaspartate; Suc: succinate; Tau: taurine.

Figure 3: ^{13}C-NMR spectrum of a brain tissue extract

Segment of a ^{13}C-NMR spectrum of an extract from medial thalamus of a control rat. Peak assignments: Ala: alanine; GABA: γ-aminobutyric acid; Glu: glutamate; Gln: glutamine; NAA: N-acetylaspartate.

Figure 4: Effect of thiamine deficiency on de novo synthesis of glutamate, GABA and aspartate

Changes in the fractional ^{13}C-enrichments in individual carbon positions of a) glutamate, b) GABA and c) aspartate labeled 60 min after administration of either 200 mg/kg (no glucose load) or 500 mg/kg [1-^{13}C]glucose (glucose load) in thiamine-deficient rats. Values were calculated from ^{13}C-NMR spectra obtained from extracts of the non-vulnerable frontal cortex and the vulnerable medial thalamus and expressed as percentages of control values (see Table 3). Values represent means ± SD for n = 5 (no glucose load) or n = 4 (glucose load). Data were analyzed using ANOVA and post-hoc Tukey`s test († significantly different from control values; § effect of thiamine deficiency significantly different after administration of a glucose load; *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$; ns, not significant).

Figure 5: ^{13}C-NMR spectra of brain extracts

Segments of ^{13}C-NMR spectra of extracts from medial thalamus of a control rat compared to a thiamine-deficient rat, showing the chemical shift region and multiplet pattern of glutamate (Glu) C3 and C4.

Figure 6: TCA cycling ratio and ^{13}C-enrichment in acetyl-CoA

The ratios of double-/mono-labeled isotopomers of glutamate (Glu) have been deduced from ^{13}C-NMR spectra (see Fig. 5) of extracts from frontal cortex and medial thalamus of control and thiamine-deficient rats. The ratio of double/mono-labeled [3-^{13}C]glutamate reflects the ^{13}C-enrichment in acetyl-CoA entering the TCA cycle. The ratio of double/mono-labeled [4-^{13}C]glutamate reflect the number of TCA cycle turns that the label from [1-^{13}C]glucose has passed through before [4-^{13}C]glutamate is synthesized. Values represent means ± SD for n = 5 (no glucose load) or n = 4 (high glucose load). Data were analyzed using ANOVA and post-hoc Tukey`s test († significantly different from control values; § effect of thiamine deficiency significantly different after administration of a high glucose load; *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$; ns, not significant).

Table 1: Concentrations of brain amino acids

The concentrations of the amino acids were calculated by integration of the respective signals in ^{1}H-NMR spectra of brain tissue extracts obtained from frontal cortex and medial thalamus of control rats and thiamine-deficient (TD) rats with and without administration of a glucose load. Values represent means ± SD for n = 5 (no glucose load) or n = 4 (glucose load). Since no significant differences were found between control rats with and without glucose loading, these data were combined. NAA: N-acetylaspartate. Data between all experimental groups were analyzed using ANOVA and post-hoc Tukey`s test († significantly different from controls; § effect of thiamine deficiency significantly altered following administration of a glucose load; *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$; ns, not significant).

Table 2: Incorporation of [1-13C]glucose into brain aspartate, glutamate and GABA of control rats: Baseline values (%)

The percentage ^{13}C-enrichments in individual carbon positions of brain aspartate, glutamate and GABA, labeled 60 min after injection of either 200 mg/kg (no glucose load) or 500 mg/kg (glucose load) [1-^{13}C]glucose, were calculated from ^{13}C-NMR spectra of extracts from the frontal cortex and medial thalamus of control rats. Values represent means ± SD for n=5 (no glucose load) or n=4 (glucose load). Data between all experimental groups were analyzed using ANOVA and post-hoc Tukey`s test († significantly different following administration of glucose load; * $p < 0.05$; ns, not significant).

Figure 1: Metabolic fate of ^{13}C-label from [1-^{13}C]glucose (Navarro et al., 2008)

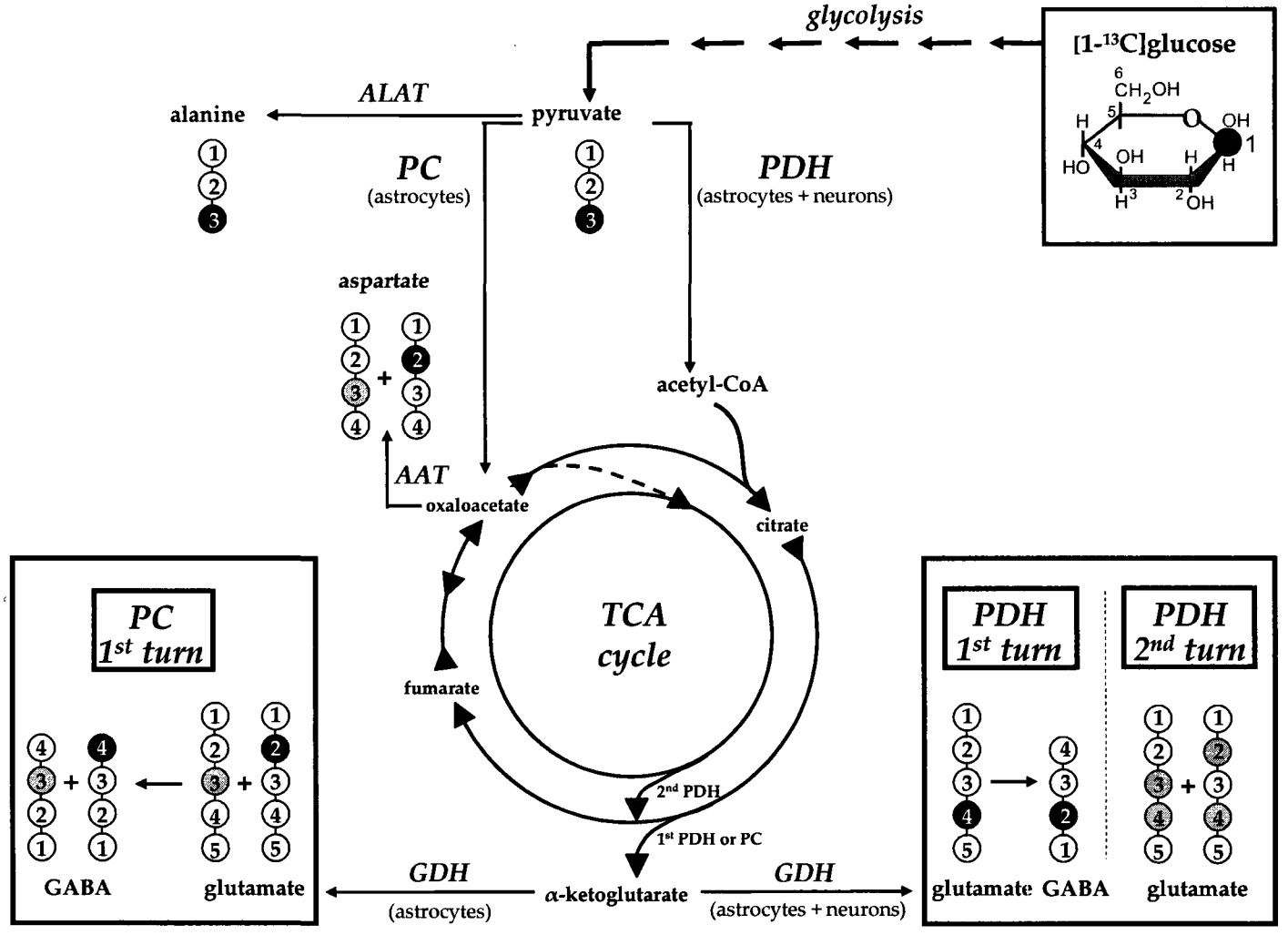


Figure 2: ^{1}H-NMR spectra of extracts from medial thalamus (Navarro et al., 2008)

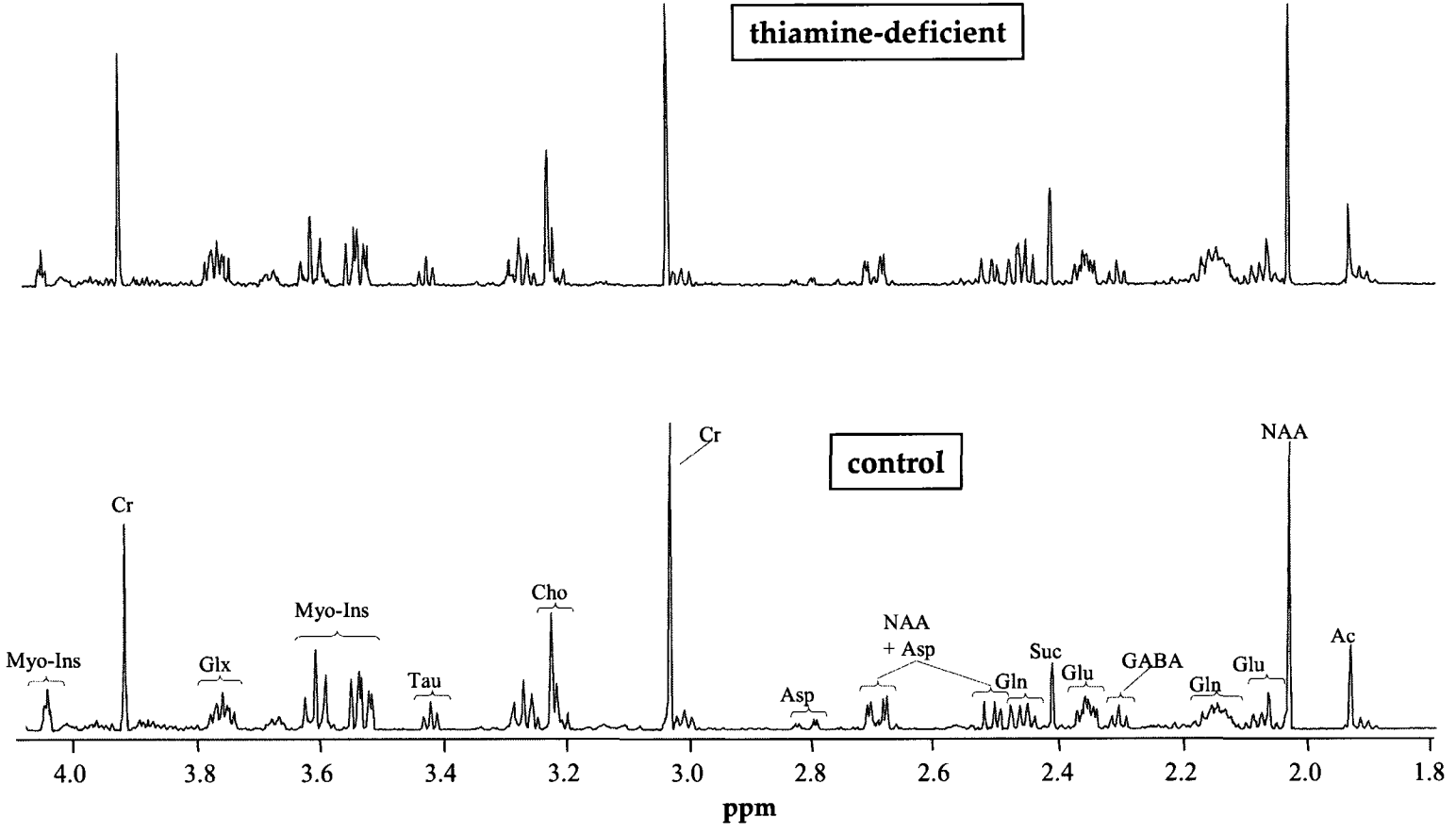


Figure 3: ^{13}C-NMR spectrum of a brain tissue extract (Navarro et al., 2008)

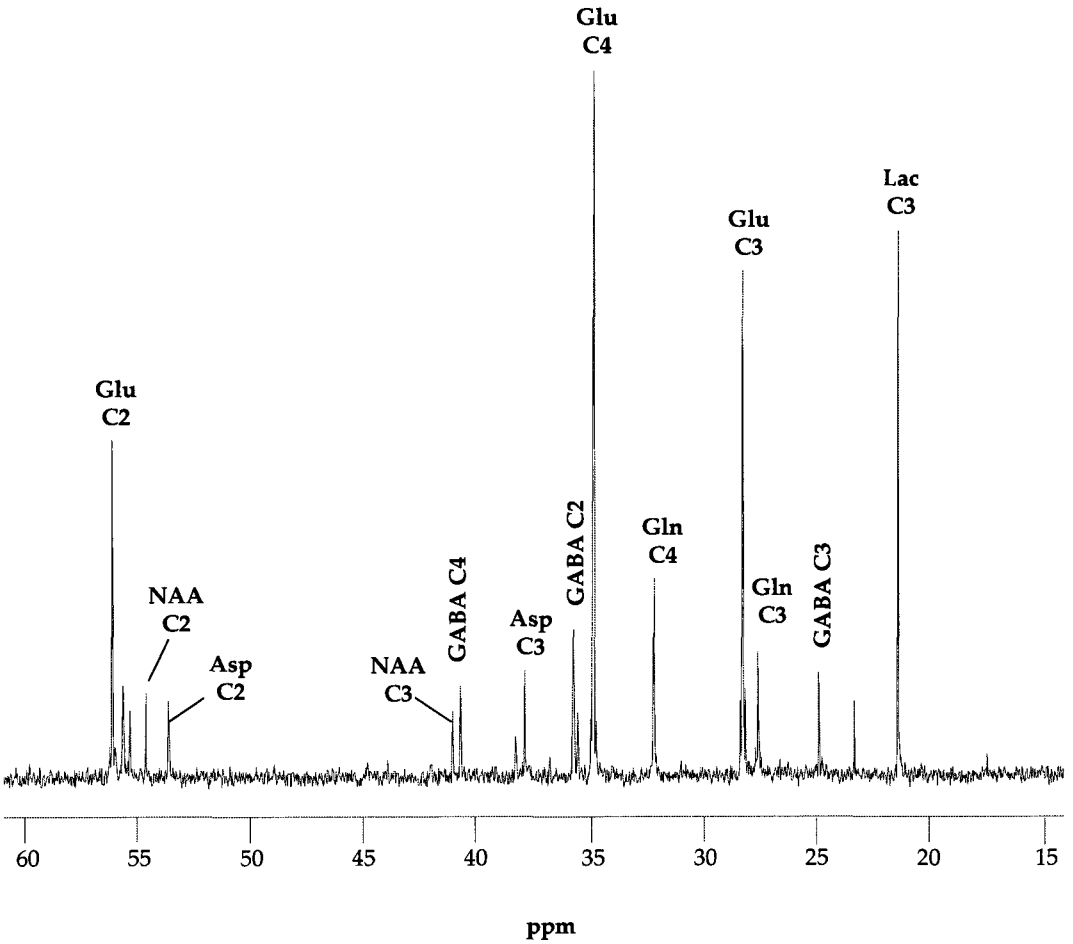


Figure 4: Effect of thiamine deficiency on *de novo* synthesis of glutamate, GABA and aspartate (Navarro et al., 2008)

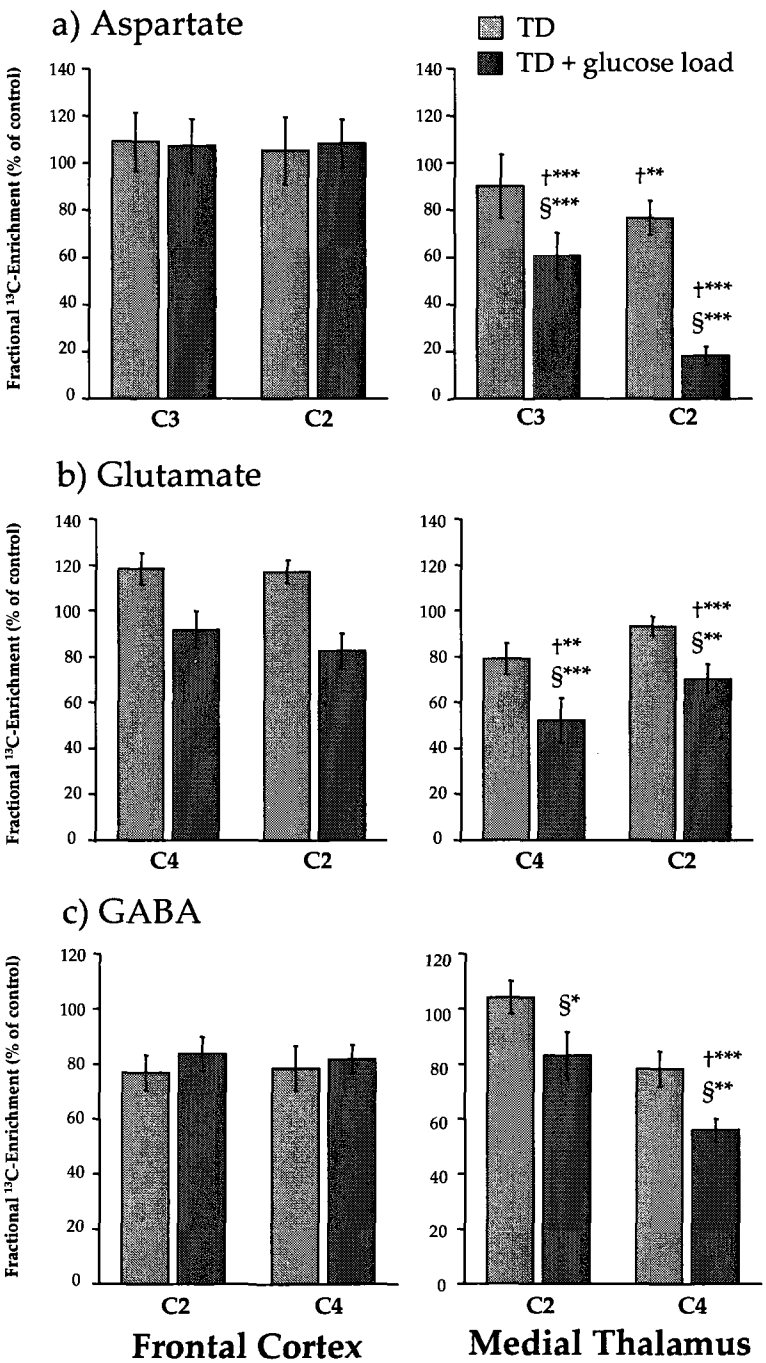


Figure 7: ^{13}C-NMR spectra of brain extracts (Navarro et al., 2008)

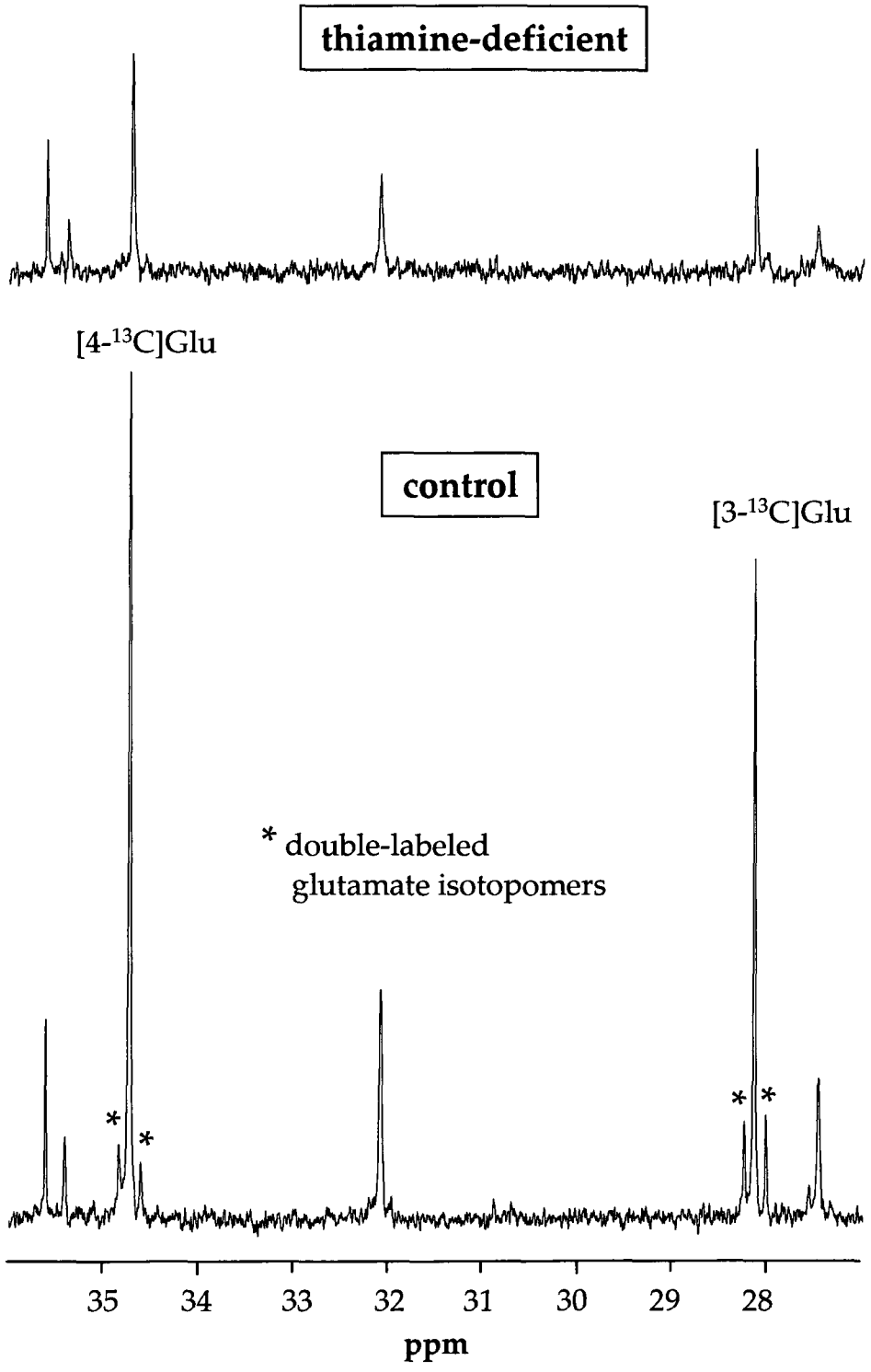


Figure 8: ^{13}C-NMR spectra of brain extracts (Navarro et al., 2008)

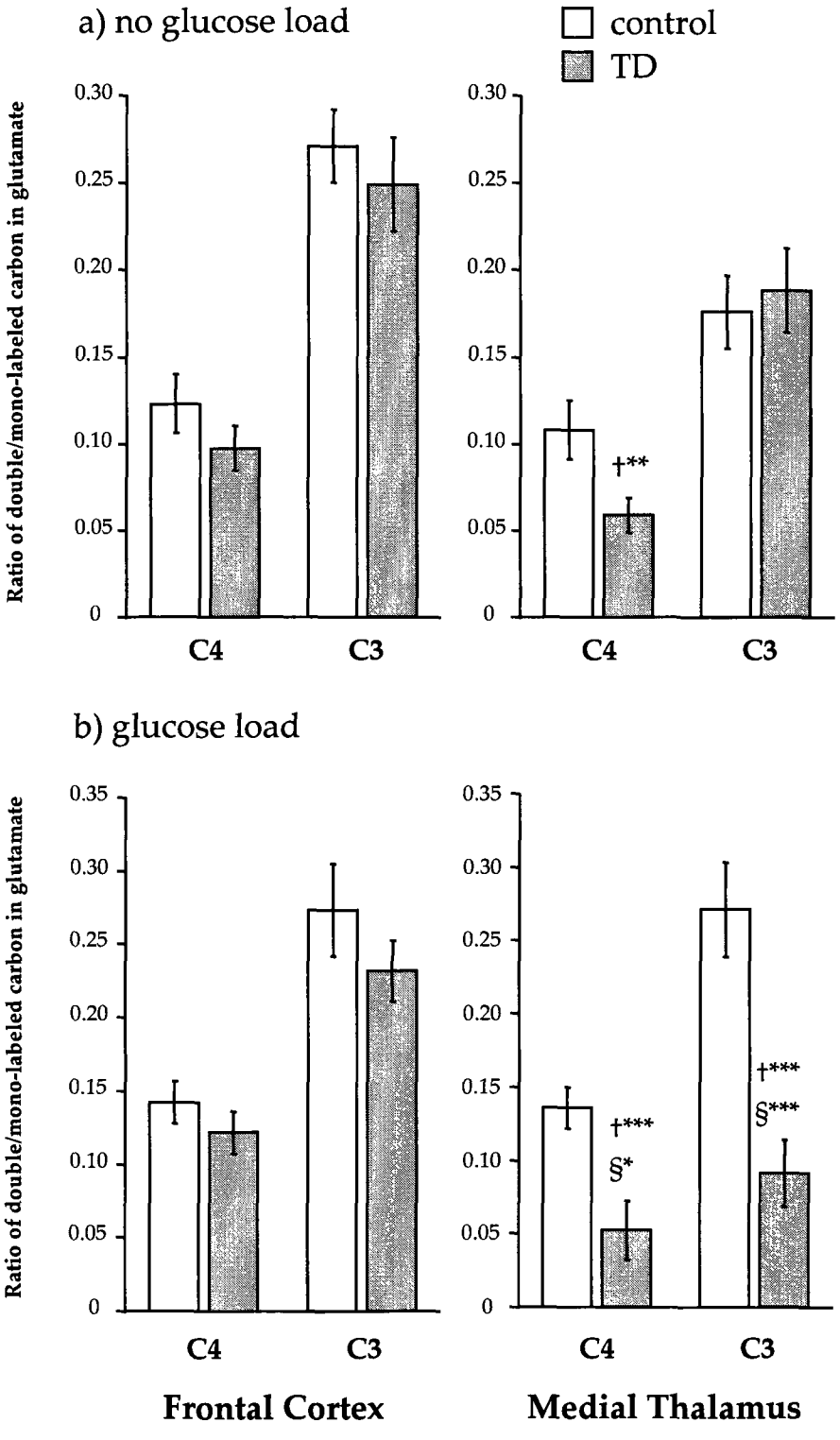


Table 1: Concentrations of brain amino acids

Frontal Cortex	PFC (μmol/g)	TD (μmol/g)		TD + Glucose (μmol/g)		
alanine	0.63 ± 0.06	0.66 ± 0.11	†ns	0.72 ± 0.11	†ns	§ns
glutamine	4.29 ± 0.40	4.92 ± 0.64	†ns	6.54 ± 0.62	†ns	§ns
glutamate	9.14 ± 1.03	10.24 ± 1.12	†ns	13.41 ± 2.17	†ns	§ns
GABA	2.03 ± 0.24	2.29 ± 0.34	†ns	2.49 ± 0.36	†ns	§ns
aspartate	1.21 ± 0.08	1.31 ± 0.15	†ns	1.23 ± 0.19	†ns	§ns
NAA	12.48 ± 0.87	13.06 ± 1.56	†ns	12.88 ± 0.97	†ns	§ns

Medial Thalamus	PFC (μmol/g)	TD (μmol/g)		TD + Glucose (μmol/g)		
alanine	0.44 ± 0.03	0.61 ± 0.05	†*	0.48 ± 0.03	†ns	§ns
glutamine	4.32 ± 0.46	4.76 ± 0.41	†ns	6.05 ± 0.84	†*	§ns
glutamate	8.97 ± 0.70	8.82 ± 1.02	†ns	8.47 ± 0.52	†**	§**
GABA	3.21 ± 0.36	3.48 ± 0.39	†ns	4.03 ± 0.34	†*	§ns
aspartate	1.01 ± 0.12	0.81 ± 0.06	†*	0.66 ± 0.06	†***	§**
NAA	11.34 ± 0.76	10.31 ± 0.57	†ns	10.70 ± 0.73	†ns	§ns

Table 2: Incorporation of [1-^{13}C]glucose into brain aspartate, glutamate and GABA of control rats: Baseline values (%)

Frontal Cortex	No glucose load (%)	Glucose load (%)	
Asp C3	3.93 ± 0.31	4.49 ± 0.55	†ns
Asp C2	3.70 ± 0.33	4.31 ± 0.28	†ns
Glu C4	8.62 ± 1.23	11.65 ± 1.83	†*
Glu C2	5.76 ± 0.61	4.82 ± 0.46	†ns
GABA C2	4.96 ± 0.66	6.71 ± 0.65	†*
GABA C4	5.90 ± 0.88	5.52 ± 0.64	†ns

Medial Thalamus	No glucose load (%)	Glucose load (%)	
Asp C3	3.88 ± 0.51	4.72 ± 0.38	†ns
Asp C2	3.21 ± 0.55	4.55 ± 0.52	†*
Glu C4	8.23 ± 1.26	11.51 ± 1.92	†*
Glu C2	4.01 ± 0.41	5.45 ± 0.25	†*
GABA C2	9.29 ± 0.97	8.63 ± 1.14	†ns
GABA C4	3.79 ± 0.51	5.31 ± 0.67	†*

ARTICLE 4

The previous three articles have explored some of the metabolic consequences of impaired glucose metabolism in brains of TD rats. Less established, however, are the effects of thiamine deficiency on the metabolism of branched-chain amino acids within the brain. The following article investigates, for the first time, the effects of thiamine deficiency on the regional activity of thiamine-dependent, branched-chain α-ketoacid dehydrogenase (BCKDH) in the brain of TD rats; and explores the possible involvement of altered BCAA metabolism in the biochemical lesions observed in the MT.

IMPAIRED OXIDATION OF BRANCHED-CHAIN AMINO ACIDS IN THE MEDIAL THALAMUS OF THIAMINE-DEFICIENT RATS

Darren Navarro, Claudia Zwingmann and Roger F. Butterworth

Neuroscience Research Unit, Hôpital Saint-Luc,
Montreal, Quebec, Canada.

Running Title: Branched-chain amino acids in the thiamine-deficient rat brain

Keywords: Thiamine deficiency, Branched-chain amino acid, Branched-chain α-ketoacid dehydrogenase, Wernicke's encephalopathy

Address for reprints and correspondence:
Roger. F. Butterworth, Ph.D, D.Sc.
Neuroscience Research Unit
CHUM (Campus Saint-Luc)
1058 Saint-Denis Street
Montreal, Quebec, Canada H2X 3J4
Phone: (514) 890-8310 ext. 35759
FAX: (514) 412-7314

Email: roger.butterworth@umontreal.ca

ABSTRACT

Thiamine, in its diphosphate form, is a required cofactor for enzymes of glucose metabolism and the branched-chain α-ketoacid dehydrogenase complex (BCKDH). Although metabolic impairments in glucose metabolism have been found to occur in selectively vulnerable brain regions of the thiamine-deficient (TD) brain, the effects of thiamine deficiency on BCKDH have not been elucidated.

BCAA metabolism and BCKDH activity were assayed by 1H-NMR analysis and by radiochemical assay, respectively, in brain extracts of vulnerable (medial thalamus; MT) versus non-vulnerable (frontal cortex; FC) brain regions of rats made TD by administration of the central thiamine antagonist, pyrithiamine. A significant regional variation in BCKDH within the TD rat brain was noted, with a higher capacity for branched-chain amino acids oxidation in FC compared with MT: BCKDH activity was significantly reduced in MT of TD rats, resulting in selective accumulation of BCAAs and α-ketoisocaproate (α-KIC) in this brain region. In particular, leucine concentrations were elevated over 5-fold in the MT of symptomatic TD rats, compared with pair-fed control (PFC) rats. Impaired branched-chain α-ketoacid (BCKA) metabolism in rats may contribute to the neuronal dysfunction and ultimate cell death observed in the MT of TD rats.

INTRODUCTION

Thiamine diphosphate (TDP) is a known cofactor for four enzymes including branched-chain α-ketoacid dehydrogenase complex (BCKDH; EC 1.2.4.4). Pyrithiamine-induced thiamine deficiency in rats, a well characterized animal model of Wernicke's encephalopathy, leads to neuronal cell death in medial thalamus (MT), while other brain regions, such as the frontal cortex (FC), remain relatively spared (Butterworth & Heroux, 1989). Although the effects of thiamine deficiency on regional cerebral glucose metabolism have been well-documented, the effects of thiamine deficiency on BCKDH in the brain have not been previously evaluated.

Branched-chain amino acids (BCAAs) have important biochemical functions in the brain including protein synthesis, maintenance of nitrogen balance, and energy production (Hutson *et al.*, 1998; Yudkoff, 1997; Yudkoff *et al.*, 1994). The initial step of BCAA metabolism is transamination to form the cognate α-ketoacid, which in the case of leucine is α-ketoisocaproate (α-KIC). In a multi-step reaction that is comparable to the decarboxylation of pyruvate or α–ketoglutarate, BCKAs are decarboxylated by BCKDH. This decarboxylation reaction, which is TDP-dependent, is the rate-limiting step of BCAA oxidation within the brain (Pardridge, 1983).

In the present study, the effects of thiamine deficiency on regional BCKDH activities and BCAA concentrations were measured by radiochemical assay and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy, respectively, during the progression of neurological symptoms in TD rats. Brain regions previously shown to be selectively lesioned, such as the MT, were compared with FC, a brain structure

shown to be largely spared following pyrithiamine treatment (Troncoso et al., 1981).

MATERIALS AND METHODS

Animal Model

Adult male Sprague Dawley rats weighing 200-225g obtained from Charles River (St. Constant, Quebec, Canada) were used in all experiments. Rats were housed individually under constant conditions of temperature, humidity and 12 hour light/dark cycles and had free access to water at all times. Rats were allowed to adapt to their environment for 3 days prior to the initiation of treatments. Animals were assigned to either thiamine-deficient (TD) or pair-fed control (PFC) groups. TD rats were further divided into presymptomatic (PS) and symptomatic (SYM) groups.

Thiamine Deficiency Protocol

Rats in the TD group were fed a diet deficient in thiamine (ICN Nutritional Biochemicals, Cleveland, OH, USA) and administered daily with the thiamine antagonist pyrithiamine hydrobromide (0.5 mg per kg body weight) intraperitoneally (i.p.). Control rats were pair-fed to equal food consumption with the TD rats using the same TD diet but with supplemental daily i.p. injections of thiamine (0.1 mg per kg body weight) (Troncoso et al. 1981; Butterworth and Heroux 1989). Rats in the PS group were sacrificed on day 11 of treatment, a time-point characterized by the onset of ataxia, at which no neuronal cell death had occurred in the vulnerable brain region. After 12 days of treatment, rats were assessed twice daily for neurological abnormalities. When rats displayed loss of righting reflex (usually between days 13-15) they were considered to be at the SYM stage. Any rats exhibiting spontaneous seizures were eliminated from the protocols. Animals were sacrificed by decapitation and the brains

were promptly removed and flash frozen and stored at –80°C. FC and MT were dissected on ice according to the coordinates described in the rat brain atlas of Paxinos and Watson (1982). All animal treatment procedures were approved by the Animal Ethics Committee of Saint-Luc Hospital (CHUM) and the University of Montreal.

Sample preparation for Nuclear Magnetic Resonance (NMR) spectroscopic studies

Brains from rats in each group (PFC, PS, SYM) were rapidly frozen in isopentane over dry ice, and dissected on ice to provide samples of FC and MT. Dissected tissue samples were immediately snap-frozen by immersion in liquid nitrogen and kept at –80 °C. Samples were ground over liquid nitrogen and homogenized in 12% PCA at 0°C using a motor-driven polished glass tube-Teflon homogenizer. Homogenates were centrifuged at 40,000 g for 15 min and this procedure was repeated once. The supernatants were combined and neutralized on ice with KOH. The precipitated KClO4 was sedimented by centrifugation (40,000 g, 15 min). Arterial blood was immediately mixed with 20% perchloric acid (PCA), centrifuged (40,000 g, 40 min), neutralized with KOH, and centrifuged again (40,000 g, 15 min) to precipitate $KClO_4$. The supernatant of each sample was lyophilized.

NMR spectroscopy

Lyophilized PCA extracts of the blood and the brain tissues were redissolved in 0.6 ml deuterium oxide (D_2O; Merck, Darmstadt, Germany) and centrifuged. Prior to NMR analysis, the pH was adjusted to 7.0 with

DCl and NaOD. 1H-NMR spectra were recorded on a Bruker DRX 600 spectrometer, operating at a frequency of 600 MHz.

^{1}H-NMR spectra were recorded with a 5-mm H,C,N inverse triple resonance probe (5-mm HX probe), 400 accumulations, repetition time 15 s, spectral width 7183 Hz. Chemical shifts were measured with reference to lactate at 1.33 ppm.

Measurement of metabolite concentrations

The concentrations (μmol/g tissue) of amino acids were determined from fully relaxed ^{1}H-NMR spectra of brain extracts using (trimethylsilyl)propionic-2,2,3,3,d_4-acid (TSP) as external standard (Zwingmann *et al.*, 2003).

Preparation of Brain Tissue Extracts for BCKDH Activity Assays

Animals were sacrificed by decapitation and the brains were promptly removed and flash frozen in liquid nitrogen and stored at –80°C. FC and MT were dissected (approximately 100–125 mg tissue per sample) on ice according to the coordinates described in the rat brain atlas of Paxinos and Watson (1982). Brain homogenates were prepared in the presence of reagents that minimize kinase, phosphatase and protease activities, according to Aftring et al. (1986).

BCKDH Activity Assay: Actual, Total and Latent Activity

Actual and total BCKDH activity was assessed by radiochemical assay, via the production of $^{14}CO_2$ from α-keto[1-^{14}C]isocaproate (55mCi/mmol, Amersham Biosciences Ltd, Buckinghamshire, UK) at 30°C, where one unit (U) of BCKDH activity catalyzes the formation of 1 μmol $^{14}CO_2$/min from α-keto[1-^{14}C]isocaproate. as previously described by Goodwin et al. (1988). The actual activity of the BCKDH complex refers to the activity as it occurs *in vivo* as a consequence of the complex existing partially in its active (dephosphorylated) state. Accurate determination of the actual activity requires preservation of the *in vivo* phosphorylation state of the complex during tissue removal, extraction and enzyme assay. This demands rapid removal and flash freezing (i.e. in liquid nitrogen) of the brain tissue followed by extraction and assay with reagents that minimize kinase, phosphatase and protease activities. One unit (U) of BCKDH enzyme activity catalyzes the formation of 1 μmol CO_2/min.

The actual activity assays were conducted with [+TDP] or without 0.5 mM TDP [-TDP] in the assay buffer in order to determine the proportion of BCKDH activity that was latent due to thiamine deficiency. Enzyme latency, or latent activity, refers to the portion of enzyme activity that requires exogenous TDP (apoenzyme) for expression of activity; it does not refer to the portion of inactivated BCKDH. Latent activity is calculated from the difference in actual activity with (holoenzyme) and without (apoenzyme) TDP in the assay, divided by the actual activity, times 100 (Pekovich *et al.*, 1996); and this is an indirect indication of the portion of BCKDH with bound TDP (holoenzyme), and not an indication of inactivated (phosphorylated) BCKDH. In other words, the enzyme activity before the addition of exogenous TDP reflects that of the existing

holoenzyme, whereas the addition of TDP further activates and reveals any apoenzyme that may be present.

Determination of total activity of the BCKDH complex requires complete activation of the enzyme prior to the activity assay, which was accomplished by treatment with phosphatase (Sigma-Aldrich Canada, Oakville, ON, Canada) as previously described (Harris *et al.*, 1982). The total activity assay was performed with TDP in the assay buffer [+TDP] because BCKDH activity state is not dependent upon whether TDP is bound, but rather the concentration of substrate BCKA (Shimomura et al., 2001). All BCKDH activity assays were shown to be linear with respect to incubation time and tissue concentration.

BCKDH Activity State

The BCKDH activity state is the ratio of actual activity (before complete activation) to total activity obtained after activation (dephosphorylation) by phosphatase treatment. This ratio enables the estimation of the proportion of the active (dephosphorylated) complex in vivo in a given tissue under different physiological states.

Statistical analysis

All NMR and BCKDH activity studies were carried out on 5-6 rats per group (PFC, PS and SYM). Data are expressed as mean ± SD values. Comparison of data from experimental groups was analyzed using ANOVA and post-hoc Tukey's test. Differences were considered significant when $p < 0.05$.

RESULTS

Effects of Thiamine Deficiency on Plasma Concentrations of BCAAs and α-KIC

In PFC rats, ^{1}H-NMR analysis revealed plasma levels of leucine, isoleucine and valine of 40.7 μM, 68.5 μM and 40.5 μM, respectively (Figure 1). After 11 days of thiamine deficiency protocol in rats (presymptomatic stage), plasma levels of leucine, isoleucine and valine were elevated to 128.1%, 139.4% and 128.8% of PFC values ($p < 0.05$), respectively; while progression to the symptomatic stage resulted in further increases to 219.8%, 199.7% and 201.8% of PFC values ($p < 0.001$), respectively (Figure 1). Similarly, plasma concentrations of α-KIC, the BCKA of leucine, increased significantly (from 57.7 nM) to 299.4% of PFC values ($p < 0.001$) at the symptomatic stage of thiamine deficiency. Concentrations of other BCKAs remained below the detection limit.

Effects of Thiamine Deficiency on Regional Brain Concentrations of BCAAs

The concentrations of leucine, isoleucine and valine were calculated from the 0.8–1.2 ppm region of ^{1}H-NMR spectra of brain extracts. The values measured in both the FC and the MT of TD rats were compared to those of PFC rats (Figure 2). In the FC, levels of leucine, isoleucine and valine remained unchanged in both presymptomatic and symptomatic TD rats. In the MT, however, concentrations of leucine, isoleucine and valine were increased significantly at the presymptomatic stage to 162.5%, 168.0% and 176.8% of PFC values ($p < 0.05$), respectively. Further increases of these metabolites to 512.5%, 279.6% and 296.4% of PFC values ($p < 0.001$), respectively, occurred in the MT at symptomatic time points.

While concentrations of brain α-KIC remained ^{1}H-NMR undetectable in FC and MT of PFC rats, in symptomatic rats, α-KIC accumulated to 171 nmoL/g wet weight selectively in the MT (Figure 2).

Effects of Thiamine Deficiency on BCKDH Activity

To determine the actual activity of the enzyme, BCKDH activity was assayed from tissue extracts with the in vivo phosphorylation (activity) state preserved. In TD rats, BCKDH actual activity decreased in the FC to 75.5% of PFC values ($p < 0.01$) at the symptomatic stage; while in the MT, actual activities were decreased significantly to 53.4% and 33.5% of PFC values in presymptomatic and symptomatic rats, respectively ($p < 0.001$). In order to assay total BCKDH activity, measurements were performed in separate aliquots of tissue extracts (in the presence of exogenous TDP) following maximal activation of the enzyme complex (dephosphorylation) with a broad specificity phosphatase (Table 1). Because the activity state of BCKDH is controlled by a kinase, which itself is influenced primarily by the local concentration of BCKA (Shimomura et al. 2001) and not by the availability of TDP, determination of BCKDH activity state was assayed in the presence of TDP [+TDP] (Table 1). Total BCKDH activities were greater in FC than in MT of PFC (54.4 and 35.8mU/g wet weight, respectively). Thiamine deficiency had no effect on total BCKDH activities in either the FC or MT.

Effects of Thiamine Deficiency on BCKDH Activity State

In the FC of PFC rats, 56.1% of BCKDH was present in the dephosphorylated state; and this was not significantly altered by thiamine deficiency; whereas in the MT, thiamine deficiency resulted in significant increases in the BCKDH activity state from 67.9% in PFC rats to 76.8% and 88.5% in presymptomatic and symptomatic rats, respectively.

Effects of Thiamine Deficiency on BCKDH Latency

Actual activity was measured both in the absence [-TDP] and in the presence [+TDP] in the assay buffer in order to estimate the portion of BCKDH activity which was latent due to thiamine deficiency. In PFC rats, 18.1% and 21.6% of BCKDH activity was latent in the FC and MT, respectively. In the FC, BCKDH latent activity increased significantly to 34.9% at SYM stages of thiamine deficiency; while in the MT, thiamine deficiency resulted in earlier and relatively larger increases of BCKDH latency to 63.7% and 79.1% in PS and SYM rats, respectively.

DISCUSSION

TDP is an essential cofactor for three enzymes involved in brain glucose metabolism, namely, the pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase, and transketolase. Less studied is a fourth TDP-dependent enzyme, BCKDH, which is involved in the oxidative metabolism of BCAAs. The effects of thiamine deficiency on BCKDH in the brain have not been previously explored.

BCAAs, particularly leucine, play an important role in nitrogen balance and the synthesis of glutamate in the brain (Bixel *et al.*, 1997; Hutson et al., 1998; Yudkoff, 1997; Yudkoff *et al.*, 1996b; Zielke *et al.*, 1997), with approximately 30–50% of all α-amino groups of brain glutamate and glutamine being derived from the BCAAs (Yudkoff *et al.*, 1993).

Regional levels of BCAAs in rat brain were differentially affected by thiamine deficiency, with increases in BCAA levels in the vulnerable MT of TD rats. Increases of leucine concentrations, in particular, were elevated over 5-fold in MT of SYM TD rats compared to PFC rats, concomitant with 3-fold elevations in levels of isoleucine and valine. No increases in BCAA concentrations, however, were observed in the FC, a brain region that is spared in thiamine deficiency. This apparent higher capacity for BCAA oxidation in the FC is consistent with previous studies demonstrating regional variations in BCKDH distribution in the rat brain (Brosnan *et al.*, 1985). Previous studies in rats have shown that brain concentrations of the intermediate ketoacid, α-KIC, are normally maintained at extremely low (<1 μM) concentrations (Keen *et al.*, 1993; Matsuo *et al.*, 1993) suggesting rapid conversion of this potentially toxic metabolite. Indeed, previous microdialysis studies have demonstrated an 11-fold increase in leucine concentrations following the infusion of α-KIC

into the brain of awake animals (Zielke *et al.*, 1996), suggesting that this BCKA is rapidly transaminated back to the BCAA. Together, the aberrant accumulation of α-KIC in MT, driven by the 5-fold greater concentration of leucine selectively in this brain region, implies impaired α-KIC oxidation within the MT of TD rats. Elevated concentrations of α-KIC, have been shown to cause neurological dysfunction in a rare metabolic disorder known as maple syrup urine disease (MSUD), which results from a congenital deficiency of BCKDH (Chuang *et al.*, 2006; Morton *et al.*, 2002).

Thiamine deficiency in rats caused significant reductions in BCKDH actual activity (in the absence of exogenously added TDP in the assay; [TDP]) in both the FC and MT rats; however, these activity reductions were less pronounced and had later onset in the FC, a region of the brain that is typically spared from lesions in thiamine deficiency (see Table 1). The apparent increase in BCKDH actual activity (in the presence of exogenously added TDP in the assay; [+TDP]) in the MT of SYM rats is likely due to the increased amount of BCKDH in the active, dephosphorylated form observed at this time-point.

Previous studies have demonstrated regional variations in the distribution of total BCKDH complex within the rat brain, with brain stem, midbrain and thalamic regions possessing lower total BCKDH activity compared with the cerebral cortex and cerebellum (Brosnan et al., 1985). Results of this study confirmed these previous investigations, suggesting an inherently greater oxidative capacity in the cerebral cortex compared to thalamic structures.

Latent BCKDH activity represents the portion of enzyme activity that requires exogenous TDP (apoenzyme) for expression of activity. As

expected, thiamine deficiency resulted in increases in BCKDH latent activity in brain tissue, reflecting the diminishing amounts of TDP available to be used as cofactor; however, increased BCKDH latency occurred earlier and to a greater extent in the vulnerable MT compared with the FC. Together, these results indicate a greater sensitivity of BCKDH to thiamine deficiency in the MT, where availability of cofactor TDP may be more limiting and/or less tightly bound to the enzyme complex.

The selective decrease in activity of BCKDH in the MT of TD rats was commensurate with increased BCAA concentrations. Previous studies have demonstrated that BCKAs inhibit the activity of BCKDH kinase, the enzyme responsible for inactivation of the BCKDH complex by phosphorylation (Paxton & Harris, 1984). Indeed, α-KIC is the most potent inhibitor of BCKDH kinase activity; thus, the accumulation of BCAAs and their cognate α-ketoacids likely contribute to the thiamine deficiency-induced decrease of BCKDH observed in MT.

Insufficient BCKDH oxidative capacity in the MT of TD rat brain leads to the accumulation of BCAAs and their cognate α-ketoacids, which may ultimately contribute to the neuronal death and mitochondrial dysfunction that have been observed in this vulnerable brain region. Excessively elevated concentrations of BCKAs, particularly α-KIC, have been shown to cause neurological dysfunction in patients with congenital deficiencies in BCKDH activity (Chuang *et al.*, 2006; Morton *et al.*, 2002). Accordingly, levels of this metabolite are actively minimized within the brain via transamination back to leucine; a process which likely takes place in neurons *in vivo* (Sweatt *et al.*, 2004). Consequently, the increased α-ketoglutarate/glutamate ratio resulting from α-KIC transamination back

to leucine, combined with the metabolic block at α-KGDH in the MT of TD rats, could promote further aspartate consumption via aspartate aminotransferase, thereby contributing to the depletion of neuronal aspartate pools. Similarly, previous ^{13}C-NMR studies performed on cultured astrocytes exposed to α-KIC in the presence of [U-^{13}C]glutamate resulted in significant reductions of internal aspartate concentrations, concomitant with two-fold increases in [^{13}C]lactate production (McKenna et al. 1998). Indeed, the MT of TD rats has been characterized by marked decreases in aspartate concentrations (Butterworth & Heroux, 1989; Gaitonde et al., 1975; Plaitakis et al., 1979), with concomitant increases lactate synthesis and accumulation (McCandless et al., 1968; Munujos et al., 1996; Navarro et al., 2005).

Results of the present study provide the first direct evidence of regional effects of thiamine deficiency on BCKDH activity in the rat brain. Insufficient BCKDH oxidative capacity in the MT of TD rat brain leads to the accumulation of BCAAs and their cognate α-ketoacids, which may ultimately contribute to the neuronal dysfunction and ultimate cell death observed in this brain region.

ACKNOWLEDGMENTS

The studies from the authors' research unit were funded by the Canadian Institutes for Health Research (CIHR). Claudia Zwingmann is a recipient of research awards from the Quebec Ministry of Education and Deutsche Forschungsgemeinschaft, Germany. We thank Professor Dieter Leibfritz, University of Bremen, for the generous availability of the NMR laboratory.

REFERENCES

Aftring, R. P., Block, K. P., & Buse, M. G. (1986). Leucine and isoleucine activate skeletal muscle branched-chain alpha-keto acid dehydrogenase in vivo. *Am J Physiol, 250*(5 Pt 1), E599-604.

Bixel, M. G., Hutson, S. M., & Hamprecht, B. (1997). Cellular distribution of branched-chain amino acid aminotransferase isoenzymes among rat brain glial cells in culture. *J Histochem Cytochem, 45*(5), 685-694.

Brosnan, M. E., Lowry, A., Wasi, Y., Lowry, M., & Brosnan, J. T. (1985). Regional and subcellular distribution of enzymes of branched-chain amino acid metabolism in brains of normal and diabetic rats. *Can J Physiol Pharmacol, 63*(10), 1234-1238.

Butterworth, R. F., & Héroux, M. (1989). Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acids and thiamine-dependent enzymes. *J Neurochem, 52*(4), 1079-1084.

Butterworth, R. F., Kril, J. J., & Harper, C. G. (1993). Thiamine-dependent enzyme changes in the brains of alcoholics: Relationship to the Wernicke-Korsakoff syndrome. *Alcohol Clin Exp Res, 17*(5), 1084-1088.

Chuang, D. T., Chuang, J. L., & Wynn, R. M. (2006). Lessons from genetic disorders of branched-chain amino acid metabolism. *J Nutr, 136*(1 Suppl), 243S-249S.

Gaitonde, M. K., Fayein, N. A., & Johnson, A. L. (1975). Decreased metabolism in vivo of glucose into amino acids of the brain of thiamine-deficient rats after treatment with pyrithiamine. *J Neurochem, 24*(6), 1215-1223.

Goodwin, G. W., Zhang, B., Paxton, R., & Harris, R. A. (1988). Determination of activity and activity state of branched-chain alpha-keto acid dehydrogenase in rat tissues. *Methods Enzymol, 166,* 189-201.

Harper, A. E., Miller, R. H., & Block, K. P. (1984). Branched-chain amino acid metabolism. *Annu Rev Nutr, 4,* 409-454.

Harris, R. A., Paxton, R., & Parker, R. A. (1982). Activation of the branched-chain alpha-ketoacid dehydrogenase complex by a broad specificity protein phosphatase. *Biochem Biophys Res Commun, 107*(4), 1497-1503.

Hutson, S. M., Berkich, D., Drown, P., Xu, B., Aschner, M., & LaNoue, K. F. (1998). Role of branched-chain aminotransferase isoenzymes and gabapentin in neurotransmitter metabolism. *J Neurochem, 71*(2), 863-874.

Hutson, S. M., & Harper, A. E. (1981). Blood and tissue branched-chain amino and alpha-keto acid concentrations: Effect of diet, starvation, and disease. *Am J Clin Nutr, 34*(2), 173-183.

Hutson, S. M., Wallin, R., & Hall, T. R. (1992). Identification of mitochondrial branched chain aminotransferase and its isoforms in rat tissues. *J Biol Chem, 267*(22), 15681-15686.

Keen, R. E., Nissenson, C. H., & Barrio, J. R. (1993). Analysis of femtomole concentrations of alpha-ketoisocaproic acid in brain tissue by precolumn fluorescence derivatization with 4,5-dimethoxy-1,2-diaminobenzene. *Anal Biochem, 213*(1), 23-28.

Mac, M., & Nalecz, K. A. (2003). Expression of monocarboxylic acid transporters (mct) in brain cells. Implication for branched chain

alpha-ketoacids transport in neurons. *Neurochem Int, 43*(4-5), 305-309.

Matsuda, T., & Cooper, J. R. (1983). Inhibition of neuronal sodium and potassium ion activated adenosinetriphosphatase by pyrithiamin. *Biochemistry, 22*(9), 2209-2213.

Matsuo, Y., Yagi, M., & Walser, M. (1993). Arteriovenous differences and tissue concentrations of branched-chain ketoacids. *J Lab Clin Med, 121*(6), 779-784.

McCandless, D. W., Schenker, S., & Cook, M. (1968). Encephalopathy of thiamine deficieny: Studies of intracerebral mechanisms. *J Clin Invest, 47*(10), 2268-2280.

McKenna, M. C., Sonnewald, U., Huang, X., Stevenson, J., Johnsen, S. F., Sande, L. M., et al. (1998). Alpha-ketoisocaproate alters the production of both lactate and aspartate from [u-13c]glutamate in astrocytes: A ^{13}C-NMR study. *J Neurochem, 70*(3), 1001-1008.

Morton, D. H., Strauss, K. A., Robinson, D. L., Puffenberger, E. G., & Kelley, R. I. (2002). Diagnosis and treatment of maple syrup disease: A study of 36 patients. *Pediatrics, 109*(6), 999-1008.

Munujos, P., Coll-Canti, J., Beleta, J., Gonzalez-Sastre, F., & Gella, F. J. (1996). Brain pyruvate oxidation in experimental thiamin-deficiency encephalopathy. *Clin Chim Acta, 255*(1), 13-25.

Navarro, D., Zwingmann, C., Hazell, A. S., & Butterworth, R. F. (2005). Brain lactate synthesis in thiamine deficiency: A re-evaluation using ^{1}H-^{13}C nuclear magnetic resonance spectroscopy. *J Neurosci Res, 79*(1-2), 33-41.

Pardridge, W. M. (1983). Brain metabolism: A perspective from the blood-brain barrier. *Physiol Rev, 63*(4), 1481-1535.

Paxinos, G., & Watson, C. (1982). *The rat brain in stereotaxic coordinates.* New York: Academic Press, Inc.

Paxton, R., & Harris, R. A. (1984). Regulation of branched-chain alpha-ketoacid dehydrogenase kinase. *Arch Biochem Biophys, 231*(1), 48-57.

Pekovich, S. R., Martin, P. R., & Singleton, C. K. (1996). Thiamine pyrophosphate-requiring enzymes are altered during pyrithiamine-induced thiamine deficiency in cultured human lymphoblasts. *J Nutr, 126*(7), 1791-1798.

Plaitakis, A., Nicklas, W. J., & Berl, S. (1979). Alterations in uptake and metabolism of aspartate and glutamate in brain of thiamine deficient animals. *Brain Res, 171*(3), 489-502.

Shimomura, Y., Obayashi, M., Murakami, T., & Harris, R. A. (2001). Regulation of branched-chain amino acid catabolism: Nutritional and hormonal regulation of activity and expression of the branched-chain alpha-keto acid dehydrogenase kinase. *Curr Opin Clin Nutr Metab Care, 4*(5), 419-423.

Sweatt, A. J., Garcia-Espinosa, M. A., Wallin, R., & Hutson, S. M. (2004). Branched-chain amino acids and neurotransmitter metabolism: Expression of cytosolic branched-chain aminotransferase (bcatc) in the cerebellum and hippocampus. *J Comp Neurol, 477*(4), 360-370.

Troncoso, J. C., Johnston, M. V., Hess, K. M., Griffin, J. W., & Price, D. L. (1981). Model of Wernicke's Encephalopathy. *Arch Neurol, 38*(6), 350-354.

Yudkoff, M. (1997). Brain metabolism of branched-chain amino acids. *Glia, 21*(1), 92-98.

Yudkoff, M., Daikhin, Y., Lin, Z. P., Nissim, I., Stern, J., Pleasure, D., et al. (1994). Interrelationships of leucine and glutamate metabolism in cultured astrocytes. *J Neurochem, 62*(3), 1192-1202.

Yudkoff, M., Daikhin, Y., Nelson, D., Nissim, I., & Erecinska, M. (1996). Neuronal metabolism of branched-chain amino acids: Flux through the aminotransferase pathway in synaptosomes. *J Neurochem, 66*(5), 2136-2145.

Yudkoff, M., Nissim, I., Daikhin, Y., Lin, Z. P., Nelson, D., Pleasure, D., et al. (1993). Brain glutamate metabolism: Neuronal-astrocytic relationships. *Dev Neurosci, 15*(3-5), 343-350.

Zielke, H. R., Huang, Y., Baab, P. J., Collins, R. M., Jr., Zielke, C. L., & Tildon, J. T. (1997). Effect of alpha-ketoisocaproate and leucine on the in vivo oxidation of glutamate and glutamine in the rat brain. *Neurochem Res, 22*(9), 1159-1164.

Zielke, H. R., Huang, Y., Tildon, J. T., Zielke, C. L., & Baab, P. J. (1996). Elevation of amino acids in the interstitial space of the rat brain following infusion of large neutral amino and keto acids by microdialysis: Alpha-ketoisocaproate infusion. *Dev Neurosci, 18*(5-6), 420-425.

Zwingmann, C., Leibfritz, D., & Hazell, A. S. (2003). Energy metabolism in astrocytes and neurons treated with manganese: Relation among cell-specific energy failure, glucose metabolism, and intercellular trafficking using multinuclear nmr-spectroscopic analysis. *J Cereb Blood Flow Metab, 23*(6), 756-771.

LEGENDS

Figure 1: Effects of thiamine deficiency on plasma concentrations of leucine, isoleucine, valine and α-KIC

Metabolite concentrations were calculated by integration of the respective signals in 1H-NMR spectra obtained from blood plasma samples of pair-fed control (PFC) rats compared with rats at the presymptomatic (PS) and symptomatic (SYM) stages of thiamine deficiency. Values represent means ± SD for n = 5 animals.

Figure 2: Effects of thiamine deficiency on regional BCAA concentrations in rat brain

BCAA and α-KIC concentrations were calculated by integration of the respective signals in 1H-NMR spectra of brain tissue extracts obtained from the frontal cortex and medial thalamus of pair-fed control (PFC), presymptomatic (PS) and symptomatic (SYM) TD rats. Values represent means ± SD for n = 5 animals. Note: Other BCKAs remained undetectable. n.d.: not detectable.

Table 1: Regional effects of thiamine deficiency on BCKDH activity in rat brain

BCKDH activity was assayed radiochemically via the production of 14CO2 from α-keto[1-14C]isocaproate, where one unit (U) of BCKDH activity catalyzes the formation of 1μmol CO2/min. Actual BCKDH was measured with the in vivo phosphorylation state of the complex preserved; while total BCKDH activity, an indication of full enzyme capacity, was determined (in the presence of exogenous TDP [+TDP]) from separate aliquots of brain tissue extracts following complete activation (dephosphorylation) of the enzyme complex with a broad specificity phosphatase. Latent BCKDH activity (calculated from the difference in actual activity with [+TDP] and without [-TDP] exogenously added TDP in the assay, divided by the actual activity, times 100) signifies the proportion of BCKDH that requires TDP (apoenzyme) for the expression of activity. Activity state represents the ratio of actual activity [+TDP] to total activity, times 100. Values represent means ± SD for n =6. Data between all experimental groups was analyzed using ANOVA and post-hoc Tukey's test († significantly different from pair-fed control (PFC) rats; ‡ significantly different at symptomatic (SYM) versus presymptomatic (PS) stage of thiamine deficiency; * $p < 0.01$; ** $p < 0.001$).

Figure 1:

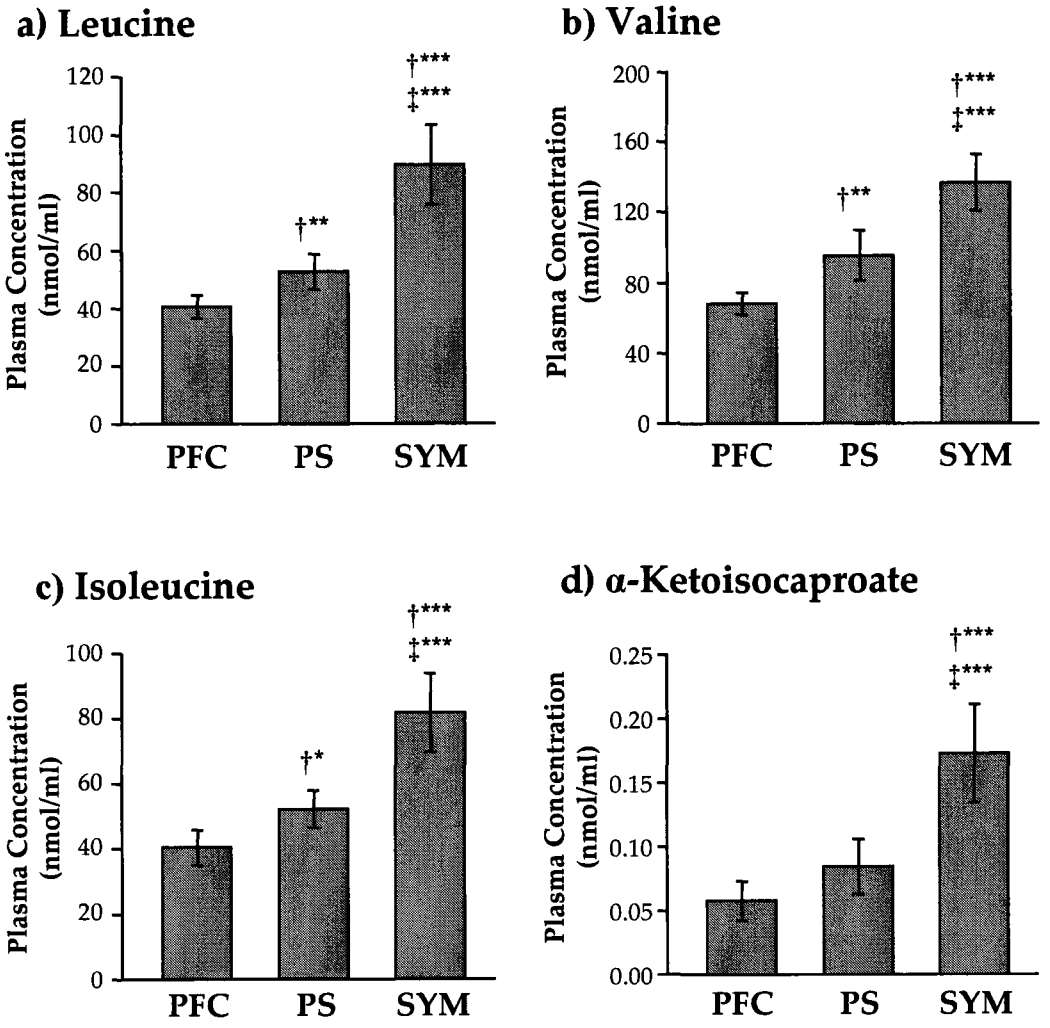

a) Leucine
b) Valine
c) Isoleucine
d) α-Ketoisocaproate
Plasma Concentration (nmol/ml)
PFC
PS
SYM
†**
†***
‡***
†*

Figure 2:

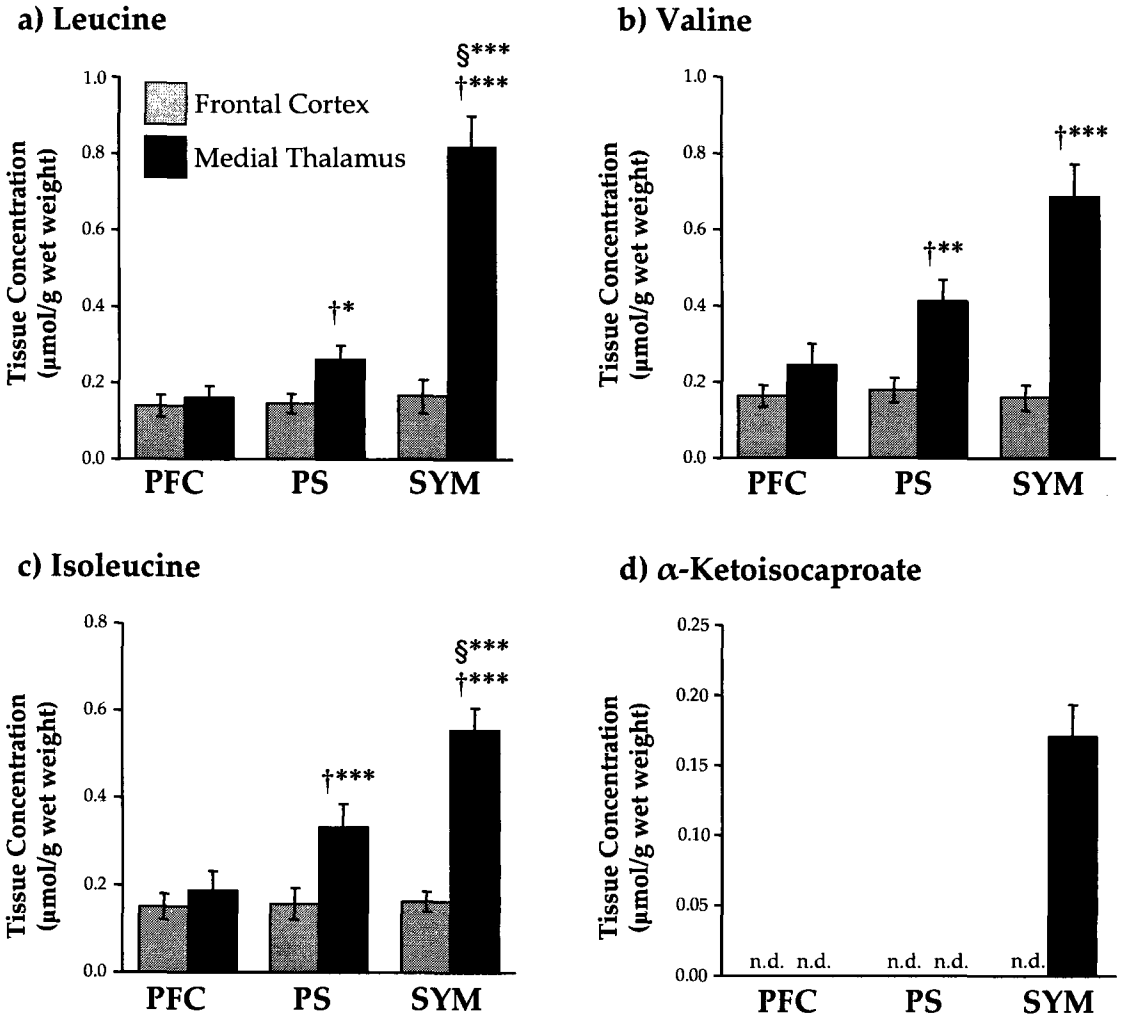

a) Leucine
b) Valine
c) Isoleucine
d) α-Ketoisocaproate
Frontal Cortex
Medial Thalamus
Tissue Concentration (µmol/g wet weight)
PFC
PS
SYM
§***
†***
†*
†**
n.d.

Table 1:

Brain Region	Group	BCKDH Actual Activity		Total Activity	Latent	Activity
		[-TDP]	[+TDP]	[+TDP]	Activity	State
		(mU/g wet weight)	(mU/g wet weight)	(mU/g wet weight)	(%)	(%)
Frontal Cortex	PFC	24.1 ± 3.4	30.5 ± 2.8	54.4 ± 3.1	19.1 ± 4.6	56.1 ± 2.4
	PS	21.9 ± 2.8	27.5 ± 2.7	50.4 ± 2.8	20.7 ± 4.9	54.6 ± 3.5
	SYM	18.2 ± 3.1 †*	26.9 ± 4.6	52.5 ± 2.6	34.9 ± 6.3 †** ‡*	53.4 ± 7.5
Medial Thalamus	PFC	19.1 ± 2.4	24.3 ± 2.6	35.8 ± 3.1	21.6 ± 2.8	67.9 ± 2.2
	PS	10.2 ± 2.6 †**	27.8 ± 2.4	36.2 ± 2.9	63.7 ± 6.8 †**	76.8 ± 3.1 †*
	SYM	6.4 ± 2.8 †**	29.9 ± 2.6 †*	33.8 ± 3.2	79.1 ± 7.8 †** ‡*	88.5 ± 5.5 †** ‡**

DISCUSSION

The following section of this thesis project constitutes a discussion and overview of the main findings of each article followed by a discussion of the significance of the results as a whole.

Effects of thiamine deficiency on lactate de novo synthesis in brains of thiamine-deficient (TD) rats

Lactate accumulation in brain is a consistent observation in experimental and clinical thiamine deficiency; however, the exact origin of this lactate has not been previously established. The data presented in Article 1 (Navarro et al., 2005) demonstrate that pyrithiamine-induced thiamine deficiency in rats results in increased production of lactate from the precursor [1-^{13}C]glucose in the medial thalamus (MT) of TD rats, indicating increased de novo synthesis of lactate via glycolysis. These findings confirm and extend earlier observations of lactate accumulation in selectively vulnerable brain regions, such as the MT, which ultimately exhibit neuronal cell death and mitochondrial dysfunction (McCandless, 1982; Munujos *et al.*, 1993).

Pyruvate recycling, which preferentially occurs within the astrocytic rather than the neuronal compartment, brought about only minor contributions to the accumulation of lactate within the TD rat brain. It has been suggested that astrocyte-derived lactate, formed via glycolysis, may serve as an energy substrate for neurons, particularly in situations of impaired glucose metabolism (Pellerin & Magistretti, 1994; Pellerin *et al.*, 1998). Lactate concentrations were also elevated in the blood plasma;

however, contributions of peripheral lactate to the accumulations observed within the TD rat brain were nominal.

Immunohistochemical analysis of lactate dehydrogenase (LDH) expression in the TD rat brain sections revealed increased immunolabeling of both LDH-1 and LDH-5 isoenzymes in the MT; although the mechanism by which TD results in the upregulation of these isoenzymes has not been established. In the CNS, the LDH-5 isoenzyme is preferentially localized in astrocytes and favours the formation of lactate from pyruvate; while LDH-1 is inhibited by pyruvate and preferentially drives the enzyme activity towards formation of pyruvate (Cahn *et al.*, 1962; Markert *et al.*, 1975). It is likely that LDH-1 and LDH-5 are induced in response to the impaired pyruvate metabolism in the MT of TD rats; however, the metabolically compromised neurons in the TD brain are unable to effectively utilize this metabolite for energy production, thus promoting lactate accumulation.

Effects of glucose loading on local cerebral pH in the MT of TD rats

Studies in cerebral ischemic subjects and experimental animals have shown that hyperglycemia leads to extensive structural alterations, including enhanced neuronal necrosis, glial cell damage and alterations in microvasculature. Similarly, administration of a glucose load has been shown to have a worsening effect on symptoms and pathology in brains of TD patients (Wallis et al., 1978; Watson et al., 1981). Furthermore, dietary restriction has been shown to attenuate neuronal loss and blood-brain barrier breakdown in TD mice (Calingasan & Gibson, 2000a). Together, these data suggest that in conditions of impaired cerebral

energy metabolism, excess glucose may be detrimental to the brain parenchyma and cerebral vasculature.

The mechanism by which glucose administration precipitates worsening of neurological status in TD patients has not been previously established. One suspected mechanism, however, has been the hastened onset of localized acidosis resulting from the focal accumulation of lactate in selectively vulnerable brain regions, such as the MT. Acidosis has been previously demonstrated to occur in the MT at symptomatic stages of pyrithiamine-induced thiamine deficiency in rats (Hakim, 1984), commensurate with the appearance of neuronal cell death.

In Article 2 (Navarro *et al.*, 2007), TD rats were administered a glucose load at a (presymptomatic) time-point preceding the appearance of acidosis and neuronal cell death. In order to assess whether glucose loading could precipitate the onset of acidosis observed in the MT, regional cerebral pH was measured autoradiographically at this time-point using the pH marker [^{14}C]DMO. Results of Article 2 indicate that following administration of a glucose load to presymptomatic TD rats, concentrations of lactate in the MT can be elevated as high as 21.86 ± 2.26 μmol/g (wet weight); and the local cerebral pH at this time-point was shown to decrease significantly, while no such alterations were observed in the FC. In prolonged periods of hypoxia and ischemia, brain tissue recovery has been shown to vary with the nutritional state of the animals, and previous results suggest that tissue lactate levels exceeding approximately 20μmol/g (wet weight) can be detrimental to recovery (Myers, 1979; Rehncrona et al., 1980).

Brain tissue acidosis is a result of either an increase in tissue pCO_2 or an accumulation of acids produced by metabolism; and acidosis likely

contributes to the selective lesions observed in the TD rat brain. An acidic environment can have harmful effects on the metabolic machinery of cells (Rehncrona et al., 1980; Van Nimmen et al., 1986), and may lead to deleterious hemodynamic changes (Kuschinsky et al., 1972). In addition, altered pH is known to adversely affect vascular tone (Kim et al., 2004) and calcium homeostasis (Hriciga & Lehn, 1983).

The mechanism by which acidosis brings about tissue damage in the TD brain has not been fully elucidated; however, studies by Li and Siesjö (1997) indicated that the effect of acidosis in ischemia-reperfusion brain injury is not mediated by a further perturbation of cell calcium metabolism, but more likely through the production of free radicals. In particular, acidosis is known to enhance iron-catalyzed production of reactive oxygen species, probably releasing iron from its bindings to transferrin, ferritin and other proteins. Similarly, oxidative stress is a prominent feature of TD neuropathology, as evidenced by the accumulation of heme oxygenase-1, ferritin, reactive iron and superoxide dismutase in microglia, nitrotyrosine and 4-hydroxynonenal in neurons, as well as induction of endothelial nitric oxide synthase within regions of the brain regions known to be vulnerable to thiamine deficiency (Calingasan et al., 2000; Kruse et al., 2004). Furthermore, endothelial cells are believed to be probable targets of free radicals (Kontos, 1985, 1989). Together, these data suggest that the disruptions to the blood-brain barrier which have been documented in the vulnerable regions of the TD brain are due, at least in part, to acidosis-mediated damage.

Effects of thiamine deficiency on *de novo* synthesis of neurotransmitter amino acids in the TD rat brain

Region-specific alterations in the steady state levels of cerebral amino acid neurotransmitters have been well-documented in experimental animal models of thiamine deficiency (Butterworth *et al.*, 1979; Butterworth & Heroux, 1989; Gaitonde, 1975; Gaitonde *et al.*, 1975; Plaitakis *et al.*, 1979); however, the dynamics of these changes have never been systematically explored. Application of $^{1}H/^{13}C$-NMR enables the examination of these regional changes in amino acid concentrations in relation to the relative contribution of the various competing pathways of glucose metabolism.

Article 3 (Navarro *et al.*, 2008) demonstrates that reduced aspartate concentrations are the earliest predictor of regional vulnerability in the MT of TD rats. The relative decrease in levels of aspartate observed in the absence of alterations in glutamate and GABA concentrations suggests that an early impairment in α-KGDH precedes impairments in PDH flux. ^{13}C isotopomer analysis indicated that the early reduction in aspartate concentrations is due primarily to decreased PDH-mediated synthesis of aspartate resulting from diminished TCA cycle flux at the level of α-KGDH, while the synthesis of aspartate via the more direct astrocytic anaplerotic pathway is relatively maintained. Ultimately, the metabolic block at the level of α-KGDH results in a reduced pool of oxaloacetate, a metabolite that is required for the continued oxidation of acetyl-CoA. Consequently, aspartate pools are consumed to replenish the limiting pools of oxaloacetate; and decreased pyruvate oxidation ensues.

Effects of thiamine deficiency and glucose loading on *in vivo* metabolic flux through PDH and α-KGDH

Although activities of thiamine-dependent enzymes of glucose metabolism have been studied extensively (Butterworth et al., 1985, 1986; Gaitonde *et al.*, 1975), few previous studies have examined the effects of thiamine deficiency on in vivo metabolic fluxes through PDH and α-KGDH. Furthermore, the cellular localization of these metabolic changes has not previously been elucidated. These issues were addressed in Article 3 through the application of ex vivo high-resolution ^{1}H- and ^{13}C-NMR spectroscopy.

Results of Article 3 provide the first direct evidence that an early impairment of α-KGDH is responsible for the decreased glucose (or pyruvate) oxidation in the TD rat brain. In addition, administration of a glucose load caused further decreases in TCA cycle turnover and precipitated impairments in PDH flux. Detailed ^{13}C isotopomer analysis suggests that inhibition of flux through α-KGDH in TD brain occurs primarily in the neurons, while astrocytes possess compensatory mechanisms, i.e. the anaplerotic pathway, to replenish oxaloacetate concentrations via metabolic pathways that do not involve thiamine-dependent enzymes.

PDH is a key mitochondrial enzyme regulating oxidative pyruvate metabolism and, like α-KGDH, is thiamine-dependent. Biochemical studies, however, reveal that, unlike α-KGDH, activities of PDH are unchanged in brain in thiamine deficiency (Butterworth et al., 1985, 1986; Gaitonde et al., 1975). Impaired pyruvate oxidation has been shown to occur in brain regions vulnerable to TD only at later stages of thiamine deficiency. Glucose loading of TD rats at the presymptomatic stage

exacerbated the decreases in TCA cycle flux in the MT, while precipitating a decrease in the flux through PDH. These results demonstrate a possible metabolic mechanism for the worsening of neurological status observed in both TD patients and experimental animals following parenteral administration of glucose.

Effects of thiamine deficiency on branched-chain amino acid metabolism in TD rat brain

Article 4 investigated the effects of thiamine deficiency on regional BCKDH activity and BCAA metabolism in the rat brain. Previous studies have shown that the BCAAs, particularly leucine, play an important role in nitrogen balance and the synthesis of glutamate within the brain (Bixel et al., 1997; Hutson et al., 1998; Yudkoff, 1997; Yudkoff *et al.*, 1996a; Zielke et al., 1997), with approximately 30–50% of all α-amino groups of brain glutamate and glutamine derived from the BCAAs (Yudkoff *et al.*, 1993). The present findings address the involvement of BCAA metabolism in the regional vulnerability observed in the TD rat brain.

In order to assess the effects of thiamine deficiency on BCAA metabolism in the brain, regional BCAA levels were determined, and BCKDH activity was assayed radiochemically via the production of $^{14}CO_2$ from the precursor [1-^{14}C]α-KIC. Results in Article 4 show that thiamine deficiency leads to focal accumulations of BCAAs in the MT of TD rats. Increases of leucine concentrations, in particular, were elevated over 5-fold in MT of SYM TD rats compared to PFC rats, concomitant with 3-fold elevations in levels of isoleucine and valine. BCAA concentrations, however, remained unaltered within the FC of TD rats. Data in Article 4 also provided the first direct evidence of regional effects of thiamine

deficiency on BCKDH activity in the rat brain. Thiamine deficiency in rats caused significant reductions in BCKDH actual activity in both the FC and MT rats; however, these activity reductions were less pronounced and had later onset in the FC, a region of the brain that is typically spared from lesions in thiamine deficiency. Together, these observations denote an inherent regional variation in the oxidative capacity of BCKDH within the TD rat brain, with a higher capacity for BCAA oxidation in the FC compared with the MT. These findings are in line with previous studies demonstrating regional variations in BCKDH distribution in the rat brain (Brosnan *et al.*, 1985).

Astrocytes, which are in close proximity to brain capillaries, are likely the initial site of BCAA metabolism; and mitochondrial branched-chain aminotransferase (BCAT; EC 2.6.1.42) in these cells results in the transamination of most BCAAs entering the brain (Hutson *et al.*, 1992). The product of BCAA transamination with α-ketoglutarate is glutamate and a BCKA; in the case of leucine, this α-ketoacid is α-KIC:

BCAT reaction: Leucine + α-Ketoglutarate ⇔ α-KIC + Glutamate

Rather than accumulating within the astrocytes, α-KIC has been shown to be released into the extracellular fluid (Mac & Nalecz, 2003; Yudkoff, 1997) where it can be taken up by neurons. Although the brain can oxidize α-KIC to CO_2, this BCKA is typically reaminated back to leucine, which can diffuse back to the astrocytes (Matsuda & Cooper, 1983; Pardridge, 1983) where it can act, once again, as an amino group donor for glutamate synthesis. This "reverse" transamination of α-KIC back to leucine likely takes place within neurons, which almost

exclusively possess a cytosolic form of BCAT (Sweatt et al., 2004). This cycling of amino groups between glutamate and leucine, combined with the cellular and subcellular compartmentalization of BCAA metabolism, most certainly provides an additional means of buffering glutamate levels within the brain.

The elevated cerebral BCAA concentrations combined with impaired BCKA oxidation observed in MT of TD rats would promote the accumulation of potentially toxic BCKAs, such as α-KIC. The concentration of α-KIC is maintained relatively low within the brain via transamination back to leucine (and to a lesser extent α-KIC oxidation or release from the brain), consuming glutamate and producing α-ketoglutarate in the process. Consequently, the increased α-ketoglutarate: glutamate ratio resulting from α-KIC transamination back to leucine by neuronal BCAT would intensify aspartate utilization through aspartate aminotransferase (AAT), thus contributing to the diminishing aspartate pools observed in the MT of TD rats.

AAT reaction: Aspartate + α-Ketoglutarate ⇔ Oxaloacetate + Glutamate

Declining aspartate pools would lead to the diminution of oxaloacetate pools, which would ultimately result in impaired TCA cycle flux and impaired pyruvate metabolism. In addition, aspartate depletion could compromise the malate-aspartate shuttle rendering it ineffective in transferring reducing equivalents from the cytoplasm to the mitochondria. Altogether, this would contribute to the energy failure and lactic acidosis that have been characterized in the MT of TD rats (Butterworth *et al.*, 1993; Navarro et al., 2005).

CONCLUSIONS

Conclusions from the studies reported in the four articles which constitute this thesis include the following:

1. Pyrithiamine-induced thiamine deficiency in rats results in increased de novo synthesis of lactate, and increased LDH expression, selectively in the MT.

The data presented in Article 1 clearly demonstrate that focal impairments in pyruvate metabolism lead to increased de novo synthesis of lactate via glycolysis. This lactate accumulates at detrimental concentrations within vulnerable brain structures such as the MT. Furthermore, this increased lactate synthesis and accumulation in the MT is exacerbated following the administration of a glucose load. These findings suggest that lactate accumulation is involved in the glucose-precipitated worsening of neurological status in TD patients.

Increased immunolabeling of LDH-1 and LDH-5 observed in the MT of TD rats likely represents the cellular induction response to impaired pyruvate metabolism in the MT of TD rats. The metabolically compromised neurons in the MT, however, are unable to effectively utilize this metabolite for energy production, thus promoting lactate accumulation in this vulnerable brain region.

2. Administration of a glucose load to TD rats precipitates a focal acidosis in the MT.

Results of Article 2 suggest that the untoward accumulation of lactate selectively in the MT leads to a focal acidosis, which can have detrimental effects on cellular metabolism and/or enhance iron-catalyzed production of reactive oxygen species. The present findings portend that decreased local cerebral pH plays an important role in the pathogenesis of TD encephalopathies; and that the administration of a glucose load can precipitate this localized lactic acidosis in the TD brain.

3. Pyrithiamine-induced thiamine deficiency in rats results in early impairments in TCA cycle flux selective to the MT, leading to secondary reductions in pyruvate oxidation: effects on de novo synthesis of neurotransmitter amino acids.

The multinuclear NMR studies presented in Article 3 clearly demonstrate that an initial impairments in TCA cycle flux in the MT of TD rats is responsible for subsequent impaired pyruvate oxidation. The early (presymptomatic) reductions in aspartate de novo synthesis in the absence of changes in the de novo synthesis of glutamate and GABA further substantiates the metabolic block at the level α-KGDH. Reductions in the de novo synthesis of glutamate and GABA manifest at later, symptomatic stages of thiamine deficiency, as a consequence of impaired pyruvate flux through PDH at this time-point.

Administration of a glucose load in presymptomatic TD rats precipitates a decrease in pyruvate flux through PDH, which is not typically observed at this time-point. Additionally, the reduction in PDH flux following glucose loading results in even further declines in

TCA cycle turnover, suggesting an involvement in the glucose-precipitated worsening of neurological status in TD patients.

4. Thiamine deficiency causes significant impairments in BCKDH activity in the MT of TD rats: contribution to the biochemical lesion.

Data in Article 4 indicates that focal impairments to BCAA oxidation at the level of thiamine-dependent BCKDH lead to the accumulation of BCAAs and their cognate α-ketoacids in the MT of TD rats. In particular, the BCKA of leucine, α-KIC, has been shown to be toxic to the brain at elevated concentrations; and thus, elevated levels of this metabolite may contribute to the selective vulnerability of the MT to thiamine deficiency. Analysis of regional differences in BCKDH total activity in the TD rat brain confirms an inherently lower oxidative capacity for BCAA oxidation in the MT compared with the FC. Together, these results suggest that impaired BCAA metabolism in the MT may contribute to the selective biochemical lesion observed in the TD rat brain.

SUMMARY

The data presented in the four articles comprising this thesis elucidate some of the mechanisms contributing to the manifestation of neuronal cell death in the MT of the TD rat brain following pyrithiamine-induced thiamine deficiency (see Figure 1).

Focal impairments in pyruvate metabolism, resulting largely from decreased activities of α-KGDH, lead to increased de novo synthesis of lactate which accumulates at detrimental concentrations within vulnerable brain structures such as the MT. Furthermore, this increased lactate synthesis and accumulation in the MT is exacerbated following the administration of a glucose load. A key consequence of elevated lactate levels is to make the cytosolic redox potential more reducing, which, in turn, can inhibit pyruvate oxidation. The untoward accumulation of lactate in the MT can cause focal acidosis, which can have detrimental effects on cellular metabolism and/or enhance iron-catalyzed production of reactive oxygen species. The present findings portend that decreased local cerebral pH plays an important role in the pathogenesis of TD encephalopathies; and that the administration of a glucose load can precipitate this localized lactic acidosis in the TD brain.

Further contributing to the selective vulnerability of the MT to thiamine deficiency is the early decline in neuronal oxaloacetate pools, caused by metabolic impairments to the neuronal TCA cycle at the level of α-KGDH. Declining oxaloacetate pools lead to impaired pyruvate oxidation and the diminution of aspartate pools. In addition, aspartate depletion would compromise the malate-aspartate shuttle rendering it ineffective in transferring reducing equivalents from the cytoplasm to the

mitochondria. Altogether, this would contribute to the energy failure and lactic acidosis that have been characterized in the MT of TD rats.

Focal impairments to BCAA oxidation at the level of BCKDH lead to the accumulation of BCAAs and their cognate α-ketoacids in the MT of TD rats. In particular, the BCKA of leucine, α-KIC, has been shown to be toxic to the brain at elevated concentrations; and thus, levels of this metabolite are actively minimized within the brain via neuronal transamination back to leucine. Consequently, the increased α-ketoglutarate-to-glutamate ratio resulting from α-KIC transamination back to leucine, combined with the metabolic block at α-KGDH, would drive the aspartate aminotransferase reaction, thereby intensifying consumption of neuronal aspartate pools in the MT of TD rats.

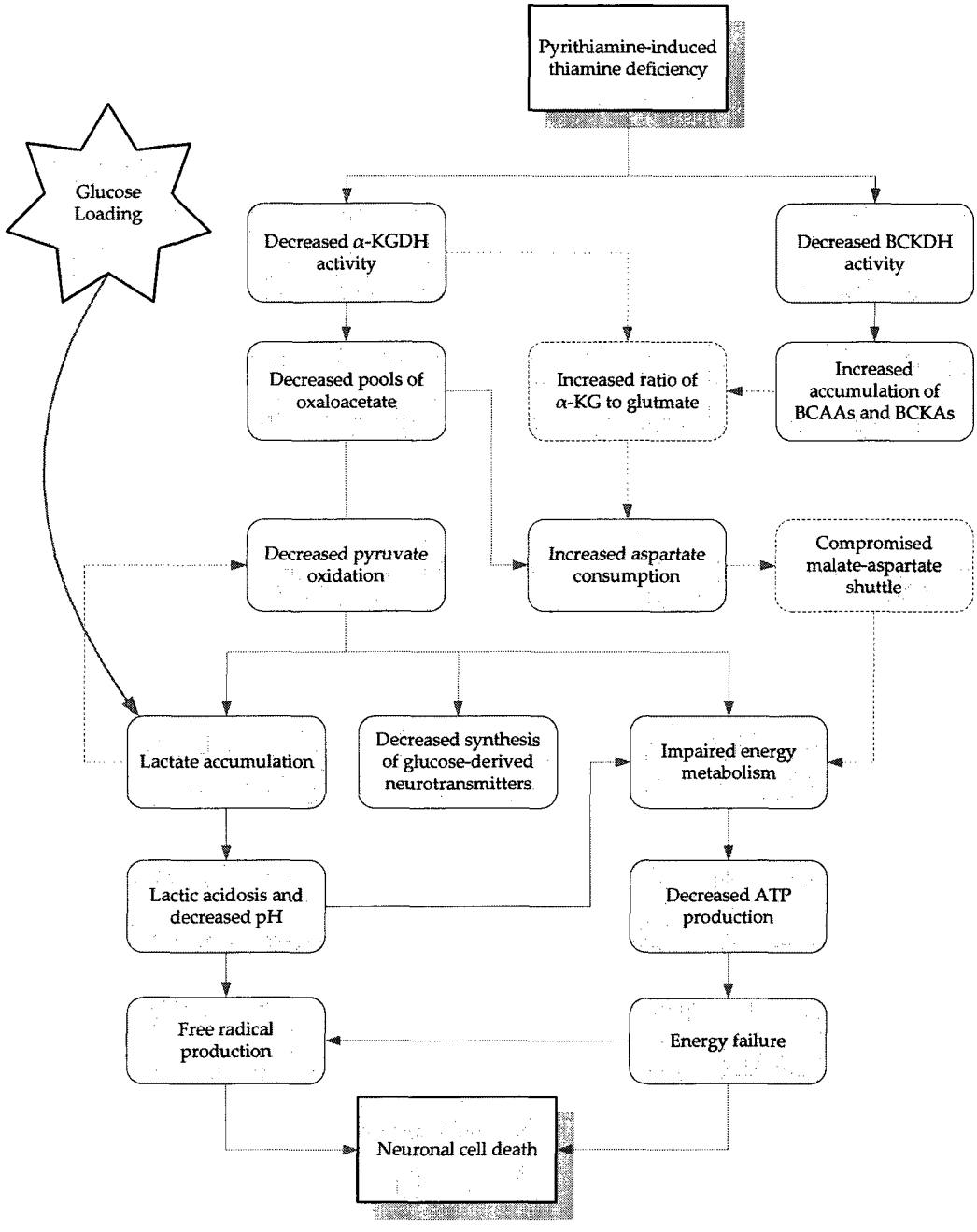


Figure 1. Integrated schematic diagram illustrating possible mechanisms of how pyrithiamine-induced thiamine deficiency leads to neuronal cell death in the medial thalamus of TD rats; and how glucose loading may precipitate worsening of neurological status. Dashed lines describe intermediate stages that may be involved but which remain unestablished at the present time. Acronyms: α-KGDH, α-ketoglutarate dehydrogenase; α-KG, α-ketoglutarate; BCAAs, branched-chain amino acids; BCKAs,

branched-chain α-ketoacids; BCKDH, branched-chain α-ketoacid dehydrogenase; ATP, adenosine triphosphate.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

These elements constitute original scholarship and advancement in knowledge in the study of thiamine deficiency in neuroscience research:

- Lactate accumulation in vulnerable regions of the pyrithiamine-induced TD rat brain results from increased lactate *de novo* synthesis via glycolysis, and to a lesser extent pyruvate recycling in astrocytes.
- Expression of both LDH-1 and LDH-5 isoenzymes are upregulated in the MT of TD rats, likely contributing to the increased synthesis of lactate in this vulnerable brain region.
- Glucose loading exacerbates lactate *de novo* synthesis and accumulation in the MT of TD rats, thereby precipitating focal acidosis.
- Thiamine deficiency diminishes *de novo* synthesis of glutamate, GABA and aspartate in the MT; however declines in *de novo* synthesis of aspartate were the earliest predictor of neuronal vulnerability. Early reductions in aspartate levels in the absence of changes to glutamate levels signify that impairments to α-KGDH precede impairments to PDH flux.
- Impaired metabolic flux through PDH occurs secondary to impairments in TCA cycle flux and early reductions in aspartate pools in the MT of TD rats.
- Detailed ^{13}C-isotopomer analysis indicates that impaired flux through α-KGDH and decreased aspartate synthesis occurs principally in neurons, while astrocytes possess compensatory mechanisms, i.e. the anaplerotic pathway, to replenish oxaloacetate concentrations via metabolic pathways that do not involve thiamine-dependent enzymes.

- Glucose loading exacerbates decreases in TCA cycle flux and precipitates impairments in pyruvate dehydrogenase flux, leading to even further reductions in *de novo* synthesis of aspartate and glutamate in the MT.
- Detailed ^{13}C-isotopomer analysis confirms that impaired metabolic flux through PDH occurs secondarily to impairments in TCA cycle flux and early reductions in aspartate pools in the MT of TD rats.
- Thiamine deficiency causes substantial impairments to BCAA metabolism in the MT. This is a result of significant decreases in BCKDH actual activity in this brain structure, compounded by the inherently lower BCAA oxidative capacity (BCKDH total activity) in the MT compared to the FC.
- Thiamine deficiency results in focal accumulation of BCAAs in the MT; while BCAA concentrations are unaffected in the FC of TD rats. In addition, notable accumulations of potentially toxic α-KIC were observed in the MT of TD rats.

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my supervisor, Dr. Roger F. Butterworth, for his role in the supervision and guidance of my thesis project during my time at the Neuroscience Research Unit at Hôpital Saint-Luc. He has been an instrumental factor in the development of my scientific thought process and my writing skills.

I would also like to acknowledge Dr. Claudia Zwingmann, Dr. Paul Desjardins and Dr. Alan Hazell, who have helped me to develop my laboratory skills and provided a constant forum for many interesting discussions. Similar appreciation is extended to my fellow colleagues who have provided a nurturing and enjoyable learning atmosphere.

Furthermore, I would like to thank my family for all the love and support they have shown me throughout the duration of this degree. In particular, I would like to give special thanks to my mother and father who have given me all the opportunities in life and have encouraged me to pursue my goals and aspirations.

Last but certainly not least, I would like to dedicate this thesis to my incredible network of friends, especially Manny Resch, Tony Saker, Jeff Dalziel, Homero Brum, Michel Sheehy and Yair Lenga, whose constant encouragement and inspiration reinforce for me what is really important in life.

LIST OF REFERENCES

Aftring, R. P., Block, K. P., & Buse, M. G. (1986). Leucine and isoleucine activate skeletal muscle branched-chain alpha-keto acid dehydrogenase in vivo. *Am J Physiol, 250*(5 Pt 1), E599-604.

Aikawa, H., Watanabe, I. S., Furuse, T., Iwasaki, Y., Satoyoshi, E., Sumi, T., et al. (1984). Low energy levels in thiamine-deficient encephalopathy. *J Neuropathol Exp Neurol, 43*(3), 276-287.

Bettendorff, L. (1994a). The compartmentation of phosphorylated thiamine derivatives in cultured neuroblastoma cells. *Biochim Biophys Acta, 1222*(1), 7-14.

Bettendorff, L. (1994b). Thiamine in excitable tissues: Reflections on a non-cofactor role. *Metab Brain Dis, 9*(3), 183-209.

Bettendorff, L., Grandfils, C., De Rycker, C., & Schoffeniels, E. (1986). Determination of thiamine and its phosphate esters in human blood serum at femtomole levels. *J Chromatogr, 382*, 297-302.

Bettendorff, L., Hennuy, B., Wins, P., & Schoffeniels, E. (1993a). Thiamin and derivatives as modulators of rat brain chloride channels. *Neuroscience, 52*(4), 1009-1017.

Bettendorff, L., Peeters, M., Wins, P., & Schoffeniels, E. (1993b). Metabolism of thiamine triphosphate in rat brain: Correlation with chloride permeability. *J Neurochem, 60*(2), 423-434.

Bettendorff, L., Weekers, L., Wins, P., & Schoffeniels, E. (1990). Injection of sulbutiamine induces an increase in thiamine triphosphate in rat tissues. *Biochem Pharmacol, 40*(11), 2557-2560.

Bettendorff, L., & Wins, P. (1994). Mechanism of thiamine transport in neuroblastoma cells. Inhibition of a high affinity carrier by sodium channel activators and dependence of thiamine uptake on membrane potential and intracellular atp. *J Biol Chem, 269*(20), 14379-14385.

Bixel, M. G., Hutson, S. M., & Hamprecht, B. (1997). Cellular distribution of branched-chain amino acid aminotransferase isoenzymes among rat brain glial cells in culture. *J Histochem Cytochem, 45*(5), 685-694.

Boros, L. G. (2000). Population thiamine status and varying cancer rates between western, asian and african countries. *Anticancer Res, 20*(3B), 2245-2248.

Brosnan, M. E., Lowry, A., Wasi, Y., Lowry, M., & Brosnan, J. T. (1985). Regional and subcellular distribution of enzymes of branched-chain amino acid metabolism in brains of normal and diabetic rats. *Can J Physiol Pharmacol, 63*(10), 1234-1238.

Brunnekreeft, J. W., Eidhof, H., & Gerrits, J. (1989). Optimized determination of thiochrome derivatives of thiamine and thiamine phosphates in whole blood by reversed-phase liquid chromatography with precolumn derivatization. *J Chromatogr, 491*(1), 89-96.

Butterworth, R. F. (1989). Effects of thiamine deficiency on brain metabolism: Implications for the pathogenesis of the wernicke-korsakoff syndrome. *Alcohol Alcohol, 24*(4), 271-279.

Butterworth, R. F. (1993). Pathophysiologic mechanisms responsible for the reversible (thiamine-responsive) and irreversible (thiamine non-responsive) neurological symptoms of wernicke's encephalopathy. *Drug Alcohol Rev, 12*(3), 315-322.

Butterworth, R. F., Gaudreau, C., Vincelette, J., Bourgault, A. M., Lamothe, F., & Nutini, A. M. (1991). Thiamine deficiency and wernicke's encephalopathy in aids. *Metab Brain Dis, 6*(4), 207-212.

Butterworth, R. F., Giguere, J. F., & Besnard, A. M. (1985). Activities of thiamine-dependent enzymes in two experimental models of thiamine-deficiency encephalopathy: 1. The pyruvate dehydrogenase complex. *Neurochem Res, 10*(10), 1417-1428.

Butterworth, R. F., Giguere, J. F., & Besnard, A. M. (1986). Activities of thiamine-dependent enzymes in two experimental models of thiamine-deficiency encephalopathy. 2. Alpha-ketoglutarate dehydrogenase. *Neurochem Res, 11*(4), 567-577.

Butterworth, R. F., Hamel, E., Landreville, F., & Barbeau, A. (1979). Amino acid changes in thiamine-deficient encephalopathy: Some implications for the pathogenesis of friedreich's ataxia. *Can J Neurol Sci, 6*(2), 217-222.

Butterworth, R. F., & Heroux, M. (1989). Effect of pyrithiamine treatment and subsequent thiamine rehabilitation on regional cerebral amino acids and thiamine-dependent enzymes. *J Neurochem, 52*(4), 1079-1084.

Butterworth, R. F., Kril, J. J., & Harper, C. G. (1993). Thiamine-dependent enzyme changes in the brains of alcoholics: Relationship to the wernicke-korsakoff syndrome. *Alcohol Clin Exp Res, 17*(5), 1084-1088.

Cahn, R. D., Zwilling, E., Kaplan, N. O., & Levine, L. (1962). Nature and development of lactic dehydrogenases: The two major types of this enzyme form molecular hybrids which change in makeup during development. *Science, 136*(3520), 962-969.

Calingasan, N. Y., & Gibson, G. E. (2000a). Dietary restriction attenuates the neuronal loss, induction of heme oxygenase-1 and blood-brain barrier breakdown induced by impaired oxidative metabolism. *Brain Res, 885*(1), 62-69.

Calingasan, N. Y., & Gibson, G. E. (2000b). Vascular endothelium is a site of free radical production and inflammation in areas of neuronal loss in thiamine-deficient brain. *Ann N Y Acad Sci, 903*, 353-356.

Calingasan, N. Y., Huang, P. L., Chun, H. S., Fabian, A., & Gibson, G. E. (2000). Vascular factors are critical in selective neuronal loss in an animal model of impaired oxidative metabolism. *J Neuropathol Exp Neurol, 59*(3), 207-217.

Casirola, D., Ferrari, G., Gastaldi, G., Patrini, C., & Rindi, G. (1988). Transport of thiamine by brush-border membrane vesicles from rat small intestine. *J Physiol, 398*, 329-339.

Casirola, D., Patrini, C., Ferrari, G., & Rindi, G. (1990). Thiamin transport by human erythrocytes and ghosts. *J Membr Biol, 118*(1), 11-18.

Chuang, D. T., Chuang, J. L., & Wynn, R. M. (2006). Lessons from genetic disorders of branched-chain amino acid metabolism. *J Nutr, 136*(1 Suppl), 243S-249S.

Cooper, J. R., & Pincus, J. H. (1979). The role of thiamine in nervous tissue. *Neurochem Res, 4*(2), 223-239.

Davis, R. E., & Icke, G. C. (1983). Clinical chemistry of thiamin. *Adv Clin Chem, 23*, 93-140.

Deolalkar, S. T., & Sohonie, K. (1954). Thiaminase from fresh-water, brackish-water and salt-water fish. *Nature, 173*(4402), 489-490.

Deolalkar, S. T., & Sohonie, K. (1957). Studies on thiaminase from fish. I. Properties on thiaminase. *Indian J Med Res, 45*(4), 571-586.

Douthit, H. A., & Airth, R. L. (1966). Thiaminase i of bacillus thiaminolyticus. *Arch Biochem Biophys, 113*(2), 331-337.

Dreyfus, P. M., & Victor, M. (1961). Effects of thiamine deficiency on the central nervous system. *Am J Clin Nutr, 9*, 414-425.

Ferrari, G., Ventura, U., & Rindi, G. (1971). The na plus-dependence of thiamin intestinal transport in vitro. *Life Sci I, 10*(2), 67-75.

Gaitonde, M. K. (1975). Conversion of [u-14c]threonine into 14c-labelled amino acids in the brain of thiamin-deficient rats. *Biochem J, 150*(2), 285-295.

Gaitonde, M. K., Fayein, N. A., & Johnson, A. L. (1975). Decreased metabolism in vivo of glucose into amino acids of the brain of thiamine-deficient rats after treatment with pyrithiamine. *J Neurochem, 24*(6), 1215-1223.

Gibson, G. E., Ksiezak-Reding, H., Sheu, K. F., Mykytyn, V., & Blass, J. P. (1984). Correlation of enzymatic, metabolic, and behavioral deficits in thiamin deficiency and its reversal. *Neurochem Res, 9*(6), 803-814.

Gibson, G. E., & Zhang, H. (2002). Interactions of oxidative stress with thiamine homeostasis promote neurodegeneration. *Neurochem Int, 40*(6), 493-504.

Goodwin, G. W., Zhang, B., Paxton, R., & Harris, R. A. (1988). Determination of activity and activity state of branched-chain alpha-keto acid dehydrogenase in rat tissues. *Methods Enzymol, 166*, 189-201.

Greenwood, J., Luthert, P. J., Pratt, O. E., & Lantos, P. L. (1986). Transport of thiamin across the blood-brain barrier of the rat in the absence of aerobic metabolism. *Brain Res, 399*(1), 148-151.

Gubler, C. J. (1968). Enzyme studies in thiamine deficiency. *Int Z Vitaminforsch, 38*(3), 287-303.

Hakim, A. M. (1984). The induction and reversibility of cerebral acidosis in thiamine deficiency. *Ann Neurol, 16*(6), 673-679.

Harata, N., Iwasaki, Y., & Ohara, Y. (1993). Reappraisal of regional thiamine content in the central nervous system of the normal and thiamine-deficient mice. *Metab Brain Dis, 8*(1), 45-59.

Harris, R. A., Joshi, M., & Jeoung, N. H. (2004). Mechanisms responsible for regulation of branched-chain amino acid catabolism. *Biochem Biophys Res Commun, 313*(2), 391-396.

Harris, R. A., Paxton, R., & Parker, R. A. (1982). Activation of the branched-chain alpha-ketoacid dehydrogenase complex by a broad specificity protein phosphatase. *Biochem Biophys Res Commun, 107*(4), 1497-1503.

Hayashi, K., Yoshida, S., & Kawasaki, T. (1981). Thiamine transport in the brush border membrane vesicles of the guinea-pig jejunum. *Biochim Biophys Acta, 641*(1), 106-113.

Hazell, A. S., Butterworth, R. F., & Hakim, A. M. (1993). Cerebral vulnerability is associated with selective increase in extracellular glutamate concentration in experimental thiamine deficiency. *J Neurochem, 61*(3), 1155-1158.

Hazell, A. S., Rao, K. V., Danbolt, N. C., Pow, D. V., & Butterworth, R. F. (2001). Selective down-regulation of the astrocyte glutamate transporters glt-1 and glast within the medial thalamus in experimental wernicke's encephalopathy. *J Neurochem, 78*(3), 560-568.

Herve, C., Beyne, P., & Delacoux, E. (1994). Determination of thiamine and its phosphate esters in human erythrocytes by high-performance liquid chromatography with isocratic elution. *J Chromatogr B Biomed Appl, 653*(2), 217-220.

Holowach, J., Kauffman, F., Ikossi, M. G., Thomas, C., & McDougal, D. B., Jr. (1968). The effects of a thiamine antagonist, pyrithiamine, on levels of selected metabolic intermediates and on activities of thiamine-dependent enzymes in brain and liver. *J Neurochem, 15*(7), 621-631.

Hoyumpa, A. M., Jr. (1982). Characterization of normal intestinal thiamin transport in animals and man. *Ann N Y Acad Sci, 378*, 337-343.

Hoyumpa, A. M., Jr., Breen, K. J., Schenker, S., & Wilson, F. A. (1975a). Thiamine transport across the rat intestine. Ii. Effect of ethanol. *J Lab Clin Med, 86*(5), 803-816.

Hoyumpa, A. M., Jr., Middleton, H. M., 3rd, Wilson, F. A., & Schenker, S. (1975b). Thiamine transport across the rat intestine. I. Normal characteristics. *Gastroenterology, 68*(5 Pt 1), 1218-1227.

Hoyumpa, A. M., Jr., Strickland, R., Sheehan, J. J., Yarborough, G., & Nichols, S. (1982). Dual system of intestinal thiamine transport in humans. *J Lab Clin Med, 99*(5), 701-708.

Hriciga, A., & Lehn, J. M. (1983). Ph regulation of divalent/monovalent ca/k cation transport selectivity by a macrocyclic carrier molecule. *Proc Natl Acad Sci U S A, 80*(20), 6426-6428.

Hutson, S. M., Berkich, D., Drown, P., Xu, B., Aschner, M., & LaNoue, K. F. (1998). Role of branched-chain aminotransferase isoenzymes and gabapentin in neurotransmitter metabolism. *J Neurochem, 71*(2), 863-874.

Hutson, S. M., Wallin, R., & Hall, T. R. (1992). Identification of mitochondrial branched chain aminotransferase and its isoforms in rat tissues. *J Biol Chem, 267*(22), 15681-15686.

James, A. G., Cook, R. M., West, S. M., & Lindsay, J. G. (1995). The pyruvate dehydrogenase complex of saccharomyces cerevisiae is regulated by phosphorylation. *FEBS Lett, 373*(2), 111-114.

Katzman, R., & H.M., P. (1973). *Brain electrolytes and fluid metabolism.* Baltimore: Williams & Wilkins.

Keen, R. E., Nissenson, C. H., & Barrio, J. R. (1993). Analysis of femtomole concentrations of alpha-ketoisocaproic acid in brain tissue by precolumn fluorescence derivatization with 4,5-dimethoxy-1,2-diaminobenzene. *Anal Biochem, 213*(1), 23-28.

Kimura, M., Fujita, T., & Itokawa, Y. (1982). Liquid-chromatographic determination of the total thiamin content of blood. *Clin Chem, 28*(1), 29-31.

Kimura, M., & Itokawa, Y. (1985). Determination of thiamine and its phosphate esters in human and rat blood by high-performance liquid chromatography with post-column derivatization. *J Chromatogr, 332,* 181-188.

Kobatake, K., Sako, K., Izawa, M., Yamamoto, Y. L., & Hakim, A. M. (1984). Autoradiographic determination of brain ph following middle cerebral artery occlusion in the rat. *Stroke, 15*(3), 540-547.

Kogure, K., Alonso, O. F., & Martinez, E. (1980). A topographic measurement of brain ph. *Brain Res, 195*(1), 95-109.

Komai, T., Kawai, K., & Shindo, H. (1974). Active transport of thiamine from rat small intestine. *J Nutr Sci Vitaminol (Tokyo), 20*(3), 163-177.

Kontos, H. A. (1985). George e. Brown memorial lecture. Oxygen radicals in cerebral vascular injury. *Circ Res, 57*(4), 508-516.

Kontos, H. A. (1989). Oxygen radicals in cns damage. *Chem Biol Interact, 72*(3), 229-255.

Kruse, M., Navarro, D., Desjardins, P., & Butterworth, R. F. (2004). Increased brain endothelial nitric oxide synthase expression in thiamine deficiency: Relationship to selective vulnerability. *Neurochem Int, 45*(1), 49-56.

Kuschinsky, W., Wahl, M., Bosse, O., & Thurau, K. (1972). Perivascular potassium and ph as determinants of local pial arterial diameter in cats. A microapplication study. *Circ Res, 31*(2), 240-247.

Laforenza, U., Gastaldi, G., & Rindi, G. (1993). Thiamine outflow from the enterocyte: A study using basolateral membrane vesicles from rat small intestine. *J Physiol, 468*, 401-412.

Laforenza, U., Orsenigo, M. N., & Rindi, G. (1998). A thiamine/h+ antiport mechanism for thiamine entry into brush border membrane vesicles from rat small intestine. *J Membr Biol, 161*(2), 151-161.

Langlais, P. J., Anderson, G., Guo, S. X., & Bondy, S. C. (1997). Increased cerebral free radical production during thiamine deficiency. *Metab Brain Dis, 12*(2), 137-143.

Langlais, P. J., & Mair, R. G. (1990). Protective effects of the glutamate antagonist mk-801 on pyrithiamine-induced lesions and amino acid changes in rat brain. *J Neurosci, 10*(5), 1664-1674.

Li, P. A., & Siesjo, B. K. (1997). Role of hyperglycaemia-related acidosis in ischaemic brain damage. *Acta Physiol Scand, 161*(4), 567-580.

Lowry, O. H. (1952). Biochemical evidence of nutritional status. *Physiol Rev, 32*(4), 431-448.

Luxemburger, C., White, N. J., ter Kuile, F., Singh, H. M., Allier-Frachon, I., Ohn, M., et al. (2003). Beri-beri: The major cause of infant mortality in karen refugees. *Trans R Soc Trop Med Hyg, 97*(2), 251-255.

Mac, M., & Nalecz, K. A. (2003). Expression of monocarboxylic acid transporters (mct) in brain cells. Implication for branched chain alpha-ketoacids transport in neurons. *Neurochem Int, 43*(4-5), 305-309.

Markert, C. L., Shaklee, J. B., & Whitt, G. S. (1975). Evolution of a gene. Multiple genes for ldh isozymes provide a model of the evolution of gene structure, function and regulation. *Science, 189*(4197), 102-114.

Matsuda, T., & Cooper, J. R. (1983). Inhibition of neuronal sodium and potassium ion activated adenosinetriphosphatase by pyrithiamin. *Biochemistry, 22*(9), 2209-2213.

Matsuo, Y., Yagi, M., & Walser, M. (1993). Arteriovenous differences and tissue concentrations of branched-chain ketoacids. *J Lab Clin Med, 121*(6), 779-784.

McCandless, D. W. (1982). Energy metabolism in the lateral vestibular nucleus in pyrithiamin-induced thiamin deficiency. *Ann N Y Acad Sci, 378*, 355-364.

McCandless, D. W., Schenker, S., & Cook, M. (1968). Encephalopathy of thiamine deficieny: Studies of intracerebral mechanisms. *J Clin Invest, 47*(10), 2268-2280.

McGready, R., Simpson, J. A., Cho, T., Dubowitz, L., Changbumrung, S., Bohm, V., et al. (2001). Postpartum thiamine deficiency in a karen displaced population. *Am J Clin Nutr, 74*(6), 808-813.

Morton, D. H., Strauss, K. A., Robinson, D. L., Puffenberger, E. G., & Kelley, R. I. (2002). Diagnosis and treatment of maple syrup disease: A study of 36 patients. *Pediatrics, 109*(6), 999-1008.

Munujos, P., Coll-Canti, J., Beleta, J., Gonzalez-Sastre, F., & Gella, F. J. (1996). Brain pyruvate oxidation in experimental thiamin-deficiency encephalopathy. *Clin Chim Acta, 255*(1), 13-25.

Munujos, P., Vendrell, M., & Ferrer, I. (1993). Proto-oncogene c-fos induction in thiamine-deficient encephalopathy. Protective effects of nicardipine on pyrithiamine-induced lesions. *J Neurol Sci, 118*(2), 175-180.

Murdock, D. S., & Gubler, C. J. (1973). Effects of thiamine deficiency and treatment with the antagonists, oxythiamine and pyrithiamine, on the levels and distribution of thiamine derivatives in rat brain. *J Nutr Sci Vitaminol (Tokyo), 19*(3), 237-249.

Myers, R. E. (1979). A unitary theory of causation of anoxic and hypoxic brain pathology. *Adv Neurol, 26*, 195-213.

Navarro, D., Zwingmann, C., & Butterworth, R. F. (2008). Region-selective alterations of glucose oxidation and amino acid synthesis in the thiamine-deficient rat brain: A re-evaluation using 1h/13c nmr spectroscopy. *Journal of Neurochemistry,* (Accepted with revisions).

Navarro, D., Zwingmann, C., Chatauret, N., & Butterworth, R. F. (2007). Glucose loading precipitates focal lactic acidosis in the vulnerable medial thalamus of thiamine-deficient rats. *Metab Brain Dis.*

Navarro, D., Zwingmann, C., Hazell, A. S., & Butterworth, R. F. (2005). Brain lactate synthesis in thiamine deficiency: A re-evaluation using 1h-13c nuclear magnetic resonance spectroscopy. *J Neurosci Res, 79*(1-2), 33-41.

Pardridge, W. M. (1983). Brain metabolism: A perspective from the blood-brain barrier. *Physiol Rev, 63*(4), 1481-1535.

Park, L. C., Calingasan, N. Y., Uchida, K., Zhang, H., & Gibson, G. E. (2000). Metabolic impairment elicits brain cell type-selective changes in oxidative stress and cell death in culture. *J Neurochem, 74*(1), 114-124.

Paxinos, G., & Watson, C. (1982). *The rat brain in stereotaxic coordinates.* New York: Academic Press, Inc.

Paxton, R., & Harris, R. A. (1984). Regulation of branched-chain alpha-ketoacid dehydrogenase kinase. *Arch Biochem Biophys, 231*(1), 48-57.

Pekovich, S. R., Martin, P. R., & Singleton, C. K. (1996). Thiamine pyrophosphate-requiring enzymes are altered during pyrithiamine-induced thiamine deficiency in cultured human lymphoblasts. *J Nutr, 126*(7), 1791-1798.

Pellerin, L., & Magistretti, P. J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: A mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A, 91*(22), 10625-10629.

Pellerin, L., Pellegri, G., Bittar, P. G., Charnay, Y., Bouras, C., Martin, J. L., et al. (1998). Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. *Dev Neurosci, 20*(4-5), 291-299.

Peters, R. A. (1936). The biochemical lesion in vitamin b1 deficiency. *Lancet, 1*, 1161-1165.

Pincus, J. H., & Grove, I. (1970). Distribution of thiamine phosphate esters in normal and thiamine-deficient brain. *Exp Neurol, 28*(3), 477-483.

Plaitakis, A., Nicklas, W. J., & Berl, S. (1979). Alterations in uptake and metabolism of aspartate and glutamate in brain of thiamine deficient animals. *Brain Res, 171*(3), 489-502.

Rehncrona, S., Rosen, I., & Siesjo, B. K. (1980). Excessive cellular acidosis: An important mechanism of neuronal damage in the brain? *Acta Physiol Scand, 110*(4), 435-437.

Rehncrona, S., Rosen, I., & Siesjo, B. K. (1981). Brain lactic acidosis and ischemic cell damage: 1. Biochemistry and neurophysiology. *J Cereb Blood Flow Metab, 1*(3), 297-311.

Rindi, G., De Giuseppe, L., & Sciorelli, G. (1968). Thiamine monophosphate, a normal constituent of rat plasma. *J Nutr, 94*(4), 447-454.

Rindi, G., Ferrari, G., & Ventura, U. (1963). [influence of amprolium on the thiamine content of rat tissues.]. *Farmaco [Sci], 18*, 1001-1004.

Rindi, G., Patrini, C., Comincioli, V., & Reggiani, C. (1980). Thiamine content and turnover rates of some rat nervous regions, using labeled thiamine as a tracer. *Brain Res, 181*(2), 369-380.

Rindi, G., & Perri, V. (1961). Uptake of pyrithiamine by tissue of rats. *Biochem J, 80*, 214-216.

Rindi, G., & Ventura, U. (1969). Thiamine countertransport in rat small intestine. *Pflugers Arch, 310*(2), 185-188.

Roos, A. (1971). Intracellular ph and buffering power of rat brain. *Am J Physiol, 221*(1), 176-181.

Schroth, G., Wichmann, W., & Valavanis, A. (1991). Blood-brain-barrier disruption in acute wernicke encephalopathy: Mr findings. *J Comput Assist Tomogr, 15*(6), 1059-1061.

Sealock, R. R., & White, H. S. (1949). Neopyrithiamine and the thiaminase of fish tissues. *J Biol Chem, 181*(1), 393-403.

Sen, I., Cooper JR. (1976). The turnover of thiamin and its phosphate esters in rat organs. *Neurochem Int, 1*, 65-71.

Sharma, S. K., & Quastel, J. H. (1965). Transport and metabolism of thiamine in rat brain cortex in vitro. *Biochem J, 94*, 790-800.

Shimomura, Y., Obayashi, M., Murakami, T., & Harris, R. A. (2001). Regulation of branched-chain amino acid catabolism: Nutritional and hormonal regulation of activity and expression of the branched-chain alpha-keto acid dehydrogenase kinase. *Curr Opin Clin Nutr Metab Care, 4*(5), 419-423.

Sklan, D., & Trostler, N. (1977). Site and extent of thiamin absorption in the rat. *J Nutr, 107*(3), 353-356.

Smidt, L. J., Cremin, F. M., Grivetti, L. E., & Clifford, A. J. (1991). Influence of thiamin supplementation on the health and general well-being of an elderly irish population with marginal thiamin deficiency. *J Gerontol, 46*(1), M16-22.

Spector, R. (1976). Thiamine transport in the central nervous system. *Am J Physiol, 230*(4), 1101-1107.

Stryer, L. (1997). *Biochemistry* (4 ed.). New York, U.S.A.: W.H. Freeman and Company.

Sweatt, A. J., Garcia-Espinosa, M. A., Wallin, R., & Hutson, S. M. (2004). Branched-chain amino acids and neurotransmitter metabolism: Expression of cytosolic branched-chain aminotransferase (bcatc) in the cerebellum and hippocampus. *J Comp Neurol, 477*(4), 360-370.

Tallaksen, C. M., Bell, H., & Bohmer, T. (1992). The concentration of thiamin and thiamin phosphate esters in patients with alcoholic liver cirrhosis. *Alcohol Alcohol, 27*(5), 523-530.

Tallaksen, C. M., Bell, H., & Bohmer, T. (1993). Thiamin and thiamin phosphate ester deficiency assessed by high performance liquid chromatography in four clinical cases of wernicke encephalopathy. *Alcohol Clin Exp Res, 17*(3), 712-716.

Tallaksen, C. M., Bohmer, T., Bell, H., & Karlsen, J. (1991). Concomitant determination of thiamin and its phosphate esters in human blood and serum by high-performance liquid chromatography. *J Chromatogr, 564*(1), 127-136.

Thomson, A. D., Baker, H., & Leevy, C. M. (1970). Patterns of 35s-thiamine hydrochloride absorption in the malnourished alcoholic patient. *J Lab Clin Med, 76*(1), 34-45.

Thomson, A. D., & Leevy, C. M. (1972). Observations on the mechanism of thiamine hydrochloride absorption in man. *Clin Sci, 43*(2), 153-163.

Todd, K., & Butterworth, R. F. (1999). Mechanisms of selective neuronal cell death due to thiamine deficiency. *Ann N Y Acad Sci, 893*, 404-411.

Todd, K. G., & Butterworth, R. F. (1998a). Evaluation of the role of nmda-mediated excitotoxicity in the selective neuronal loss in experimental wernicke encephalopathy. *Exp Neurol, 149*(1), 130-138.

Todd, K. G., & Butterworth, R. F. (1998b). Increased neuronal cell survival after l-deprenyl treatment in experimental thiamine deficiency. *J Neurosci Res, 52*(2), 240-246.

Troncoso, J. C., Johnston, M. V., Hess, K. M., Griffin, J. W., & Price, D. L. (1981). Model of wernicke's encephalopathy. *Arch Neurol, 38*(6), 350-354.

Van Nimmen, D., Weyne, J., Demeester, G., & Leusen, I. (1986). Local cerebral glucose utilization during intracerebral ph changes. *J Cereb Blood Flow Metab, 6*(5), 584-589.

Ventura, U., & Rindi, G. (1965). Transport of thiamine by the small intestine in vivo. *Experientia, 21*(11), 645-646.

Vimokesant, S. L., Hilker, D. M., Nakornchai, S., Rungruangsak, K., & Dhanamitta, S. (1975). Effects of betel nut and fermented fish on the thiamin status of northeastern thais. *Am J Clin Nutr, 28*(12), 1458-1463.

Voet, D., & Voet, J. G. (1990). *Biochemistry*. New York, U.S.A.: John Wiley & Sons, Inc.

Wallis, W. E., Willoughby, E., & Baker, P. (1978). Coma in the wernicke-korsakoff syndrome. *Lancet, 2*(8086), 400-401.

Watson, A. J., Walker, J. F., Tomkin, G. H., Finn, M. M., & Keogh, J. A. (1981). Acute wernickes encephalopathy precipitated by glucose loading. *Ir J Med Sci, 150*(10), 301-303.

Weber, W., & Kewitz, H. (1985). Determination of thiamine in human plasma and its pharmacokinetics. *Eur J Clin Pharmacol, 28*(2), 213-219.

Wieland, O. H. (1983). The mammalian pyruvate dehydrogenase complex: Structure and regulation. *Rev Physiol Biochem Pharmacol, 96*, 123-170.

Wielders, J. P., & Mink, C. J. (1983). Quantitative analysis of total thiamine in human blood, milk and cerebrospinal fluid by reversed-phase ion-pair high-performance liquid chromatography. *J Chromatogr, 277*, 145-156.

Yamamoto, S., Koyama, S., & Kawasaki, T. (1981). Properties of the active thiamine transport system in ehrlich ascites tumor cells. *J Biochem (Tokyo), 89*(3), 809-816.

Yoshioka, K. (1984). Some properties of the thiamine uptake system in isolated rat hepatocytes. *Biochim Biophys Acta, 778*(1), 201-209.

Yudkoff, M. (1997). Brain metabolism of branched-chain amino acids. *Glia, 21*(1), 92-98.

Yudkoff, M., Daikhin, Y., Grunstein, L., Nissim, I., Stern, J., Pleasure, D., et al. (1996a). Astrocyte leucine metabolism: Significance of branched-chain amino acid transamination. *J Neurochem, 66*(1), 378-385.

Yudkoff, M., Daikhin, Y., Lin, Z. P., Nissim, I., Stern, J., Pleasure, D., et al. (1994). Interrelationships of leucine and glutamate metabolism in cultured astrocytes. *J Neurochem, 62*(3), 1192-1202.

Yudkoff, M., Daikhin, Y., Nelson, D., Nissim, I., & Erecinska, M. (1996b). Neuronal metabolism of branched-chain amino acids: Flux through the aminotransferase pathway in synaptosomes. *J Neurochem, 66*(5), 2136-2145.

Yudkoff, M., Nissim, I., Daikhin, Y., Lin, Z. P., Nelson, D., Pleasure, D., et al. (1993). Brain glutamate metabolism: Neuronal-astroglial relationships. *Dev Neurosci, 15*(3-5), 343-350.

Zelaya, F. O., Rose, S. E., Nixon, P. F., Wholohan, B. T., Bower, A. J., Zimitat, C., et al. (1995). Mri demonstration of impairment of the blood-csf barrier by glucose administration to the thiamin-deficient rat brain. *Magn Reson Imaging, 13*(4), 555-561.

Zielke, H. R., Huang, Y., Baab, P. J., Collins, R. M., Jr., Zielke, C. L., & Tildon, J. T. (1997). Effect of alpha-ketoisocaproate and leucine on the in vivo oxidation of glutamate and glutamine in the rat brain. *Neurochem Res, 22*(9), 1159-1164.

Zielke, H. R., Huang, Y., Tildon, J. T., Zielke, C. L., & Baab, P. J. (1996). Elevation of amino acids in the interstitial space of the rat brain following infusion of large neutral amino and keto acids by microdialysis: Alpha-ketoisocaproate infusion. *Dev Neurosci, 18*(5-6), 420-425.

Zwingmann, C., Leibfritz, D., & Hazell, A. S. (2003). Energy metabolism in astrocytes and neurons treated with manganese: Relation among cell-specific energy failure, glucose metabolism, and intercellular trafficking using multinuclear nmr-spectroscopic analysis. *J Cereb Blood Flow Metab, 23*(6), 756-771.

APPENDIX

Appendix 1: List of Acronyms and Initialisms

Acronym/Initialism	Definition
AAT	Aspartate Aminotransferase
ADP	Adenine 5′-Diphosphate
AIDS	Acquired Immunodeficiency Syndrome
ALAT	Alanine Aminotransferase
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
Asp	Aspartate
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BCAA	Branched-Chain Amino Acid
BCAT	Branched-Chain Aminotransferase
BCKA	Branched-Chain α-Ketoacid
BCKDH	Branched-Chain α-Ketoacid Dehydrogenase
CoA	Coenzyme A
CoA-SH	Reduced Coenzyme A
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DAB	3-3′-Diaminobenzidine
DMO	5,5-dimethyloxazolidine-2, 4-dione
eNOS	Endothelial Nitric Oxide Synthase
EW	l'Encéphalopathie de Wernicke
GFAP	Glial Fibrillary Acidic Protein
FAD	Flavin Adenine Dinucleotide

$FADH_2$	1,5-Dihydro-Flavin Adenine Dinucleotide
FC	Frontal Cortex
GABA	γ-Aminobutyric Acid
GDH	Glutamate Dehydrogenase
Gln	Glutamine
Glu	Glutamate
HIV	Human Immunodeficiency Virus
IB-CoA	Isobutyryl-CoA
Acronym/Initialism	**Definition**
iNOS	Inducible Nitric Oxide Synthase
IV	Intravenous
IV-CoA	Isovaleryl-CoA
KGDH	α-Ketoglutarate Dehydrogenase
KIC	α-Ketoisocaproate
KIV	α-Ketoisovalerate
KMV	α-Keto-β-methylvalerate
KOH	Potassium Hydroxide
LCpH	Local Cerebral pH
LDH	Lactate Dehydrogenase
MB-CoA	α-Methylbutyryl-CoA
ME	Malic Enzyme
MRI	Magnetic Resonance Imaging
MT	Medial Thalamus
NAA	N-Acetyl Aspartate
NAD^+	Nicotinamide Adenine Dinucleotide

NADH	Nicotinamide Adenine Dinucleotide, reduced form
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NMDA	N-Methyl-D-Aspartate
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Enhancement
PC	Pyruvate Carboxylase
PCA	Perchloric Acid
PDH	Pyruvate Dehydrogenase
PEPCK	Phosphoenolpyruvate Carboxykinase
PFC	Pair-Fed Control
pH_P	Plasma pH
P_i	Inorganic Phosphate
pH_t	Tissue pH
PS	Presymptomatic
PTD	Pyrithiamine-induced Thiamine Deficiency
R-CoA	Acyl-CoA
RDA	Recommended Daily Allowance
SD	Standard Deviation
SYM	Symptomatic
TCA	Tricarboxylic Acid
TD	Thiamine-Deficient
TDP	Thiamine Diphosphate
TMP	Thiamine Monophosphate
TTP	Thiamine Triphosphate

U	Unit
WE	Wernicke's Encephalopathy
WKS	Wernicke-Korsakoff Syndrome

Certificate of Rat Methodology Workshop (McGill University)

Permit for the Use of Radioisotopes (CHUM)

Comité Institutionnel de Protection des Animaux (CIPA)

4. ÉVALUATION PAR DES PAIRS/ SOURCES DE FINANCEMENT (Voir Directives aux chercheurs)

Organisme de pairs ☒ Entreprise ☐	Demande de subvention	Période de subvention
IRSC	☐ Nouvelle ☒ En cours ☐ Renouvellement	2001-2007
IRSC	☐ Nouvelle ☒ En cours ☐ Renouvellement	1999-2004

Pour un protocole n'ayant pas fait l'objet d'une évaluation par un comité de pairs reconnu, s'il vous plaît nous suggérer des évaluateurs affiliés à l'Université de Montréal :

Nom :	Téléphone : ( )
Nom :	Téléphone : ( )
Nom :	Téléphone : ( )

5. PERSONNEL QUI UTILISERA DES ANIMAUX (Compétences)

Chaque utilisateur doit avoir complété une seule fois le formulaire : Compétences professionnelles de l'utilisateur d'animaux.

Type[1]	Nom	Prénom	Expérience pour ce type de technique / procédure		Diplôme	Tél.
P	CHAN	Helen	☒ Oui	☐ Non	MSC	35740
P	BÉLANGER	MIREILLE	☒ Oui	☐ Non	M Sc	35740
C	ZWINGMANN	CLAUDIA	☒ Oui	☐ Non	PH D	35740
P	NAVARRO	DARREN	☒ Oui	☐ Non	M Sc	35740
			☐ Oui	☐ Non		
			☐ Oui	☐ Non		
			☐ Oui	☐ Non		
			☐ Oui	☐ Non		

[1] **P** : Principal utilisateur **C** : Chercheur associé **A** : Assistant de recherche **E** : Étudiant **T** : Technicien

Dans le cas où les individus n'ont aucune expérience d'une technique ou d'une procédure particulière au protocole, indiquez comment ils obtiendront l'expertise :

6. TYPE DE PROJET

☐ projet pilote ☒ recherche ☐ enseignement N° cours :

7. RÉSUMÉ VULGARISÉ ET CONCIS DU PROJET

Ce résumé doit pouvoir servir à la préparation d'un document de presse pour les médias. Expliquez de façon à être **compris par un public non scientifique**, le but général de votre recherche, les objectifs spécifiques que vous entendez poursuivre avec ce protocole et la contribution aux connaissances scientifiques ou à la santé de l'homme et/ou des animaux :

7. RÉSUMÉ VULGARISÉ ET CONCIS DU PROJET

Des cultures cellulaires astrocytaires et neuronales seront obtenues à partir de tissus cérébral d'embryons et de nouveaux nés (1 et 7 jours) de rats afin d'étudier les mécanismes responsables d'encéphalopathies métaboliques causées par l'hyperammonémie ou la déficience en thiamine. Les cultures cellulaires sont donc exposées à des concentrations physiopathologiques d'ammoniaque ou à un inhibiteur compétitif de la thiamine, la pyrithiamine, et l'effet de ces substances sur les voies biochimiques et sur l'expression génique est étudiée.

8. RÉSUMÉ SCIENTIFIQUE

Ce résumé permet aux membres du CIPA de comprendre les buts, les objectifs de votre recherche ainsi que la pertinence de l'utilisation d'animaux pour votre projet.

Les cultures cellulaire neurales constituent un important outil d'étude des mécanismes physiopathologiques impliqués dans les encéphalopathies métaboliques. En effet, ces désordres neurologiques affectent souvent un type cellulaire bien précis et les cultures cellulaires permettent l'étude d'un système dont nous pouvons contrôler les conditions afin d'isoler les mécanismes d'intérêt.

Lors de la dysfonction hépatique, certaines neurotoxines telles que l'ammoniaque s'accumulent dans le sang avec plusieurs conséquence telles que l'oedème cérébral, une altération du métabolisme énergétique ainsi que l'altérations de l'expression génique de plusieurs protéines. Nos études porteront d'abord sur les effets de l'ammoniaque sur le métabolisme énergétique. Pour ce faire, l'effet de sources externe d'énergie (créatine) ou de cofacteurs métaboliques (coenzyme Q) sur la fonction cellulaire sera évalué. De plus, l'effet de l'ammoniaque sur les voies métaboliques ainsi que sur les niveaux de substrats énergétiques sera évalué grâce à des études de Résonnance Magnétique Nucléaire. L'activité, l'expression et le niveau de phosphorylation d'enzymes clés du métabolisme énergétique (lactate déshydrogénase, alpha-kétoglutarate déshydrogénase, transkétolase) seront également étudiés lors de l'exposition de cultures cellulaires à l'ammoniaque. D'autre part, certaines études indiquent une implication possible du stress oxidatif lors de la neurotoxicité due à l'ammoniaque. Des études seront donc menées afin d'évaluer la présence de produits du stress oxydatif. De plus, l'effet de l'exposition de cultures cellulaires à des sources de radicaux libres sur la fonction cellulaire (recapture de glutamate, production de lactate, régulation du volume cellulaire) sera évalué.

Notre laboratoire s'intéresse également à l'étude de la déficience en thiamine, un cofacteur essentiel à certains enzymes mitochondriaux impliqués dans le métabolisme énergétique. Des observations cliniques ont démontré qu'une administration de glucose à des patients déficients en thiamine s'avère néfaste. Nous étudierons donc l'effet du glucose sur les voies métaboliques ainsi que les niveaux de molécules énergétiques riches en phosphates par Résonnance Magnétique Nucléaire. D'autre part, nous évaluerons le rôle du stress oxidatif dans la déficience en thiamine. Le stress oxidatif peut induire la formation du pore de transition de perméabilité dans les mitochindries et ainsi contribuer à la mort programée cellulaire. Nous utiliserons donc des agents pharmacologiques inhibant la formation du mégapore afin d'évaluer la possibilité de son implication dans les mécanismes de mort cellulaire causée par la déficience en thiamine. L'effet neuroprotecteur de substances antioxidantes sera également évalué.

9. NATURE DU PROJET

Afin de faciliter l'examen de votre demande par le CIPA, veuillez cocher les manipulations utilisées ou les conditions prévalant au cours de l'expérimentation :

- ☒ Aiguë
- ☐ Chronique
- ☐ Non traumatique
- ☐ Traumatique

9. NATURE DU PROJET

☐ Immunisation	☐ Étude de comportement
☐ Chirurgie/ anesthésie	☐ Induction au stress
☐ Colonie de production d'animaux	☐ Immobilisation
☐ Privation. Spécifiez :	☐ Autre. Spécifiez :

10. REMPLACEMENT, RÉDUCTION, RAFFINEMENT

« Des animaux ne devraient être utilisés que si le chercheur a tenté en vain, par tous les moyens possibles, de trouver une solution de rechange » [1]

10.1 Lors de la conception de ce projet, avez-vous considéré le remplacement des animaux par une méthode alternative comme :

☐ Un modèle utilisant la culture cellulaire ☐ Un modèle non vivant

☐ L'emploi d'un animal de phylum taxinomique inférieur

Pour les protocoles d'enseignement :

☐ Un document audiovisuel ☐ Un logiciel de simulation

Expliquez pourquoi vous ne pouvez utiliser une de ces alternatives :

« Ceux qui utilisent des animaux doivent recourir aux méthodes les plus humaines et ce, sur le plus petit nombre possible d'animaux appropriés requis pour obtenir des renseignements valables». [2]

10.2 Lors de la conception de ce projet, avez-vous considéré le NOMBRE MINIMAL d'animaux nécessaires pour obtenir des résultats valables? Expliquez :

Oui, le nombre de portées nécessaire constitue le minimum nécessaire afin d'obtenir une analyse statistique valable de nos résultats. Les études utilisant les cellules en culture nécessitent de deux à trois réplicats afin de s'assurer de la reproductibilité d'un lot à l'autre.

10.3 Lors de la conception de ce projet, avez-vous considéré le raffinement (analgésie, euthanasie humanitaire, anesthésie, enrichissement du milieu, etc....)? Expliquez :

Les cultures cellulaires nécessitent de très jeunes animaux, il sont donc gardés en animalerie très peu de temps. De plus, l'étendue des manipulations est très restreinte puisqu'il s'agit d'un sacrifice par décapitation.

10.4 Avez-vous considéré le niveau de douleur et d'inconfort de l'animal en rapport avec l'importance des résultats escomptés? Expliquez :

Le niveau d'inconfort est minimisé autant qu'il se peut. Cet inconfort est nécessaire à l'obtention de résultats pouvant contribuer à développer de nouvelles voies thérapeutiques pour l'encéphalopathie hépatique et la déficience en thiamine.

10.5 Quels sont les organes des animaux utilisés qui peuvent servir à un autre protocole? Indiquez les organes :

Tout sauf le cerveau

[1] Manuel sur le soin et l'utilisation des animaux d'expérimentation. Volume I, CCPA, 1993, page 219

[2] Manuel sur le soin et l'utilisation des animaux d'expérimentation. Volume I, CCPA, 1993, page 219

11. PRODUCTION D'ANTICORPS

Demande de production d'anticorps : oui ☐ **non** ☒
Dans le cas d'un protocole de production d'anticorps, veuillez indiquer les démarches entreprises pour déterminer si l'anticorps est disponible commercialement ou auprès d'un autre groupe de recherche.

Sites Internet visités :

Personnes contactées :

12. ANIMAUX REQUIS (Vous pouvez utiliser les lignes avec ou sans les listes déroulantes)

Espèce (Ex: rat, chien)	Souche (C57Bl/6, Yucatán)	Âge / poids	Sexe M / F	Fournisseur	Nb demandé en 1 fois(1)	Nb de cage (1)	Nb pour année	Total projet
rat	Sprague-Dawley	gestante	F	Charles River	2	2	162	
				Charles River				
				Charles River				
				Charles River				
				Charles River				

(1) Estimation

N.B. ANIMAUX TRANSGÉNIQUES (Les utilisateurs de souris TG doivent remplir annuellement la fiche d'utilisation des animaux transgéniques.)

13. DURÉE DE L'ÉTUDE

Date prévue du début de l'expérience : 05/2003 (mois/ année) Date prévue de la fin : 05/2006 (mois/ année)

Veuillez indiquer la période maximale durant laquelle l'animal sera gardé pour l'étude :

1 jour

14. JUSTIFICATION DU NOMBRE D'ANIMAUX

Les techniques expérimentales utilisées doivent être faites sur le plus petit nombre requis d'animaux pour obtenir des données valables. La justification du nombre d'animaux doit comprendre une description claire du modèle expérimental incluant **un tableau résumant vos groupes expérimentaux et contrôles**. Indiquez également le rationnel statistique qui vous a permis de déterminer le nombre d'animaux par groupe. **Pour les protocoles d'élevage, veuillez fournir le nombre d'animaux requis pour faire et maintenir la colonie.**

Tableau

Tour d'abord, il faut préciser que chaque expérience doit être effectuée en double, avec des cultures cellulaires provenant de petits de deux portées différentes, pour que les résultats soient exploitables statistiquement. Il faut donc deux femelles par expérience.

-Effets de l'application de sources extérieures d'énegie (créatine) sur la fonction cellulaire (recapture de glutamate et régulation du volume cellulaire) en présence ou en absence d'ammoniaque: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)

-Effets de l'application de cofacteurs métaboliques (coenzyme Q) sur la fonction cellulaire (recapture de glutamate et régulation du volume cellulaire) en présence ou en absence d'ammoniaque: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)

-Études par résonnance magnétique nucléaire des voies métaboliques ainsi que des niveaux d'ATP et de phosphocréatine lors de l'exposition à l'ammoniaque. Les études de Résonnamce Magnétique Nucléaire nécessitent de très gros échantillons. Il est donc nécessaire de "pooler" plusieurs cultures afin d'obtenir un échantillon: 24 portées: (8 cultures d'astrocytes, 8 cultures de neurones, 8 cultures de cellules granulaires)

-Étude de l'activité et de l'expression d'enzymes du métabolismes énergétique en présence d'ammoniaque
- activité enzymatique: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)
- expression de l'ARNm codant pour ces enzymes: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)
- expression protéinique: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)
- études de l'état de phosphorylation de ces enzymes par immunoprécipitation: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)

-Étude de l'effet de l'exposition à l'ammoniaque sur la production de dommage oxydatif:
- étude de la nitrosylation des protéines par immunoprécipitation: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)
- étude de la péroxydation des lipides: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)

-Étude de l'effet de substances induisant un stress oxydatif (peroxynitrite) sur:
-la recapture du glutamate: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)
- la production de lactate: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)
- la régulation du volume cellulaire: 6 portées (2 cultures d'astrocytes, 2 cultures de neurones, 2 cultures de cellules granulaires)

TOTAL AMMONIAQUE: 90 portées

-Études par Résonnance Magnétique Nucléaire des voies métaboliques ainsi que des niveaux d'ATP et de phosphocréatine lors de l'exposition au glucose de cellules déficientes en thiamine. 24 portées: (8 cultures d'astrocytes, 8 cultures de neurones, 8 cultures de cellules granulaires)

-Études par résonnance magnétique nucléaire des voies métaboliques ainsi que des niveaux d'ATP et de phosphocréatine dans un système de coculture: 16 portées: (8 cultures d'astrocytes, 8 cultures de neurones)

- Effet de la cyclosporine A (inhibiteur du pore de transition de perméabilité) sur la déficience en thiamine
- Effet sur la protection celluaire (viabilité): 2 portées (cellules granulaires)
- Effet sur l'activité de l'alpha-kétoglutarate déshydrogénase: 2 portées (cellules granulaires)

- Effet du trifluoroperazine (inhibiteur de la phospholipase A2) sur la déficience en thiamine
- Effet sur la protection celluaire (viabilité): 2 portées (cellules granulaires)
- Effet sur l'activité de l'alpha-kétoglutarate déshydrogénase: 2 portées (cellules granulaires)

- Effet combiné de la cyclosporine A et du du trifluoroperazine sur la déficience en thiamine
- Effet sur la protection celluaire (viabilité): 2 portées (cellules granulaires)
- Effet sur l'activité de l'alpha-kétoglutarate déshydrogénase: 2 portées (cellules granulaires)

- Effet de la N-acetyl-cysteine (antioxydant) sur la déficience en thiamine
- Effet sur la protection celluaire (viabilité): 2 portées (cellules granulaires)
- Effet sur l'activité de l'alpha-kétoglutarate déshydrogénase: 2 portées (cellules granulaires)

-Évaluer si la mort cellulaire causée par SIN-1 (un donneur de NO) peut être atténuée par la présence d'astrocytes (cocultures). 4 portées (2 cultures d'astrocytes; 2 cultures de neurones corticaux) et évaluer l'activité de l'alpha-kétoglutarate déshydrogénase dans ces conditions: 4 portées (2 cultures d'astrocytes; 2 cultures de neurones corticaux)

- Afin de déterminer la présence de facteurs solubles/relâchés par les astrocytes impliqués dans la neuroprotection des neurones déficients en thiamine, des astrocytes seront mis en culture en parallèle avec des cellules granulaires. Les neurones seront ensuite mis en contact avec le milieu de culture provenant des astrocytes et:
  - la viablité cellulaire des neurones sera évaluée: 4 portées (2 cultures d'astrocytes; 2 cultures de neurones)
  - l'activité de l'alpha-kétoglutarate déshydrogénase sera évaluée: 4 portées (2 cultures d'astrocytes; 2 cultures de neurones)

TOTAL DÉFICIENCE EN THIAMINE: 72
GRAND TOTAL: 162

15. HÉBERGEMENT ET SOINS AUX ANIMAUX (Donnez les explications ci-dessous)

☐ Hôtel-Dieu ☐ Hôpital Notre-Dame ☒ Hôpital Saint-Luc

☐ Manipulations dans les locaux de l'animalerie
☒ Manipulations à l'extérieur
☐ Retour à l'animalerie
☐ Régimes alimentaires spéciaux
☐ Équipement particulier
☐ Installations pour animaux immunodéficients
☐ Assistance technique par le personnel de l'animalerie
☐ Autre Justifiez et / ou spécifiez :

16. ENRICHISSEMENT DU MILIEU

Selon les directives du CCPA [3], on se doit de fournir un milieu stimulant approprié pour chaque espèce animale mise en cage. Des stratégies d'enrichissement de l'environnement sont donc systématiquement effectuées pour les animaux utilisés dans **tous** les protocoles approuvés par le CIPA (référence : Politique et procédure C-1 ENRICHISSEMENT DU MILIEU) à moins que les objectifs de l'étude n'en interdisent l'usage.

☒ Non. Certaines stratégies d'enrichissement de l'environnement ne peuvent être utilisées :
Précisez lesquelles.

[3] Manuel sur le soin et l'utilisation des animaux d'expérimentation, CCPA, Volume 1, 1993, page 58

17. PROCÉDURE D'UTILISATION DES ANIMAUX DE PROTOCOLE EXPÉRIMENTAL

Décrire ci-dessous les procédures expérimentales, la description chirurgicale et toutes les manipulations pratiquées chez l'animal (à l'aide des « Procédures normalisées de fonctionnement » (PNF) du Service des animaleries du CRCHUM, ou, si possible, d'articles publiés, de livres de références). Lors d'utilisation de médicaments, de produits biologiques ou d'autres agents administrés aux animaux, inclure également une justification du choix de l'agent et des doses, les effets secondaires et de quelle façon les animaux seront surveillés pour assurer le suivi du protocole à l'intérieur des points limites proposés.

Les numéros des PNF utilisées :

1.Pour les cultures astrocytaires, des rats nouveaux nés (moins de 24 heures) sont sacrifiés par décapitation. Le cortex cérébral est prélevé, nettoyé des débris vasculaires, et dissocié mécaniquement. La préparation en résultant est ensuite mise en culture sur un pétri et maintenue dans une solution de DMEM contenant 10% de sérum fœtal bovin jusqu'à ce que les cellules atteignent la confluence.

2. Pour les cultures de cellules granulaires cerebelleuses, des rat sont sacrifiés par décapitation 7 jours après la naissance. Le cervelet est prélevé et les régions appropriées sont disséquées. Le tissus obtenu est nettoyé des débris vasculaires, et dissocié mécaniquement. La préparation en résultant est ensuite mise en culture sur un pétri et maintenue dans une solution de DMEM contenant 10% de sérum fœtal bovin supplémenté de cytosine arabinoside afin d'inhiber la croissance de cellules gliales.

3. Pour les cultures de neurones corticaux, une rate gestante est anesthésiée (1mL/kg somnotol), la cavité abdominale est ouverte et le placenta et les embryons (17 jours embryonnaires) sont rapidement prélevés. La rate est sacrifiée au moyen d'une injection intracardiaque de chlorure de potassium. Les cortex cérébraux des embryons sont prélevés et dissociés mécaniquement. La préparation en résultant est ensuite mise en culture sur un pétri et maintenue dans une solution de DMEM contenant 10% de sérum fœtal bovin pour 5 jours avant d'être utilisée.

Ces cultures cellulaires sont utilisées pour étudier l'encéphalopathie causée par l'hyperammonémie ainsi que l'encéphalopathie causée par la déficience en thiamine. Pour les études des effets de l'ammonique, les cultures cellulaires sont exposées à des concentrations d'ammoniaque allant jusqu'à 5 mM pour différentes périodes de temps. Pour les études portant sur la déficience en thiamine, les cultures sont exposées à des concentrations allant jusqu'à 10 μM de pyrithiamine pour différentes périodes de temps.

18. ANALGÉSIE

Dans toute situation où il est possible que l'animal ressente de la douleur, une analgésie adéquate doit être utilisée, de même qu'après toute chirurgie ou manipulation invasive.

☐ Oui. Un analgésique sera utilisé — Nom de l'agent utilisé :

Dosage : — Voie :

Nom du responsable : — Fréquence : — Durée :

☐ Contre-indiquée. Justifiez de façon détaillée les interférences rapportées dans la littérature :

☒ Non requise. Justifiez :

Pas d'intervention chirurgicale

19. ANESTHÉSIE

☒ Oui. Une anesthésie est nécessaire ☐ Avec réveil ☒ Sans réveil (terminal)

Nom du responsable : HELEN CHAN Durée totale de l'anesthésie : 10 MINUTES

☐ Description du protocole anesthésique en annexe (**fait avec la collaboration du personnel de l'animalerie**)

☐ Selon les « Procédures normalisées de fonctionnement » (**PNF**) du Service des animaleries N°

☒ **Sinon, veuillez remplir le tableau ci-dessous**

Anesthésie	Agent	Dose (mg/kg)	Voie
Pré-anesthésique			
Induction	Somnotol	1 mL/kg	i.p.
Maintien			
Traitement de support			
Fluides			
Antibiothérapie			

☐ Contre-indiquée. Justifiez :

☐ Non requise. Justifiez :

20. CHIRURGIE / MANIPULATIONS SOUS ANESTHÉSIE

La procédure doit être décrite en détails à la section 17, Procédures d'utilisation des animaux de protocole expérimental.
Toute procédure chirurgicale ou manipulation sous anesthésie doit être effectuée conformément aux Politiques et procédures du CIPA.

☒ Non, allez à la section 22

☐ Oui, veuillez cocher les cases appropriées :

- ☐ Procédure terminale, l'animal sera euthanasié avant le réveil.
- ☐ Chirurgie majeure : accès à une cavité corporelle ou altération permanente de la physiologie ou de l'anatomie de l'animal.
- ☐ Chirurgie mineure
- ☐ Manipulations non chirurgicales sous anesthésie générale

21. SOINS PEROPÉRATOIRES ET POSTOPÉRATOIRES

Chez les rongeurs, on doit assurer la surveillance des signes vitaux et des réflexes.
Chez les grands animaux, on devrait assurer la surveillance des signes vitaux et des réflexes (durant la chirurgie). Un technicien expérimenté devrait assurer le « monitoring ». Les soins postopératoires doivent être effectués conformément aux Politiques et procédures du CIPA.

Surveillance peropératoire. Décrire les paramètres à observer :

☐ Température ☐ ECG ☐ Pression sanguine ☐ SPO_2

☐ Pincement de l'orteil ☐ Pincement de la queue ☐ Réflexe palpébral

☐ Relaxation musculaire ☐ Couleur des muqueuses ☐ Pouls

Veuillez spécifier les soins peropératoires spéciaux, si requis :

Nom du responsable : Ou personnel de l'animalerie : ☐

L'animal devra être surveillé durant la période de réveil au moins toutes les 15 minutes. Il devra être retourné à sa cage lorsqu'il aura été extubé, qu'il aura atteint une température corporelle de 37°C et qu'il aura fait des efforts pour se mettre en décubitus sternal.

Surveillance postopératoire. Décrire les paramètres à observer :

☐ Température ☐ ECG ☐ Pression sanguine ☐ SPO_2

Veuillez spécifier les soins postopératoires spéciaux, si requis :

Nom du responsable : Ou personnel de l'animalerie : ☐

22. POINTS LIMITES

« Il faut éviter de soumettre les animaux à des souffrances ou à des angoisses inutiles. » [4,5]

Voici une liste des points limites les plus fréquemment observés en recherche animale et requérant qu'un animal soit traité ou euthanasié si sa condition se détériore au point où il ne peut plus être traité.

Veuillez indiquer quelles conditions suivantes peuvent être provoquées par vos manipulations et expliquez a) à quel point limite la décision sera prise de sacrifier l'animal et b) les traitements appropriés à administrer.

Changements physiologiques :

☐ Hypothermie ☐ Période de fièvre prolongée ☐ Troubles nerveux

☐ Perte de poids ☐ Déshydratation ☐ Hypoxie

☐ Autre Expliquez :

Changements comportementaux

☐ Anorexie prolongée ☐ Agressivité reliée à la douleur

☐ Prostration ☐ Douleur incontrôlable

☐ Autre Expliquez :

[4] Manuel sur le soin et l'utilisation des animaux d'expérimentation, CCPA, Volume 1, 1993, page 219

[5] Lignes directrices : choisir un point limite approprié pour les expériences faisant appel à l'utilisation des animaux en recherche, en enseignement et dans les tests, CCPA, 1998

22. POINTS LIMITES

Traitements :

23. CAS D'URGENCE

Dans l'impossibilité de rejoindre le chercheur ou un membre de son équipe, le CIPA peut autoriser l'euthanasie d'un animal de façon humanitaire, s'il est impossible de soulager la douleur ou l'angoisse que l'animal ressent.

Afin de prévenir la perte de vos données scientifiques, veuillez indiquer :

☐ Tissus à prélever. Spécifiez :

Méthode de conservation :

☐ Sang

Couleur du tube : Quantité :

Méthode de conservation :

☐ Autre. Spécifiez :

24. EUTHANASIE (Indiquez la méthode d'euthanasie pour chaque espèce)

Pour connaître les méthodes d'euthanasie acceptables, veuillez vous référer au Manuel du CCPA [6].

Espèce N° 1 : rats

☐ Surdosage d'anesthésique

☐ Barbiturique ☐ Gaz. Agent :

☒ Autre. Spécifiez : pour la rate gestante: injection intracardiaque de KCl (la rate est alors sous anesthésie)

☐ Moyen physique. Ce choix doit être exigé par le protocole. Un sédatif ou un tranquillisant doit être administré, si possible.

☐ Décapitation Sédatif : **Justifiez :**

☐ Dislocation cervicale Sédatif : **Justifiez :**

☐ Exsanguination sous anesthésie. Agent anesthésique :

☐ CO_2

☐ Autre. Précisez :

☐ Les animaux seront réutilisés dans un autre protocole N°

Espèce N° 2 : rats

☐ Surdosage d'anesthésique

☐ Barbiturique ☐ Gaz. Agent :

☐ Autre. Spécifiez :

☒ Moyen physique. Ce choix doit être exigé par le protocole. Un sédatif ou un tranquillisant doit être administré, si possible.

[6] Manuel sur le soin et l'utilisation des animaux d'expérimentation, CCPA, Volume 1, 1993, page 161.

24. EUTHANASIE (Indiquez la méthode d'euthanasie pour chaque espèce)

☒	Décapitation	Sédatif :	**Justifiez :** pour prélèvement du cerveau
☐	Dislocation cervicale	Sédatif :	**Justifiez :**

☐ Exsanguination sous anesthésie. Agent anesthésique :
☐ CO_2
☐ Autre. Précisez :
☐ Les animaux seront réutilisés dans un autre protocole N°

25. RISQUES POUR LE PERSONNEL ET LES ANIMAUX

Type de risque :

☒ Aucun	☐ Cancérigène	☐ Infectieux
☐ Vecteur viral	☐ Chimique	☐ ADN/ARN recombinant
☐ Transplantation de tumeurs	Spécifiez :	
☐ Radio-isotopes / radiation	Nom de l'isotope :	Activité totale par animal :

Administration :

Agent :	Quantité :	Voie d'administration :
Fréquence :	Durée totale :	
Durée du risque de contamination :		

Voie prévue d'excrétion par l'animal :

☐ Aérosol	☐ Sang	☐ Urine
☐ Fèces	☐ Toutes sécrétions	☐ Autre. Spécifiez :

Mesures à prendre pour réduire le(s) risque(s) :

Niveau de confinement : minimum

26. ANNEXE

4. ÉVALUATION PAR DES PAIRS/ SOURCES DE FINANCEMENT (Voir Directives aux chercheurs)

Organisme de pairs ☒ Entreprise ☐	Demande de subvention	Période de subvention
IRSC	☐ Nouvelle ☒ En cours ☐ Renouvellement	1999-2005
	☐ Nouvelle ☐ En cours ☐ Renouvellement	

Pour un protocole n'ayant pas fait l'objet d'une évaluation par un comité de pairs reconnu, s'il vous plaît nous suggérer des évaluateurs affiliés à l'Université de Montréal :

Nom :	Téléphone : ( )
Nom :	Téléphone : ( )
Nom :	Téléphone : ( )

5. PERSONNEL QUI UTILISERA DES ANIMAUX (Compétences)

Chaque utilisateur doit avoir complété une seule fois le formulaire : Compétences professionnelles de l'utilisateur d'animaux.

Type[1]	Nom	Prénom	Expérience pour ce type de technique / procédure	Diplôme	Tél.
P	NAVARRO	DARREN	☒ Oui ☐ Non	MSC	35740
E	KRUSE-WHATLEY	Milarca	☒ Oui ☐ Non	BSC	35740
C	ZWINGMANN	CLAUDIA	☒ Oui ☐ Non	PH D	35740
			☐ Oui ☐ Non		
			☐ Oui ☐ Non		
			☐ Oui ☐ Non		
			☐ Oui ☐ Non		
			☐ Oui ☐ Non		

[1] **P** : Principal utilisateur **C** : Chercheur associé **A** : Assistant de recherche **E** : Étudiant **T** : Technicien

Dans le cas où les individus n'ont aucune expérience d'une technique ou d'une procédure particulière au protocole, indiquez comment ils obtiendront l'expertise :

6. TYPE DE PROJET

☐ projet pilote ☒ recherche ☐ enseignement N° cours : _____

7. RÉSUMÉ VULGARISÉ ET CONCIS DU PROJET

Ce résumé doit pouvoir servir à la préparation d'un document de presse pour les médias. Expliquez de façon à être **compris par un public non scientifique**, le but général de votre recherche, les objectifs spécifiques que vous entendez poursuivre avec ce protocole et la contribution aux connaissances scientifiques ou à la santé de l'homme et/ou des animaux :

7. RÉSUMÉ VULGARISÉ ET CONCIS DU PROJET

Le but principal de ce projet est d'examiner les mécanismes qui mènent aux dommages cérébraux observés dans la déficience en thiamine expérimentale. Ce désordre s'apparente à l'encéphalopathie de Wernicke chez l'humain qui fait souvent suite à l'alcoolisme chronique. Notre but est de comprendre les événements conduisant aux dommages cérébraux observés suite à une déficience en thiamine et par le fait même, mieux comprendre ce type de maladie neurodégénérative.

8. RÉSUMÉ SCIENTIFIQUE

Ce résumé permet aux membres du CIPA de comprendre les buts, les objectifs de votre recherche ainsi que la pertinence de l'utilisation d'animaux pour votre projet.

Notre projet comporte deux axes de recherche majeurs:

1. L'inflammation

2. Études du métabolisme énergétique et de neuroprotection par Résonnance Magnétique Nucléaire

1. INFLAMMATION

Plusieurs études récentes sur la déficience en thiamine ont souligné une progression temporelle de la mort neuronale dans les régions vulnérable à la déficience en thiamine dans le cerveau de rats et de souris. La raison pour laquelle certaines régions sont sélectivement touchées est toujours inconnue. Il est toutefois admis que la mort neuronale sélective dans ces régions est précédée par la disruption de la barrière hémato-encéphalique en raison d'une réponse inflammatoire localisée dans ces régions. Des changements d'activité d'enzymes dépendents de la thiamine (par exemple, l'alpha-kétoglutarate déshydrogénase) surviennent également dans ces régions. Nous proposons donc d'évaluer la présence de neuroprotection suite au bloquage de la réponse inflammatoire à différentes étapes de son développement lors de la déficience en thiamine chez le rat. Ces études pourraient nous aider à déterminer si l'utilisation d'immunosuppressants ou de drogues anti-inflammatoire pourrait s'avérer bénéfique lors de la déficience en thiamine.

L'effet neuroprotecteur de différents agents pharmacologiques sera donc évalué:

a) Indométhacine (anti-inflammatoire non-stéroidien): il s'agit d'un inhibiteur non-spéifique de la cycloxygénase

b) Le Vioxx est un inhibiteur spécifique de la COX-2. Des études préliminaires ont démontré une hausse d'expression de l'ARNm et de la protéine COX-2 dans les régions vulnérable à la déficience en thiamine au stade présymptomatique.

c) L'inflammation de la barrière hémato-encéphalique survient tôt lors de la déficience en thiamine. L'effet de la cyclosporine A. (immunosuppressant et inhibiteur des cytokines pro-inflammatoires) sur l'intégrité de la barrière hémato-encéphalique et la neuropathologie sera étudié.

d) Des études préliminaires ont également démontré une augmentation de l'expression de eNOS dans les régions vulnérables lors de la déficience en thiamine. Nous utiliserons donc le L-NIO, un inhibiteur de eNOS, afin de déterminer si cette induction de eNOS est protectrice ou néfaste.

2. Études du métabolisme énergétique et de neuroprotection par Résonnance Magnétique Nucléaire

La déficience en thiamine induite par la pyrithiamine résulte en une baisse réversible de l'activité de l'enzyme dépendent de la thiamine alpha-ketoglutarate déshydrogénase, ainsi que des changements concomitants des concentrations d'acides aminés tels que le glutamate, le GABA et l'aspartate. Nous proposons donc qu'une dysfonction du métabolisme énergétique constitue la "lésion biochimique" propre à la déficience en thiamine.

Contrairement aux autres organes, le métabolisme énergétique cérébral dépent presque exclusivement de

8. RÉSUMÉ SCIENTIFIQUE

l'oxidation du glucose. À ce jour, il n'y a cependent toujours pas d'études démontrant une corrélation entre la dysfonction du métabolisme énergétique et le métabolisme du glucose lors de la déficience en thiamine. Nous évaluerons les voies métaboliques spécifiques aux différents types cellulaires dans les régions vulnérables (thalamus médian et collicule inférieur) et non-vulnérables (cortex frontal) de rats ayant une déficience en thiamine causée par l'antagoniste de la thiamine, la pyrithiamine. Ces études seront effectuées grâce à la technique de Rénonnance Magnétique Nucléaire à haute résolution ex vivo, combinée à une injection in vivo de [1-13C]glucose. Cette technique nous permettra de mesurer la concentration de métabolites et leur synthèse de novo ainsi que les flux métaboliques intra- et extra-cellulaires, ce qui n'est pas possible grâce aux techniques traditionnelles faisant usage de radiologands.

a) Des études préliminaires démontrent que les niveaux sanguins de glucose sont augmentés jusqu'à 200% lors de la déficience en thiamine. Une première étude évaluera donc l'effet du glucose sur les symptomes neurologiques et l'activité métabolique lors de la déficience en thiamine

b) Les effets de l'insuline (afin de réduire les niveaux sanguins de glucose) sur les symptomes neurologiques et l'activité métabolique lors de la déficience en thiamine sera évalué.

c) Des études préliminaires ont démontré une consommation accrue de corps cétonique au stade présymptomatique après quoi la consommation diminue. Puisque les corps cétoniques peuvent être neuroprotectifs lors d'autres encéphalopathies métaboliques et maladies neurodégénératives, nous nous proposons d'évaluer l'effet des corps cétoniques sur les symptomes neurologiques et l'activité métabolique lors de la déficience en thiamine

d) D'autres études ont démontré une récupération presque complète du métabolisme énergétique suite à l'administration de N-acétyl-cystéine (NAC), un antioxydant. Nous effecterons donc une étude plus poussée du NAC sur les symptomes neurologiques et l'activité métabolique lors de la déficience en thiamine

9. NATURE DU PROJET

Afin de faciliter l'examen de votre demande par le CIPA, veuillez cocher les manipulations utilisées ou les conditions prévalant au cours de l'expérimentation :

- ☐ Aiguë
- ☒ Non traumatique
- ☐ Immunisation
- ☐ Chirurgie/ anesthésie
- ☐ Colonie de production d'animaux
- ☐ Privation. Spécifiez : légère restriction de nourriture (pair-feeding)
- ☒ Chronique
- ☐ Traumatique
- ☐ Étude de comportement
- ☐ Induction au stress
- ☐ Immobilisation
- ☐ Autre. Spécifiez :

10. REMPLACEMENT, RÉDUCTION, RAFFINEMENT

« Des animaux ne devraient être utilisés que si le chercheur a tenté en vain, par tous les moyens possibles, de trouver une solution de rechange » [1]

10.1 Lors de la conception de ce projet, avez-vous considéré le remplacement des animaux par une méthode alternative comme :

- ☐ Un modèle utilisant la culture cellulaire
- ☐ Un modèle non vivant
- ☐ L'emploi d'un animal de phylum taxinomique inférieur

[1] Manuel sur le soin et l'utilisation des animaux d'expérimentation. Volume 1, CCPA, 1993, page 219

10. REMPLACEMENT, RÉDUCTION, RAFFINEMENT

Pour les protocoles d'enseignement :

☐ Un document audiovisuel ☐ Un logiciel de simulation

Expliquez pourquoi vous ne pouvez utiliser une de ces alternatives : La déficience en thiamine est une maladie multifactorielle ayant un effet sur l'organisme entier, mais touchant très spécifiquement certaines régions cérébrales. Il est donc nécessaire d'utiliser un modèle animal. Le rat est un animal du phylum taxinomique le plus inférieur que nous puissions utiliser pour nos expériences.

« Ceux qui utilisent des animaux doivent recourir aux méthodes les plus humaines et ce, sur le plus petit nombre possible d'animaux appropriés requis pour obtenir des renseignements valables». [2]

10.2 Lors de la conception de ce projet, avez-vous considéré le **NOMBRE MINIMAL** d'animaux nécessaires pour obtenir des résultats valables? Expliquez :

Oui, nous utilisons un n de 5 rats par groupe étudié, ce qui représente le nombre minimal de rats nécessaire afin que nos études soient exploitables statistiquement. D'autre part, nous tentons, lorsque possible, d'effectuer le plus de mesures possibles sur chaque animal afin de minimiser le nombre d'animaux requis. Cependent, les régions cérébrales vulnérables à la déficience en thiamine sont très petites, ce qui rend difficile la mesure de plusieurs paramètres à la fois.

10.3 Lors de la conception de ce projet, avez-vous considéré le raffinement (analgésie, euthanasie humanitaire, anesthésie, enrichissement du milieu, etc....)? Expliquez :

Les rats qui subiront une perfusion-fixation seront préalablement anesthésiés grâce à du pentobarbital

10.4 Avez-vous considéré le niveau de douleur et d'inconfort de l'animal en rapport avec l'importance des résultats escomptés? Expliquez :

Le niveau d'inconfort est minimisé autant qu'il se peut. Cet inconfort est nécessaire à l'obtention de résultats pouvant contribuer à développer de nouvelles voies thérapeutiques pour la déficience en thiamine.

10.5 Quels sont les organes des animaux utilisés qui peuvent servir à un autre protocole? Indiquez les organes :

Tout sauf le cerveau

11. PRODUCTION D'ANTICORPS

Demande de production d'anticorps : oui ☐ **non** ☒

Dans le cas d'un protocole de production d'anticorps, veuillez indiquer les démarches entreprises pour déterminer si l'anticorps est disponible commercialement ou auprès d'un autre groupe de recherche.

Sites Internet visités :

Personnes contactées :

12. ANIMAUX REQUIS (Vous pouvez utiliser les lignes avec ou sans les listes déroulantes)

Espèce (Ex: rat, chien)	Souche (C57Bl/6, Yucatán)	Âge / poids	Sexe M / F	Fournisseur	Nb demandé en 1 fois[1]	Nb de cage [1]	Nb pour année	Total projet
rat	Sprague-Dawley	200-225 fg	M	Charles River	15	1	384	

[2] Manuel sur le soin et l'utilisation des animaux d'expérimentation. Volume 1, CCPA, 1993, page 219

12. ANIMAUX REQUIS (Vous pouvez utiliser les lignes avec ou sans les listes déroulantes)

Espèce (Ex: rat, chien)	Souche (C57Bl/6, Yucatán)	Âge / poids	Sexe M / F	Fournisseur	Nb demandé en 1 fois[1]	Nb de cage [1]	Nb pour année	Total projet
				Charles River				
				Charles River				
				Charles River				
				Charles River				

[1] Estimation

N.B. ANIMAUX TRANSGÉNIQUES (Les utilisateurs de souris TG doivent remplir annuellement la fiche d'utilisation des animaux transgéniques.)

13. DURÉE DE L'ÉTUDE

Date prévue du début de l'expérience : 06/2003 (mois/ année) Date prévue de la fin : 06/2005 (mois/ année)

Veuillez indiquer la période maximale durant laquelle l'animal sera gardé pour l'étude :

16 jours

14. JUSTIFICATION DU NOMBRE D'ANIMAUX

Les techniques expérimentales utilisées doivent être faites sur le plus petit nombre requis d'animaux pour obtenir des données valables. La justification du nombre d'animaux doit comprendre une description claire du modèle expérimental incluant **un tableau résumant vos groupes expérimentaux et contrôles**. Indiquez également le rationnel statistique qui vous a permis de déterminer le nombre d'animaux par groupe. **Pour les protocoles d'élevage, veuillez fournir le nombre d'animaux requis pour faire et maintenir la colonie.**

Tableau
Étude # 1: inflammation

Il y aura trois groupes par agent pharmacologique utilisé:
1. contrôle + drogue
2. déficient en thiamine + drogue
3. Déficient en thiamine + saline

Chaque groupe comporte 5 animaux, le nombre minimal nécessaire afin de pouvoir faire une analyse statistique valable. Un groupe d'étude comporte donc 15 animaux

Les rats recevant des agents pharmacologiques seront sacrifiés au moment où les rats déficients en thiamine atteindront le stade symptomatique (perte du réflexe de redressement).

Quatre groupes d'étude de 15 animaux seront nécessaires afin d'effectuer les expériences suivantes:
a) étude de l'expression protéique (Western blots de HO-1, COX-2, GFAP, etc.)
b) études de l'expression de l'ARNm codant pour ces mêmes protéines
c) études de neuropathologie cellulaires (coupes histologiques révélant l'immunomarquage de NeuN (un marqueur neuronal)
d) étude de l'activité d'enzymes dépendents de la thiamine (alpha-KGDH)

Comme nous l'avons mentionnné plus tôt, nous utiliserons quatre drogues différentes. Le nombre total d'animaux requis est donc de 4(drogues) X 4 (paramètres à étudier) X 15 animaux par groupe = 240 rats

Étude # 2. Études du métabolisme énergétique et de neuroprotection par Résonnance Magnétique Nucléaire

Il y aura trois goupes par étude:
1. contrôles
2. pré-symptomatiques
3. symptomatiques

chaque groupe est composé d'un n de 4. Cependant, pour les études de Résonnance Magnétique Nucléaire une très grande quantité de tissus est nécessaire, ce qui nous oblige à "pooler" trois cerveaux par échantillon. Chaque étude nécessite donc 3 (groupes) X 4 (échantillons (n)) X 3 (cerveaux par échantillons)= 36 rats

Comme nous l'avons mentionné plus tôt, nous effectuerons quatre études
1) effet du glucose
2) effet de l'insuline
3) effet des corps cétoniques
4) effet de NAC

L'étude # 2 demande donc 4 X 36 = 144 rats
Pour un total de 144 + 240 = 384 rats

15. HÉBERGEMENT ET SOINS AUX ANIMAUX (Donnez les explications ci-dessous)

☐ Hôtel-Dieu ☐ Hôpital Notre-Dame ☒ Hôpital Saint-Luc

☒ Manipulations dans les locaux de l'animalerie
☐ Manipulations à l'extérieur
☐ Retour à l'animalerie
☒ Régimes alimentaires spéciaux
☐ Équipement particulier
☐ Installations pour animaux immunodéficients
☐ Assistance technique par le personnel de l'animalerie
☐ Autre Justifiez et / ou spécifiez :

16. ENRICHISSEMENT DU MILIEU

Selon les directives du CCPA [3], on se doit de fournir un milieu stimulant approprié pour chaque espèce animale mise en cage. Des stratégies d'enrichissement de l'environnement sont donc systématiquement effectuées pour les animaux utilisés dans **tous** les protocoles approuvés par le CIPA (référence : Politique et procédure C-1 ENRICHISSEMENT DU MILIEU) à moins que les objectifs de l'étude n'en interdisent l'usage.

Non. Certaines stratégies d'enrichissement de l'environnement ne peuvent être utilisées :

☒ Précisez lesquelles. L'utilisation des cylindres en plastique serait à éviter puisque nous avons observés que les rats les grignotaient, et avalaient les morceaux de plastique.

17. PROCÉDURE D'UTILISATION DES ANIMAUX DE PROTOCOLE EXPÉRIMENTAL

Décrire ci-dessous les procédures expérimentales, la description chirurgicale et toutes les manipulations pratiquées chez l'animal (à l'aide des « Procédures normalisées de fonctionnement » (PNF) du Service des animaleries du CRCHUM, ou, si possible, d'articles publiés, de livres de références). Lors d'utilisation de médicaments, de produits biologiques ou d'autres agents administrés aux animaux, inclure également une justification du choix de l'agent et des doses, les effets secondaires et de quelle façon les animaux seront surveillés pour assurer le suivi du protocole à l'intérieur des points limites proposés.

Les numéros des PNF utilisées :

La déficience en thiamine est obtenue grâce à une diète déficiente en thiamine combinée à l'administration de pyrithiamine 50 ug/kg, i.p., une fois par jour, sur une durée totale de 14 jours, moment où les animaux deviennent symptomatiques et sont sacrifiés.

Tous les agents pharmacologiques (sauf le Vioxx; voir plus bas) seront administrés par voie intrapéritonéale, une fois par jour, selon les doses et le début du traitement suivants :

Indométhacine : 5 mg/kg/jour ; débutant au jour 7

Cyclosporin A : 10 mg/kg/jour ; débutant au jour 7

L-NIO : 50 mg/kg/jour ; débutant au jour 9

Glucose : 1 g/kg/jour ; débutant au jour 1

Insuline : la dose sera ajustée afin d'obtenir les mêmes niveaux de glucose sanguin que les contrôles

Beta-hydroxybutyrate (Corps cétonique) : 500 mg/kg/jour ; débutant au jour 6

NAC : 500 mg/kg/jour ; débutant au jour 7

Le Vioxx sera administré par gavage. En résumé, un comprimé de Vioxx est pulvérisé à l'aide d'un pilon et d'un mortier. La poudre est ensuite resuspendue dans de la saline. Une dose de 10 mg/kg/jour est ensuite administrée par gavage à l'aide d'une seryngue à gavage. Ce traitement est effectué une fois par jour à partir du jour 8.

Pour les études de Résonnance Magnétique Nucléaire, les rats recevront une dose de 200mg/kg de [U-13C]glucose une heure avant d'être sacrifiés.

Dans le cas d'études d'immunohistochimie la technique de perfusion-fixation sera effectuée tel que décrit dans le protocole S98048RBr.

[3] Manuel sur le soin et l'utilisation des animaux d'expérimentation, CCPA, Volume 1, 1993, page 58

18. ANALGÉSIE

Dans toute situation où il est possible que l'animal ressente de la douleur, une analgésie adéquate doit être utilisée, de même qu'après toute chirurgie ou manipulation invasive.

☐ Oui. Un analgésique sera utilisé — Nom de l'agent utilisé : _____

Dosage : _____ — Voie : _____

Nom du responsable : _____ — Fréquence : _____ — Durée : _____

☒ Contre-indiquée. Justifiez de façon détaillée les interférences rapportées dans la littérature :

Les analgésiques neuroactifs

☐ Non requise. Justifiez :

19. ANESTHÉSIE

☒ Oui. Une anesthésie est nécessaire ☐ Avec réveil ☒ Sans réveil (terminal)

Nom du responsable : DARREN NAVARRO — Durée totale de l'anesthésie : 20 MINUTES

☐ Description du protocole anesthésique en annexe (**fait avec la collaboration du personnel de l'animalerie**)

☐ Selon les « Procédures normalisées de fonctionnement » (**PNF**) du Service des animaleries N° _____

☒ **Sinon, veuillez remplir le tableau ci-dessous**

Anesthésie	Agent	Dose (mg/kg)	Voie
Pré-anesthésique			
Induction	pentobarbital	1.6 mL/kg	I.P.
Maintien	pentabarbital		
Traitement de support			
Fluides			
Antibiothérapie			

☐ Contre-indiquée. Justifiez :

☐ Non requise. Justifiez :

20. CHIRURGIE / MANIPULATIONS SOUS ANESTHÉSIE

La procédure doit être décrite en détails à la section 17, Procédures d'utilisation des animaux de protocole expérimental.
Toute procédure chirurgicale ou manipulation sous anesthésie doit être effectuée conformément aux Politiques et procédures du CIPA.

☒ Non, allez à la section 22

☐ Oui, veuillez cocher les cases appropriées :

- ☐ Procédure terminale, l'animal sera euthanasié avant le réveil.
- ☐ Chirurgie majeure : accès à une cavité corporelle ou altération permanente de la physiologie ou de l'anatomie de l'animal.
- ☐ Chirurgie mineure
- ☐ Manipulations non chirurgicales sous anesthésie générale

21. SOINS PEROPÉRATOIRES ET POSTOPÉRATOIRES

Chez les rongeurs, on doit assurer la surveillance des signes vitaux et des réflexes.
Chez les grands animaux, on devrait assurer la surveillance des signes vitaux et des réflexes (durant la chirurgie). Un technicien expérimenté devrait assurer le « monitoring ». Les soins postopératoires doivent être effectués conformément aux Politiques et procédures du CIPA.

Surveillance peropératoire. Décrire les paramètres à observer :

☐ Température ☐ ECG ☐ Pression sanguine ☐ SPO_2

☐ Pincement de l'orteil ☐ Pincement de la queue ☐ Réflexe palpébral

☐ Relaxation musculaire ☐ Couleur des muqueuses ☐ Pouls

Veuillez spécifier les soins peropératoires spéciaux, si requis :

Nom du responsable : Ou personnel de l'animalerie : ☐

L'animal devra être surveillé durant la période de réveil au moins toutes les 15 minutes. Il devra être retourné à sa cage lorsqu'il aura été extubé, qu'il aura atteint une température corporelle de 37°C et qu'il aura fait des efforts pour se mettre en décubitus sternal.

Surveillance postopératoire. Décrire les paramètres à observer :

☐ Température ☐ ECG ☐ Pression sanguine ☐ SPO_2

Veuillez spécifier les soins postopératoires spéciaux, si requis :

Nom du responsable : Ou personnel de l'animalerie : ☐

22. POINTS LIMITES

« Il faut éviter de soumettre les animaux à des souffrances ou à des angoisses inutiles. » [4,5]

Voici une liste des points limites les plus fréquemment observés en recherche animale et requérant qu'un animal soit traité ou euthanasié si sa condition se détériore au point où il ne peut plus être traité.

Veuillez indiquer quelles conditions suivantes peuvent être provoquées par vos manipulations et expliquez a) à quel point limite la décision sera prise de sacrifier l'animal et b) les traitements appropriés à administrer.

Changements physiologiques :

- ☐ Hypothermie
- ☐ Perte de poids
- ☐ Autre Expliquez :
- ☐ Période de fièvre prolongée
- ☐ Déshydratation
- ☐ Troubles nerveux
- ☐ Hypoxie

Changements comportementaux

- ☐ Anorexie prolongée
- ☐ Prostration
- ☐ Autre Expliquez :
- ☐ Agressivité reliée à la douleur
- ☐ Douleur incontrôlable

Traitements :

23. CAS D'URGENCE

Dans l'impossibilité de rejoindre le chercheur ou un membre de son équipe, le CIPA peut autoriser l'euthanasie d'un animal de façon humanitaire, s'il est impossible de soulager la douleur ou l'angoisse que l'animal ressent.

Afin de prévenir la perte de vos données scientifiques, veuillez indiquer :

- ☒ Tissus à prélever. Spécifiez : cerveau
  Méthode de conservation : congélation dans l'isopentane
- ☐ Sang
  Couleur du tube : Quantité :
  Méthode de conservation :
- ☐ Autre. Spécifiez :

24. EUTHANASIE (Indiquez la méthode d'euthanasie pour chaque espèce)

[4] Manuel sur le soin et l'utilisation des animaux d'expérimentation, CCPA, Volume 1, 1993, page 219

[5] Lignes directrices : choisir un point limite approprié pour les expériences faisant appel à l'utilisation des animaux en recherche, en enseignement et dans les tests, CCPA, 1998

24. EUTHANASIE (Indiquez la méthode d'euthanasie pour chaque espèce)

Pour connaître les méthodes d'euthanasie acceptables, veuillez vous référer au Manuel du CCPA [6].

Espèce Nº 1 : rats

☐ Surdosage d'anesthésique
- ☐ Barbiturique ☐ Gaz. Agent :
- ☐ Autre. Spécifiez :

☒ Moyen physique. Ce choix doit être exigé par le protocole. Un sédatif ou un tranquillisant doit être administré, si possible.
- ☒ Décapitation Sédatif : isoflurane **Justifiez :** pour prélèvement du cerveau
- ☐ Dislocation cervicale Sédatif : **Justifiez :**

☐ Exsanguination sous anesthésie. Agent anesthésique :

☐ CO_2

☐ Autre. Précisez :

☐ Les animaux seront réutilisés dans un autre protocole Nº _____

Espèce Nº 2 :

☐ Surdosage d'anesthésique
- ☐ Barbiturique ☐ Gaz. Agent :
- ☐ Autre. Spécifiez :

☐ Moyen physique. Ce choix doit être exigé par le protocole. Un sédatif ou un tranquillisant doit être administré, si possible.
- ☐ Décapitation Sédatif : **Justifiez :**
- ☐ Dislocation cervicale Sédatif : **Justifiez :**

☐ Exsanguination sous anesthésie. Agent anesthésique :

☐ CO_2

☐ Autre. Précisez : _____

☐ Les animaux seront réutilisés dans un autre protocole Nº _____

25. RISQUES POUR LE PERSONNEL ET LES ANIMAUX

Type de risque :

☒ Aucun	☐ Cancérigène	☐ Infectieux
☐ Vecteur viral	☐ Chimique	☐ ADN/ARN recombinant
☐ Transplantation de tumeurs	Spécifiez :	
☐ Radio-isotopes / radiation	Nom de l'isotope :	Activité totale par animal :

Administration :

Agent : Quantité : Voie d'administration :

[6] Manuel sur le soin et l'utilisation des animaux d'expérimentation, CCPA, Volume 1, 1993, page 161.

25. RISQUES POUR LE PERSONNEL ET LES ANIMAUX

Fréquence : Durée totale :

Durée du risque de contamination :

Voie prévue d'excrétion par l'animal :

☐ Aérosol ☐ Sang ☐ Urine

☐ Fèces ☐ Toutes sécrétions ☐ Autre. Spécifiez :

Mesures à prendre pour réduire le(s) risque(s) :

Niveau de confinement : minimum

26. ANNEXE

MRCRM

Medical Research Council of Canada / Conseil de recherches médicales du Canada

AUTHORIZATION FOR FUNDING

The Medical Research Council has approved funding as detailed below. Subject to the approbation of funding by Parliament, these funds will be made available to the business officer at the indicated institution for disbursement.

AUTORISATION DE FINANCEMENT

Le Conseil de recherches médicales vous accorde les fonds tel qu'indiqué ci-dessous. Suivant l'affectation des crédits par le Parlement du Canada, les fonds seront mis à la disposition du trésorier de l'établissement indiquée qui s'occupera des versements.

REVISED **RÉVISÉ**

199903MOP-42333-M-CFCA-11020

28/02/2000

Recipient/Bénéficiaire: Dr. Roger F. BUTTERWORTH
Medicine
Faculty of Medicine
Université de Montréal

Program/Programme: Operating Grants
Grant New

Project Title/Titre du Projet:
Further studies on the pathophysiology of selective neuronal cell death due to vitamin B1 deficiency

Co-investigator(s) & Associates/Supervisor(s)/Host/Co-chercheur(s)/Directeur(s) de Recherche/Hôte:

Industrial Partner(s)/partenaire(s) Industriel(s):

PAYMENT DETAILS/DÉTAILS DES VERSEMENTS Funding Reference Number/ No. de Référence du Financement: MOP – 36441

Period / Période	Type	Amount by Type / Montant Par Type	Total by Fiscal Year / Total Par Exercice	
01/10/1999 to 31/03/2000	Operating	$45,003	$45,003	1999-00
01/04/2000 to 31/03/2001	Operating	$73,641	$73,641	2000-01
01/04/2001 to 31/03/2002	Operating	$73,641	$73,641	2001-02
01/04/2002 to 31/03/2003	Operating	$73,641	$73,641	2002-03
01/04/2003 to 31/03/2004	Operating	$73,641	$73,641	2003-04
01/04/2004 to 30/09/2004	Operating	$36,820	$36,820	2004-05

Progress Report Required: **01/03/2002**
Rapport des Progrès Réalisés Requis:

Application to Renew Funding Required: **01/03/2004**
Demande de Renouvellement des Fonds Requis:

NOTES:

This revised AFF reflects the awarding of a supplement to the operating funds for fiscal year 1999-00. /
Cette autorisation de financement reflète l'allocation d'un supplément aux fonds de fonctionnement pour l'exercice 1999-2000.

J. Warnock for

Cindy L. Bell, Ph.D.
Deputy Director, Programs Branch
Head, Physiological Systems Unit

cc — Supervisor/Directeur
— Dean/Doyen

4. ÉVALUATION PAR DES PAIRS/ SOURCES DE FINANCEMENT (Voir Directives aux chercheurs)

Organisme de pairs ☒ Entreprise ☐	Demande de subvention	Période de subventic
CIHR	☐ Nouvelle ☐ En cours ☒ Renouvellement	2000-2005
	☐ Nouvelle ☐ En cours ☐ Renouvellement	

Pour un protocole n'ayant pas fait l'objet d'une évaluation par un comité de pairs reconnu, s'il vous plaît nous suggérer des évaluateurs affiliés à l'Université de Montréal :

Nom :	Téléphone : ( )
Nom :	Téléphone : ( )
Nom :	Téléphone : ( )

5. PERSONNEL QUI UTILISERA DES ANIMAUX (Compétences)

Chaque utilisateur doit avoir complété une seule fois le formulaire : Compétences professionnelles de l'utilisateur d'animaux

Type(1)	Nom	Prénom	Expérience pour ce type de technique / procédure		Diplôme	Tél.
E	NAVARRO	DARREN	☒ Oui	☐ Non	M.Sc.	35740
E	CHATAURET	NICOLAS	☒ Oui	☐ Non	M.SC.	35740
E	BELANGER	MIREILLE	☒ Oui	☐ Non	M.SC.	35740
			☐ Oui	☐ Non		
			☐ Oui	☐ Non		
			☐ Oui	☐ Non		
			☐ Oui	☐ Non		
			☐ Oui	☐ Non		

(1) **P** : Principal utilisateur **C** : Chercheur associé **A** : Assistant de recherche **E** : Étudiant **T** : Technicien

Dans le cas où les individus n'ont aucune expérience d'une technique ou d'une procédure particulière au protocole, indiquez comment ils obtiendront l'expertise :

6. TYPE DE PROJET

☐ projet pilote ☒ recherche ☐ enseignement N° cours :

7. RÉSUMÉ VULGARISÉ ET CONCIS DU PROJET

Ce résumé doit pouvoir servir à la préparation d'un document de presse pour les médias. Expliquez de façon à être **compris par un public non scientifique**, le but général de votre recherche, les objectifs spécifiques que vous entendez poursuivre avec ce protocole et la contribution aux connaissances scientifiques ou à la santé de l'homme et/ou des animaux :

7. RÉSUMÉ VULGARISÉ ET CONCIS DU PROJET

Le but de ce projet est d'examiner les mécanismes pathophysiologiques responsables des dommages cérébraux survenant à la suite d'une déficience en thiamine expérimentale. Ce modèle s'apparente à l'encéphalopathie de Wernicke chez l'humain, une pathologie survenant chez les patients souffrant d'alcoolisme chronique et/ou de malnutrition. L'objectif ultime de ce projet consiste à comprendre les mécanismes responsables de cette maladie neurodégérérative.

8. RÉSUMÉ SCIENTIFIQUE

Ce résumé permet aux membres du CIPA de comprendre les buts, les objectifs de votre recherche ainsi que la pertinence de l'utilisation d'animaux pour votre projet.

Nos études portant sur les mécanismes entraînant la mort neuronale dans certaines régions spécifiques du cerveau suite à la déficience en thiamine (causée par l'utilisation d'une diète déficiente en thiamine et l'administration de pyrithiamine, un antagoniste du métabolisme de la thiamine) chez le rat ont démontré que plusieurs changements surviennent de façon précoce (i.e. dans les jours précédant la mort neuronal) et par conséquent jouent très probablement un rôle important dans le développement de cette maladie neurodégénérative. Parmi ceux-ci, mentionnons i) un stress oxydatif découlant d'une augmentation rapide et importante de l'expression et de l'activation de l'isoforme endothélial de l'enzyme de synthèse de l'oxyde nitrique (eNOS) entraînant ainsi la formation de quantité considérable d'oxyde nitrique qui en réagissant avec l'anion superoxyde mènent à la production de peroxynitrite, une molécule hautement instable causant la nitration et la nitrosylation de certaines protéines de même que des dommages à l'ADN; ii) l'activation de la microglie entraînant une augmentation de iNOS, la forme inductible de cet enzyme via une augmentation de l'expression de l'interleukin-1beta et de la cyclooxygenase-2 (un mécanisme lié à l'activation du facteur de transcription NFkappaB) entraînant ainsi un stress oxydatif supplémentaire. Dans le but d'établir la contribution respective de ces évènements au processus de mort cellulaire, nous nous proposons d'utiliser des souris transgéniques (knock-out) afin d'évaluer différents paramètres qui pourraient éventuellement mener au développement de nouvelles avenues thérapeutiques. Les paramètres qui seront étudiés sont:

1. L'effet neuroprotecteur tel que démontré par le retardement de l'apparition des symptômes neurologiques et l'examen pathologique;

2. L'importance du stress oxydatif telle que démontrée par la formation de peroxynitrite et la nitration/nitrosylation de certaines protéines membranaires;

3. La rupture de la barrière hémato-encéphalique telle que démontrée par l'extravasation des immunoglobulines;

4. L'activation astrocytaire et microgliale telles que démontrées par immunohistochimie (GFAP, ED-1), la synthèse d'Interleukin-1beta, l'activation de l'enzyme Cyclooxygenase-2 (synthèse de prostaglandines), et l'activation du facteur de transcription NFkappaB.

Parmi les souris transgéniques disponibles commercialement (Jackson Laboratory, MA) et qui seront utilisées dans la présente étude mentionnons:

-Souris C57BL/6j (Stock # 000664), NOD/LtJ (Stock #001976) et B6129SF2/J (Stock # 101045) de types sauvages (à titre de contrôle)

-Souris déficientes pour l'isoforme de l'enzyme endothéliale de synthèse de l'oxyde nitrique synthétase (eNOS-/-) (Souche # B6.129P2-NOS3tm1Unc/J) (Stock # 002684) (souche contrôle: C57BL/6j);

-Souris déficientes pour l'isoforme inductible de l'enzyme de synthèse de l'oxyde nitrique synthétase (iNOS-/-) (Souche # B6.129P2-NOS2tm1Lau/J) (Stock # 002609) (souche contrôle C57BL/6j);

-Souris déficientes pour l'isoforme neuronal de l'enzyme de synthèse de l'oxyde nitrique synthétase (nNOS-/-)

8. RÉSUMÉ SCIENTIFIQUE

(Souche # B6.129S4-Nos1tm1Plh/J) (Stock # 002986) (souche contrôle C57BL/6j);

-Souris déficientes pour le récepteur de l'Interleukine de type 1 (IL-1b-/-) (Souche # B6.129S1-Il1rtm1Roml/J) (Stock # 003018) (souche contrôle: B6129SF2/j);

-Souris déficientes pour l'enzyme de conversion de l'interleukin-1beta (Casp1-/-) (Souche # NOD.129S2(B6)-Casp1tm1Sesh/Lt) (Stock # 003847) (souche contrôle: NOD/LtJ);

-Souris déficientes pour la cyclooxygénase-2 (COX2-/-) (Souche # B6.129S7-Ptgs2tm1Jed/J) (Stock # 002476) (souche contrôle: B6129SF2/j);

-Souris déficientes pour la protéine gliale acidique fibrillaire (GFAP-/-) (Souche # B6.129S-Gfaptm1Mes/J) (Stock # 002642) (souche contrôle: B6129SF/j).

Il est à noter que certaines de ces souris (ie. celles déficientes pour le recepteur de l'interleukin-1, l'enzyme de conversion de l'interleukin-1beta, iNOS, et la souche NOD/LtJ de type sauvage) présentent un phénotype d'immunodéficience.

9. NATURE DU PROJET

Afin de faciliter l'examen de votre demande par le CIPA, veuillez cocher les manipulations utilisées ou les conditions prévalant au cours de l'expérimentation :

- ☐ Aiguë
- ☒ Non traumatique
- ☐ Immunisation
- ☐ Chirurgie/ anesthésie
- ☐ Colonie de production d'animaux
- ☐ Privation. Spécifiez :
- ☒ Chronique
- ☐ Traumatique
- ☐ Étude de comportement
- ☐ Induction au stress
- ☐ Immobilisation
- ☐ Autre. Spécifiez :

10. REMPLACEMENT, RÉDUCTION, RAFFINEMENT

« Des animaux ne devraient être utilisés que si le chercheur a tenté en vain, par tous les moyens possibles, de trouver une solution de rechange » [1]

10.1 Lors de la conception de ce projet, avez-vous considéré le remplacement des animaux par une méthode alternative comme :

- ☐ Un modèle utilisant la culture cellulaire
- ☐ Un modèle non vivant
- ☐ L'emploi d'un animal de phylum taxinomique inférieur

Pour les protocoles d'enseignement :

- ☐ Un document audiovisuel
- ☐ Un logiciel de simulation

Expliquez pourquoi vous ne pouvez utiliser une de ces alternatives : La déficience en thiamine est une maladie multifactorielle ayant un effet sur l'organisme entier et touchant certaines régions spécifiques du cerveau.

« Ceux qui utilisent des animaux doivent recourir aux méthodes les plus humaines et ce, sur le plus petit nombre possible d'animaux appropriés requis pour obtenir des renseignements valables». [2]

10.2 Lors de la conception de ce projet, avez-vous considéré le **NOMBRE MINIMAL** d'animaux nécessaires pour obtenir des

[1] Manuel sur le soin et l'utilisation des animaux d'expérimentation. Volume 1, CCPA, 1993, page 219

[2] Manuel sur le soin et l'utilisation des animaux d'expérimentation. Volume 1, CCPA, 1993, page 219

10. REMPLACEMENT, RÉDUCTION, RAFFINEMENT

résultats valables? Expliquez.:

Le nombre minimal est utilisé pour pouvoir réaliser une analyse statistique convenable. De plus, le plus grand nombre de paramètres est mesuré sur chaque animal afin d'optimiser les résultats et de minimiser le nombre d'animaux requis.

10.3 Lors de la conception de ce projet, avez-vous considéré le raffinement (analgésie, euthanasie humanitaire, anesthésie, enrichissement du milieu, etc....)? Expliquez :

Les rats qui doivent subir une perfusion-fixation seront anesthésiés au pentobarbital.

10.4 Avez-vous considéré le niveau de douleur et d'inconfort de l'animal en rapport avec l'importance des résultats escomptés? Expliquez :

Le niveau de douleur et d'inconfort est maintenu à son plus bas niveau.

10.5 Quels sont les organes des animaux utilisés qui peuvent servir à un autre protocole? Indiquez les organes :

La totalité à l'exception du cerveau.

11. PRODUCTION D'ANTICORPS

Demande de production d'anticorps : oui ☐ **non** ☒

Dans le cas d'un protocole de production d'anticorps, veuillez indiquer les démarches entreprises pour déterminer si l'anticorps est disponible commercialement ou auprès d'un autre groupe de recherche.

Sites Internet visités :

Personnes contactées :

12. ANIMAUX REQUIS (Vous pouvez utiliser les lignes avec ou sans les listes déroulantes)

Espèce (Ex: rat, chien)	Souche (C57Bl/6, Yucatán)	Âge / poids	Sexe M / F	Fournisseur	Nb demandé en 1 fois(1)	Nb de cage (1)	Nb pour année	Total projet
Souris	C57BL/6J	24 sem.	M	Jackson Laboratory	24	6	72	
Souris	B6129SF2/J	24 sem.	M	Jackson Laboratory	24	6	72	
Souris	NOD/LtJ	24 sem.	M	Jackson Laboratory	24	6	24	
Souris	Transgeniques	24 sem.	M	Jackson Laboratory	24	6	168	
Souris				Charles River				
				Charles River				
				Charles River				
				Charles River				

(1) Estimation

N.B. ANIMAUX TRANSGÉNIQUES (Les utilisateurs de souris TG doivent remplir annuellement la fiche d'utilisation des animaux transgéniques.)

13. DURÉE DE L'ÉTUDE

Date prévue du début de l'expérience : 09/2004 mois/ année

Date prévue de la fin : 09/2005 mois/ année

13. DURÉE DE L'ÉTUDE

Veuillez indiquer la période maximale durant laquelle l'animal sera gardé pour l'étude :

12 jours

14. JUSTIFICATION DU NOMBRE D'ANIMAUX

Les techniques expérimentales utilisées doivent être faites sur le plus petit nombre requis d'animaux pour obtenir des données valables. La justification du nombre d'animaux doit comprendre une description claire du modèle expérimental incluant **un tableau résumant vos groupes expérimentaux et contrôles**. Indiquez également le rationnel statistique qui vous a permis de déterminer le nombre d'animaux par groupe. **Pour les protocoles d'élevage, veuillez fournir le nombre d'animaux requis pour faire et maintenir la colonie.**

Tableau obligatoire

Trois groupes de 8 animaux (type sauvage et transgénique) c'est-a-dire un groupe témoin, pré-symptomatique et symptomatique seront utilisés pour mesurer les différents paramètres mentionnés à la section #1 (total = 24 contrôles et 24 transgéniques). Au total, 7 différentes lignées de souris transgéniques seront étudiées. Le nombre total d'animaux requis sera donc de 7x 24 controles = 168 et 7x 24 transgéniques = 168.

15. HÉBERGEMENT ET SOINS AUX ANIMAUX (Donnez les explications ci-dessous)

☐ Hôtel-Dieu ☐ Hôpital Notre-Dame ☒ Hôpital Saint-Luc

☒ Manipulations dans les locaux de l'animalerie

☐ Manipulations à l'extérieur

☐ Retour à l'animalerie

☒ Régimes alimentaires spéciaux

☐ Équipement particulier

☒ Installations pour animaux immunodéficients

☐ Assistance technique par le personnel de l'animalerie

☐ Autre Justifiez et / ou spécifiez :

16. ENRICHISSEMENT DU MILIEU

Selon les directives du CCPA [3], on se doit de fournir un milieu stimulant approprié pour chaque espèce animale mise en cage. Des stratégies d'enrichissement de l'environnement sont donc systématiquement effectuées pour les animaux utilisés dans **tous** les protocoles approuvés par le CIPA (référence : Politique et procédure C-1 ENRICHISSEMENT DU MILIEU) à moins que les objectifs de l'étude n'en interdisent l'usage.

☐ Non. Certaines stratégies d'enrichissement de l'environnement ne peuvent être utilisées :
Précisez lesquelles.

17. PROCÉDURE D'UTILISATION DES ANIMAUX DE PROTOCOLE EXPÉRIMENTAL

Décrire ci-dessous les procédures expérimentales, la description chirurgicale et toutes les manipulations pratiquées chez l'animal (à l'aide des « Procédures normalisées de fonctionnement » (PNF) du Service des animaleries du CRCHUM, ou, si possible, d'articles publiés, de livres de références). Lors d'utilisation de médicaments, de produits biologiques ou d'autres agents administrés aux animaux, inclure également une justification du choix de l'agent et des doses, les effets secondaires et de quelle façon les animaux seront surveillés pour assurer le suivi du protocole à l'intérieur des points limites proposés.

Les numéros des PNF utilisées :

La déficience en thiamine est réalisée par l'administration d'une diète déficiente en thiamine combinée à l'administration de pyrithiamine 50µg/kg, i.p. une fois par jour, pour une durée maximale de 12 jours (stage symptomatique), après quoi les animaux sont sacrifiés.

Pour les études d'immunohistochimie (expression et rupture de la barrière hemato-encéphalique) la technique de perfusion-fixation sera réalisée tel que décrit dans le protocole S98048RBr.

18. ANALGÉSIE

Dans toute situation où il est possible que l'animal ressente de la douleur, une analgésie adéquate doit être utilisée, de même qu'après toute chirurgie ou manipulation invasive.

☐ Oui. Un analgésique sera utilisé — Nom de l'agent utilisé :
Dosage : — Voie :
Nom du responsable : — Fréquence : — Durée :

☐ Contre-indiquée. Justifiez de façon détaillée les interférences rapportées dans la littérature :
Action neuroactive des analgésiques.

☒ Non requise. Justifiez :
Aucune chirurgie ne sera réalisée

[3] Manuel sur le soin et l'utilisation des animaux d'expérimentation, CCPA, Volume 1, 1993, page 58

19. ANESTHÉSIE

☒ Oui. Une anesthésie est nécessaire ☐ Avec réveil ☒ Sans réveil (terminal)

Nom du responsable : DARREN NAVARRO Durée totale de l'anesthésie : 20MINUTES

☐ Description du protocole anesthésique en annexe (**fait avec la collaboration du personnel de l'animalerie**)

☐ Selon les « Procédures normalisées de fonctionnement » (**PNF**) du Service des animaleries N°

☒ **Sinon, veuillez remplir le tableau ci-dessous**

Anesthésie	Agent	Dose (mg/kg)	Voie
Pré-anesthésique			
Induction	pentobarbital	1.6ml/kg	i.p.
Maintien	pentobarbital		
Traitement de support			
Fluides			
Antibiothérapie			

☐ Contre-indiquée. Justifiez :

☐ Non requise. Justifiez :

20. CHIRURGIE / MANIPULATIONS SOUS ANESTHÉSIE

La procédure doit être décrite en détails à la section 17, Procédures d'utilisation des animaux de protocole expérimental.
Toute procédure chirurgicale ou manipulation sous anesthésie doit être effectuée conformément aux Politiques et procédures du CIPA.

☒ Non, allez à la section 22

☐ Oui, veuillez cocher les cases appropriées :

- ☐ Procédure terminale, l'animal sera euthanasié avant le réveil.
- ☐ Chirurgie majeure : accès à une cavité corporelle ou altération permanente de la physiologie ou de l'anatomie de l'animal.
- ☐ Chirurgie mineure
- ☐ Manipulations non chirurgicales sous anesthésie générale

21. SOINS PEROPÉRATOIRES ET POSTOPÉRATOIRES

Chez les rongeurs, on doit assurer la surveillance des signes vitaux et des réflexes.
Chez les grands animaux, on devrait assurer la surveillance des signes vitaux et des réflexes (durant la chirurgie). Un technicien expérimenté devrait assurer le « monitoring ». Les soins postopératoires doivent être effectués conformément aux Politiques et procédures du CIPA.

Surveillance peropératoire. Décrire les paramètres à observer :

☐ Température ☐ ECG ☐ Pression sanguine ☐ SPO_2

☐ Pincement de l'orteil ☐ Pincement de la queue ☐ Réflexe palpébral

☐ Relaxation musculaire ☐ Couleur des muqueuses ☐ Pouls

Veuillez spécifier les soins peropératoires spéciaux, si requis :

Nom du responsable : Ou personnel de l'animalerie : ☐

L'animal devra être surveillé durant la période de réveil au moins toutes les 15 minutes. Il devra être retourné à sa cage lorsqu'il aura été extubé, qu'il aura atteint une température corporelle de 37°C et qu'il aura fait des efforts pour se mettre en décubitus sternal.

Surveillance postopératoire. Décrire les paramètres à observer :

☐ Température ☐ ECG ☐ Pression sanguine ☐ SPO_2

Veuillez spécifier les soins postopératoires spéciaux, si requis :

Nom du responsable : Ou personnel de l'animalerie : ☐

22. POINTS LIMITES

« Il faut éviter de soumettre les animaux à des souffrances ou à des angoisses inutiles. » [4,5]

Voici une liste des points limites les plus fréquemment observés en recherche animale et requérant qu'un animal soit traité ou euthanasié si sa condition se détériore au point où il ne peut plus être traité.

Veuillez indiquer quelles conditions suivantes peuvent être provoquées par vos manipulations et expliquez a) à quel point limite la décision sera prise de sacrifier l'animal et b) les traitements appropriés à administrer.

Changements physiologiques :

☐ Hypothermie ☐ Période de fièvre prolongée ☐ Troubles nerveux

☐ Perte de poids ☐ Déshydratation ☐ Hypoxie

☐ Autre Expliquez :

Changements comportementaux

☐ Anorexie prolongée ☐ Agressivité reliée à la douleur

☐ Prostration ☐ Douleur incontrôlable

☐ Autre Expliquez :

[4] Manuel sur le soin et l'utilisation des animaux d'expérimentation, CCPA, Volume 1, 1993, page 219

[5] Lignes directrices : choisir un point limite approprié pour les expériences faisant appel à l'utilisation des animaux en recherche, en enseignement et dans les tests, CCPA, 1998

22. POINTS LIMITES

Traitements : Le traitement d'induction de la déficience en Thiamine est bien connu et conduit à l'apparition reproductible de symptômes neurologiques dus au manque de thiamine. Il n'y a pas d'autres effets qui nécessiteraient la définition de point limite.

23. CAS D'URGENCE

Dans l'impossibilité de rejoindre le chercheur ou un membre de son équipe, le CIPA peut autoriser l'euthanasie d'un animal de façon humanitaire, s'il est impossible de soulager la douleur ou l'angoisse que l'animal ressent.

Afin de prévenir la perte de vos données scientifiques, veuillez indiquer :

☒ Tissus à prélever. Spécifiez : CERVEAU

Méthode de conservation : Congélation dans l'isopentane et glace sèche.

☐ Sang

Couleur du tube : Quantité :

Méthode de conservation :

☐ Autre. Spécifiez :

24. EUTHANASIE (Indiquez la méthode d'euthanasie pour chaque espèce)

Pour connaître les méthodes d'euthanasie acceptables, veuillez vous référer au Manuel du CCPA [6].

Espèce N° 1 : souris

☐ Surdosage d'anesthésique

☐ Barbiturique ☐ Gaz. Agent :

☐ Autre. Spécifiez :

☒ Moyen physique. Ce choix doit être exigé par le protocole. Un sédatif ou un tranquillisant doit être administré, si possible.

☒ Décapitation Sédatif : isoflurane **Justifiez :** pour prélèvement du cerveau

☐ Dislocation cervicale Sédatif : **Justifiez :**

☐ Exsanguination sous anesthésie. Agent anesthésique :

☐ CO_2

☐ Autre. Précisez :

☐ Les animaux seront réutilisés dans un autre protocole N°

Espèce N° 2 :

☐ Surdosage d'anesthésique

☐ Barbiturique ☐ Gaz. Agent :

☐ Autre. Spécifiez :

☐ Moyen physique. Ce choix doit être exigé par le protocole. Un sédatif ou un tranquillisant doit être administré, si

[6] Manuel sur le soin et l'utilisation des animaux d'expérimentation, CCPA, Volume 1, 1993, page 161.

24. EUTHANASIE (Indiquez la méthode d'euthanasie pour chaque espèce)

possible.

☐ Décapitation Sédatif : **Justifiez :**

☐ Dislocation cervicale Sédatif : **Justifiez :**

☐ Exsanguination sous anesthésie. Agent anesthésique :

☐ CO_2

☐ Autre. Précisez :

☐ Les animaux seront réutilisés dans un autre protocole N°

25. RISQUES POUR LE PERSONNEL ET LES ANIMAUX

Type de risque :

☒ Aucun ☐ Cancérigène ☐ Infectieux

☐ Vecteur viral ☐ Chimique ☐ ADN/ARN recombinant

☐ Transplantation de tumeurs Spécifiez :

☐ Radio-isotopes / radiation Nom de l'isotope : Activité totale par animal :

Administration :

Agent : Quantité : Voie d'administration :

Fréquence : Durée totale :

Durée du risque de contamination :

Voie prévue d'excrétion par l'animal :

☐ Aérosol ☐ Sang ☐ Urine

☐ Fèces ☐ Toutes sécrétions ☐ Autre. Spécifiez :

Mesures à prendre pour réduire le(s) risque(s) :

Niveau de confinement : minimum

26. ANNEXE

Roger F. Butterworth

Further Studies on the Pathogenesis of selective neuronal cell Death Due to Vitamin B1 Deficiency

SUMMARY OF RESEARCH PROPOSAL

Vitamin B_1 (thiamine) deficiency results in one of two well-defined neurological disorders, namely a mixed sensory-motor neuropathy and Wernicke's Encephalopathy (also referred to as the Wernicke-Korsakoff Syndrome (WKS)). Neuropathologic evaluation of the brains of patients with WKS reveals a highly selective and reproducible pattern of neuronal cell loss involving primarily diencephalic and brainstem structures (Harper and Butterworth, Greenfield's Neuropathology: 601-655, 1997). In view of these observations and of our findings of selectively decreased activities of the mitochondrial enzyme αKGDH in vulnerable brain regions in thiamine deficiency, our studies have focussed on deficits in mitochondrial oxidation and their consequences as likely causes of neuronal cell death due to thiamine deficiency. Such mechanisms include impairments in cerebral energy metabolism (Navarro et al., J. Neurochem: 2004 (in press)), and damage due to reactive oxygen species (ROS). Consistent with this possibility are our findings of early activation of microglia (Todd and Butterworth, Glia: 190-198, 1999) and increased activities of the free radical scavenging enzyme superoxide dismutase (SOD) in vulnerable brain structures of thiamine-deficient rats. Microglia are a major source of ROS. More recent results from our Unit also reveal increased expression of endothelial nitric oxide synthesis (eNOS) mRNA in the medial thalamus and inferior colliculus, two vulnerable regions in thiamine-deficient rats (Kruse et al., Neurochem. Int.: 2004 (in press)).

In continuation of these studies the overall goal of the studies now proposed is to investigate the relative contribution of the vascular endothelium and activated microglia to oxidative stress and selective neuronal cell death in thiamine defiency.

Three hypotheses will be evaluated.

Hypothesis I. Induction of NO production by the vascular endothelium results in microglial activation in TD.

Hypothesis II. Cytokine (IL-1β) production by activated microglia contributes to selective neuronal cell death in TD.

Hypothesis III. These above features occur in WKS in humans.

A series of 5 specific aims will address Hypotheses I to III:

Specific Aim #1. To demonstrate that eNOS gene knockout prevents the formation of peroxynitrite/nitrotyrosine and blood brain barrier dysfunction in brain regions destined to manifest neuronal cell death due to TD.

Specific Aim #2. To demonstrate IL-1β localization to activated microglia and/or vascular endothelial cells in brain structures destined to manifest neuronal cell death in TD.

Specific Aim #3. To demonstrate that eNOS gene knockout prevents microglial activation and IL-1β production in vulnerable brain structures in TD.

Specific Aim #4. To demonstrate that the prevention of microglial activation using (+)naloxone prevents IL-1β production and attenuates neuronal cell loss in brain structures vulnerable to TD.

Specific aim #5. To demonstrate that eNOS induction, microglial activation and IL-1β production are features of the cell death cascade in WKS in humans.

In addition to the elucidation of neuronal cell death mechanisms in thiamine deficiency-related disorders, such as WKS, the proposed studies have the potential to advance our understanding of the relationship between chronic mitochondrial dysfunction and neurodegeneration.

MRCRM

Medical Research Council of Canada / Conseil de recherches médicales du Canada

AUTHORIZATION FOR FUNDING

The Medical Research Council has approved funding as detailed below. Subject to the approbation of funding by Parliament, these funds will be made available to the business officer at the indicated institution for disbursement.

AUTORISATION DE FINANCEMENT

Le Conseil de recherches médicales vous accorde les fonds tel qu'indiqué ci-dessous. Suivant l'affectation des crédits par le Parlement du Canada, les fonds seront mis à la disposition du trésorier de l'établissement indiquée qui s'occupera des verseme

REVISED — **RÉVISÉ**

199903MOP-42333-M-CFCA-11020

28/02/2000

Recipient/Bénéficiaire: Dr. Roger F. BUTTERWORTH
Medicine
Faculty of Medicine
Université de Montréal

Program/Programme: Operating Grants
Grant New

Project Title/Titre du Projet:
Further studies on the pathophysiology of selective neuronal cell death due to vitamin B1 deficiency

Co-Investigator(s) & Associates/Supervisor(s)/Host/Co-chercheur(s)/Directeur(s) de Recherche/Hôte:

Industrial Partner(s)/partenaire(s) Industriel(s):

PAYMENT DETAILS/DÉTAILS DES VERSEMENTS Funding Reference Number/ No. de Référence du Financement: **MOP – 36441**

Period / Période	Type	Amount by Type / Montant Par Type	Total by Fiscal Year / Total Par Exercice	
01/10/1999 to 31/03/2000	Operating	$45,003	$45,003	1999-00
01/04/2000 to 31/03/2001	Operating	$73,641	$73,641	2000-01
01/04/2001 to 31/03/2002	Operating	$73,641	$73,641	2001-02
01/04/2002 to 31/03/2003	Operating	$73,641	$73,641	2002-03
01/04/2003 to 31/03/2004	Operating	$73,641	$73,641	2003-04
01/04/2004 to 30/09/2004	Operating	$36,820	$36,820	2004-05

Progress Report Required: **01/03/2002**
Rapport des Progrès Réalisés Requis:

Application to Renew Funding Required: **01/03/2004**
Demande de Renouvellement des Fonds Requis:

NOTES:

This revised AFF reflects the awarding of a supplement to the operating funds for fiscal year 1999-00. /
Cette autorisation de financement reflète l'allocation d'un supplément aux fonds de fonctionnement pour l'exercice 1999-2000