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Regulation of excitotoxicity in thiamine deficiency: role of glutamate transporters.

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Cette thèse intitulée:

Regulation of excitotoxicity in thiamine deficiency: role of glutamate transporters.

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RÉSUMÉ

L'excitotoxicité est un mécanisme physiopathologique majeur impliqué dans la pathogenèse de la déficience en thiamine (DT). Dans les régions cérébrales vulnérables à la DT, on observe une mort cellulaire induite par excitotoxicité dont l'origine semble être la conséquence d'une perturbation du métabolisme énergétique mitochondrial, d'une dépolarisation membranaire soutenue et d'une diminution de l'absorption du glutamate par les astrocytes suite à la diminution de l'expression des transporteurs EAAT1 et EAAT2. Il est clairement établi que le glutamate joue un rôle central dans l'excitotoxicité lors de la DT. Ainsi, la mise en évidence des mécanismes impliqués dans la diminution de l'expression des transporteurs du glutamate est essentielle à la compréhension de la physiopathologie de la DT.

L'objectif de cette thèse consiste en l'étude de la régulation des transporteurs astrocytaires du glutamate et la mise au point de stratégies thérapeutiques ciblant la pathogenèse de l'excitotoxicité lors de l'encéphalopathie consécutive à la DT.

Les principaux résultats de cette thèse démontrent des perturbations des transporteurs du glutamate à la fois dans des modèles animaux de DT et dans des astrocytes en culture soumis à une DT. La DT se caractérise par la perte du variant d'épissage GLT-1b codant pour un transporteur du glutamate dans le thalamus et le colliculus inférieur, les régions cérébrales affectées lors d'une DT, en l'absence de modification des niveaux d'ARNm. Ces résultats suggèrent une régulation post-transcriptionnelle de l'expression des transporteurs du glutamate en condition de DT.

Les études basées sur l'utilisation d'inhibiteurs spécifiques des facteurs de transcription NFkB et de l'enzyme nucléaire poly(ADP)ribose polymérase-1 (PARP-1) démontrent que la régulation de l'expression du transporteur GLT-1 est sous le contrôle de voies de signalisation NFkB dépendantes de

PARP-1. Cette étude démontre une augmentation de l'activation de PARP-1 et de NFkB dans les régions vulnérables chez le rat soumis à une DT et en culture d'astrocytes DT. L'inhibition pharmacologique du facteur de transcription NFkB par le PDTC induit une augmentation des niveaux d'expression de GLT-1, tandis que l'inhibition de PARP-1 par le DPQ conduit à l'inhibition de l'hyperactivation de NFkB observée lors de DT. L'ensemble de ces résultats met en évidence un nouveau mécanisme de régulation des transporteurs du glutamate par l'activation de PARP-1.

L'accumulation de lactate est une caractéristique de la DT. Un traitement avec le milieu de culture d'astrocytes en condition de DT sur des cultures d'astrocytes naïfs induit une diminution de l'expression de GLT-1 ainsi qu'une inhibition de la capacité d'absorption du glutamate par les astrocytes naïfs. En revanche, l'administration de lactate exogène ne modifie pas le niveau d'expression protéique de GLT-1. Ainsi, des facteurs solubles autres que le lactate sont sécrétés par des astrocytes en condition de perturbation métabolique et peuvent potentiellement réguler l'activité des transporteurs du glutamate et contribuer à la pathogenèse du syncytium astroglial.

En outre, la ceftriaxone, un antibiotique de la famille des β-lactamines, augmente de façon différentielle l'expression du variant-d'épissage GLT-1 dans le colliculus inférieur chez le rat DT et en culture d'astrocytes DT. Ces résultats suggèrent que la ceftriaxone peut constituer une avenue thérapeutique dans la régulation de l'activité des transporteurs du glutamate lors de DT.

Pour conclure, la mort cellulaire d'origine excitotoxique lors de DT survient en conséquence d'une dysfonction mitochondriale associée à une perturbation du métabolisme énergétique cérébral. La modification de l'expression des transporteurs du gluatamate est sous le contrôle des voies de signalisation NFkB dépendantes du facteur PARP-1. De plus, l'inhibition métabolique et l'augmentation des sécrétions de lactate observées lors de DT peuvent également constituer un autre mécanisme physiopathologique expliquant la diminution d'expression des transporteurs de glutamate. Enfin, la ceftriaxone pourrait représenter une stratégie thérapeutique potentielle dans le traitement de la régulation

de l'expression des transporteurs du glutamate et de la perte neuronale associés à l'excitotoxicité observée lors de DT.

Mots-clés : Excitotoxicité, transporteurs du glutamate, syndrome de Wernicke-Korsakoff, déficience en thiamine, stress oxydatif, mort neuronale.

ABSTRACT

Excitotoxicity has been implicated as a major pathophysiological mechanism in the pathogenesis of thiamine deficiency (TD). Excitotoxic-mediated cell death is localized in areas of focal vulnerability in TD and may occur as a consequence of impairment in mitochondrial energy metabolism, sustained cell membrane depolarization and decreased uptake of glutamate by astrocytes due to the loss of excitatory amino acid transporters, (EAAT1 and EAAT2). Over the years, a number of studies have identified glutamate as being a major contributor to excitotoxicity in the pathophysiology of TD. Thus, downregulation of astrocytic glutamate transporters resulting in excitotoxicity is a key feature of TD and understanding the regulation of these transporters is essential to understanding the pathophysiology of the disorder.

The objective of the present thesis project was to examine the underlying basis of astrocytic glutamate transporter regulation during TD encephalopathy.

Major findings of the studies presented in this thesis project provide evidence for glutamate transporter abnormalities in TD animal models and astrocyte cultures exposed to TD. TD results in the loss of the glutamate transporter splice variant-1b (GLT-1b) in vulnerable areas of brain, i.e. thalamus and inferior colliculus, with no significant alteration in the mRNA levels of the transporters, suggesting that glutamate transporter regulation under conditions of TD is a posttranscriptional event.

Studies using a specific inhibitor of the transcription factor, Nuclear factor-kappa B (NF-κB) and a nuclear enzyme poly (ADP)ribose polymerase-1 (PARP-1) provided evidence for the regulation of GLT-1 by PARP-1 dependent NF-κB signalling pathways. The major findings of this study suggested an increase in the activation of PARP-1 and NF-κB molecule in the vulnerable areas of TD rat brain and TD astrocyte cultures. Pharmacological inhibition of NF-κB showed an increase in the levels of GLT-1, while inhibition of PARP-1 using a specific PARP-1 inhibitor, DPQ inhibited the increased activation of NF-κB that was observed during TD. Overall results of this finding provided evidence for a mechanism

involving PARP-1 activation in the regulation of glutamate transporters.

Given the increased lactate accumulation as a classical feature of TD, we studied the effect of soluble factors produced by astrocytes on glutamate transporter function. Treatment of naïve astrocyte cultures with TD conditioned media resulted in decreased levels of GLT-1 and inhibition of glutamate uptake capacity concomitant with a loss of mitochondrial membrane potential. Administration of exogenous lactic acid produced a similar reduction in glutamate uptake to that resulting from conditioned media. However, lactic acid treatment did not result in a change in GLT-1 protein levels. In addition, the pro-inflammatory cytokine TNF-α was shown to be increased in astrocytes treated with TD along with elevated levels of the phospho-IκB fragment, indicative of increased activation of NFκB. Inhibition of NFκB led to an amelioration of the decrease in GLT-1 that occurs in TD, along with recovery of glutamate uptake. Thus, soluble factors released from astrocytes under conditions of metabolic impairment such as lactate and TNF-α impairment appear to exert a regulatory influence on glutamate transporter function.

Ceftriaxone, a β-lactam antibiotic, has the ability to differentially stimulate GLT-1b (splice-variant) expression in the inferior colliculus in TD rats and under *in vitro* conditions with TD astrocyte cultures. Thus, ceftriaxone may be a potential therapeutic strategy in the regulation of glutamate transporter function during TD.

In summary, excitotoxic cell death in TD occurs as a consequence of mitochondrial dysfunction associated with cerebral energy impairment and abnormal glutamate transporter status. A major underlying mechanism for glutamate transporter abnormalities is mediated by PARP-1 dependent NF-κB signaling pathways. In addition, metabolic inhibition with substantial production of lactate and TNF-α may be perhaps another mechanism responsible for glutamate transporter downregulation in TD.

Keywords: Excitotoxicity, glutamate transporters, Wernicke-Korsakoff syndrome, thiamine deficiency, oxidative stress, neuronal cell death.

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LIST OF ABBREVIATIONS

AA	:	Arachidonic acid
AD	:	Alzheimer's disease
ALS	:	Amyotrophic lateral sclerosis;
AMPA	:	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid
AQP-4	:	Aquaporin-4
ATP	:	Adenosine triphosphate
Aβ	:	Amyloid-β peptide
APP	:	Amyloid precursor protein
BBB	:	Blood-brain barrier
BCSFB	:	Blood-cerebrospinal fluid barrier
bFGF	:	Basic fibroblast growth factor
4-CIN	:	alpha-Cyano-4-Hydroxycinnamate
CNQX	:	6-cyano-7-nitroquinoxaline-2,3,-dione
CNTF	:	Ciliary neurotrophic factor
CT	:	Computed tomography
DAG	:	Diacylglycerol
dBC	:	dibutyryl-cAMP
Dlg-1	:	Drosophila disc large tumor suppressor
DPQ	:	3,4-Dihydro-5[4-(1-piperindinyl)butoxy]-1(2H)-isoquinoline
EAAT-1	:	Excitatory amino acid transporter-1
EAAT-2	:	Excitatory amino acid transporter-2
EBP-β	:	Enhancer-binding protein- β

EBP-δ	:	Enhancer-binding protein beta- δ
EGF	:	Epidermal growth factor
eNOS	:	Endothelial nitric oxide synthase
Egr-1	:	Early growth response protein-1
GABA	:	γ-aminobutyric acid
GFAP	:	Glial fibrillary acidic protein
GLT-1	:	Glutamate transporter-1
GLT-1a	:	Glutamate transporter-1*splice variant a*
GLT-1b	:	Glutamate transporter-1*splice variant b*
GLAST	:	Glutamate/Aspartate transporter
GPS-1	:	G protein pathway suppressor-1
Gro1	:	Growth-regulated oncogene-1
GSH	:	Glutathione
HD	:	Huntington's disease
IEG	:	Immediate-early gene
IFNs	:	Interferon's
IFN-γ	:	Interferon gamma
IkBs	:	NFkB inhibitor proteins
IKK	:	IkB kinase
IL-1β	:	Interleukin-1β
IL-6	:	Interleukin-6
IL-18	:	Interleukin-18
iNOS	:	Inducible nitric oxide synthase

JM4	:	Jena muenchen-4
K^+	:	Potassium
KGDHC	:	α-ketoglutarate dehydrogenase complex
Kif-4	:	Kinesin superfamily proteins-4
MCP-1	:	Monocyte chemoattractant protein 1
mGluRs	:	Metabotropic glutamate receptors
MIP-1α	:	Macrophage inflammatory protein-1α
MIP-1β	:	Macrophage inflammatory protein-1 β
MK-801	:	Methyl-dihydro-dibenzocyclohepten imine hydrogen maleate
MMP-9	:	Matrix metalloproteinase-9
MPT	:	Mitochondrial permeability transition
MRI	:	Magnetic resonance imaging
NeuN	:	Neuronal nuclear antigen protein/neuronal marker
NCM	:	Neuron conditioned media
NFκB	:	Nuclear factor-kappa B
NGF	:	Nerve growth factor
NMDA	:	N-methyl-D-aspartate
NHERF-1	:	Na+/H+ exchanger regulatory factors-1
NHERF-2	:	Na+/H+ exchanger regulatory factors-2
PAR	:	Poly(ADP)Ribose
PARP-1	:	Poly(ADP)Ribose polymerase-1
PACAP	:	Pituitary adenylate cyclase-activating polypeptide
PD	:	Parkinson’s disease

PDGF	:	Platelet-derived growth factor
PDTC	:	Pyrrolidine dithiocarbamate
PET	:	Positron emission tomography
PKC	:	Protein kinase A
PKA	:	Protein kinase C
PSD	:	Post synaptic density protein
ROS	:	Reactive oxygen species
RTK	:	Receptor tyrosine kinase
SPECT	:	Single photon emission computed tomography
SNARE	:	Soluble *N*-ethylmaleimide attachment protein receptors
TCA	:	Tricarboxylic acid cycle
TD	:	Thiamine deficiency
TDP	:	Thiamine diphosphate
TGF- α	:	Transforming growth factor α
TK	:	Transketolase
TKs	:	Tyrosine kinase
TNF-α	:	Tumor necrosis factor- α
TPP	:	Thiamine pyrophosphate
TSPO	:	Translocator protein
VGLUTs	:	Vesicular glutamate transporters
VAChT	:	Vesicular acetylcholine transporters
VMATs	:	Vesicular monoamine transporters
VGATs	:	Vesicular GABA transporters

VIAAT	:	Vesicular inhibitory amino acid transporters
WE	:	Wernicke's encephalopathy
WKS	:	Wernicke-Korsakoff syndrome
ZO-1	:	zonula occludens-1 protein

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CHAPTER 1

INTRODUCTION

1.1 THIAMINE DEFICIENCY ENCEPHALOPATHY

1.1.2 Thiamine, the vitamin

1.1.2.1 History

Thiamine or "thio-vitamine" ("sulfur-containing vitamin") is a water-soluble vitamin of the B complex. Also named aneurin for its detrimental neurological effects. Phosphate derivatives of thiamine are involved in many cellular processes. The best-characterized form is thiamine pyrophosphate (TPP), a coenzyme essential for the catabolism of sugars and amino acids. Deficiency of thiamine has been implicated in the variety of metabolic and anatomic disturbances. First described in the Dutch East Indies during the 1600's, beriberi became a serious health problem in the 1900's with the advent of steam powered rice mills which produced widely consumed 'polished' rice devoid of the vitamin rich husk. A series of studies by Peters in the 1930s indicated that birds fed polished rice resulted in TD and the accumulation of lactate in the brainstem, addition of a small quantity of crystalline thiamine led to normalization of lactate levels and improved the polyneuritis in birds. This interesting finding at the time led to formulation of the concept of a biochemical lesion in TD (Kinnersley and Peters, 1930; Peters, 1936). However, administration of thiamine did not completely eliminate the features of beriberi and it was quickly realized with the neurological manifestation persisting despite supplementation with thiamine (Singleton and Martin 2001).

1.1.2.2 Chemical properties

The thiamine molecule (Fig. 1) is a water soluble, white crystalline solid. In the crystallized state or in an acid solution the stability of thiamine is good. In a neutral or alkaline solution thiamine is unstable and sensitive to heat, oxygen and ultraviolet light.

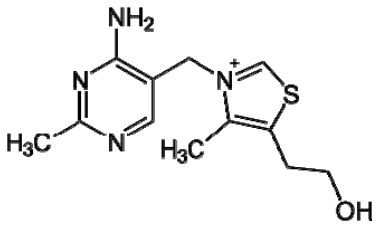

Figure1. Chemical structure of thiamine

1.1.2.3 Physiology and metabolic function

Thiamine is actively absorbed from the small intestine and is transformed within the body by phosphorylation into active co-enzyme thiamine pyrophosphate. It is because of the inability of the body to produce thiamine and can only store up to 30mg of it in tissues and also the vitamin has a high turnover rate, that a continuous supply of the vitamin is needed. The limited stores may be depleted within 9-18 days on a thiamine-free diet, with the appearance of the clinical signs (Ariaey-Nejad et. al., 1970; McCormick et. al., 1988; Rosen and Barkin, 1998). In addition, the body is readily depleted of thiamine by fever and other metabolic stress. The heart, kidney, liver and brain have the highest concentrations, followed by the leukocytes and the red blood cells (F. Hoffman-LaRoche, 1994). Dephosphorylation of thiamine occurs in kidney and excess free vitamin is rapidly excreted in the urine. The urinary excretion depends on the urine volume and during diuresis large amounts of thiamine may be lost. Small quantities of thiamine are excreted in sweat (Marks, 1975).

1.1.2.3.1 Thiamine transport and phosphorylation in brain.

Transport of thiamine across the blood-brain barrier is conducted through a carrier-mediated system (Spector 1976; Greenwood et al., 1982). This system appears to be independent of energy metabolism (Greenwood et al., 1986). Sharma and Quastel (1965) presented evidence for a saturable and energy-requiring thiamine uptake in cortex

slices, suggesting that nerve cells actively pump thiamine. Moreover, neuroblastomas as well as glial cells possess a high-affinity thiamine transport system [K_m of 35 nM for thiamine] (Bettendorff and Wins, 1994). This high affinity carrier is responsible for the transport of thiamine across the cell membrane and thus contributes to the homeostasis of intracellular thiamine at high extracellular concentrations (Bettendorff, 1994). Thiamine taken up by neuroblastoma cells is rapidly phosphorylated to TDP by thiamine pyrophosphokinase (EC 2.7.6 .2) (Bettendorff and Wins, 1994). Phosphorylation of thiamine and subsequent binding of TDP to cytoplasmic transketolase (EC 2 .2.1 .1) and mitochondrial pyruvate (PDH, EC 1 .2.4.1) and a-ketoglutarate (KGDH, EC 1.2.4.2) dehydrogenases are responsible for secondary active accumulation of thiamine compounds in neuroblastoma cells (Fig. 2).

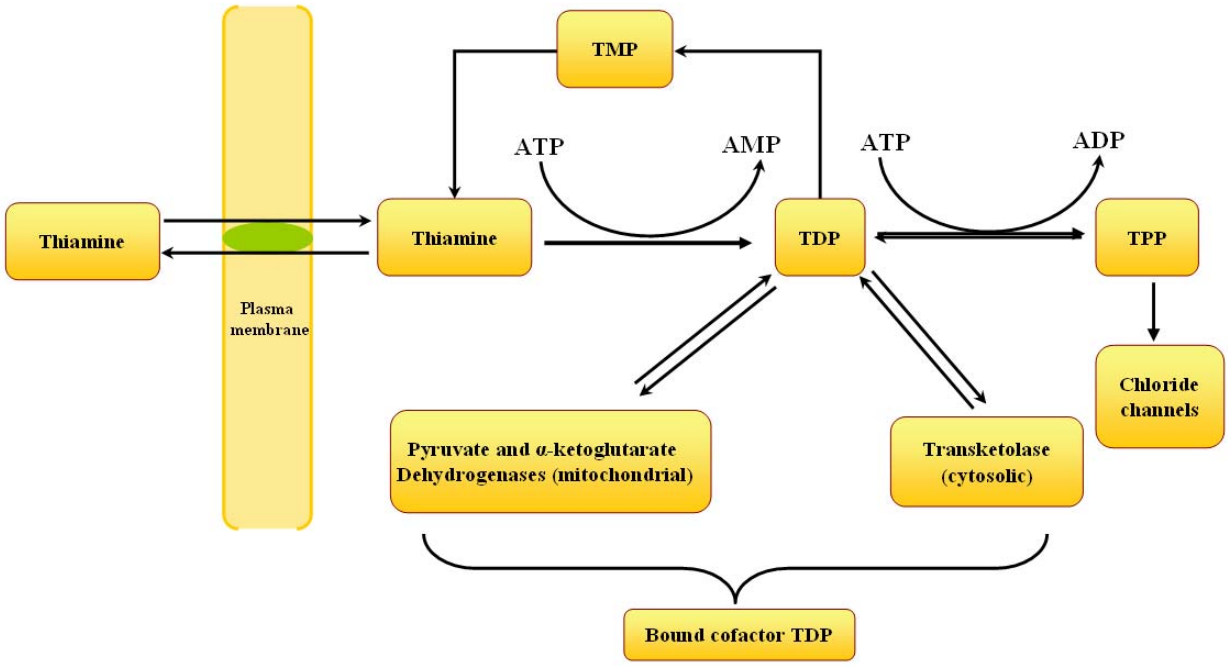

Figure 2 Thiamine metabolism in brain

1.1.2.3.2 Role of thiamine in brain metabolism

The metabolically active form of thiamine is thiamine pyrophosphate (TPP). Essentially TPP is required for several biochemical reactions involved in the breakdown of glucose to liberate energy. It acts as a co-enzyme in oxidative decarboxylation and transketolase reactions. Mainly there are three major enzyme systems that require TPP as a cofactor: pyruvate dehydrogenase (EC 1.2.4.1) complex, an organized enzyme assembly that connects glycolysis with the tricarboxylic acid (TCA) cycle, α-ketoglutarate dehydrogenase (EC 1.2.4.2) complex (KGDHC), a multicomponent enzyme complex associated with the TCA, and transketolase (EC 2.2.1.1), a key participant in the pentose phosphate shunt which is involved in nucleic acid and lipid biosynthesis. Thiamine is thus an important factor in carbohydrate metabolism and hence in thiamine deficiency (TD), blood pyruvate and often blood lactate levels rise abruptly (Combs, 1992).

1.1.3 Etiology and epidemiology of thiamine deficiency

Vitamin B_1 deficiency can occur because of many different causes. The most basic form of TD results because of impaired nutritional status associated with chronic diseases, such as alcoholism, gastrointestinal diseases, HIV-AIDS, and persistent vomiting. Following are the major factors responsible for TD:

1.1.3.1 Lack of thiamine intake

Lack of thiamine intake can occur with food containing a high level of thiaminases such as milled rice, shrimp, mussels, clams, fresh fish, and raw animal tissues, food high in anti-thiamine factors and processed food with a content high in sulfite, which destroys thiamine. In addition, diet-related factors such as alcoholic state,

starvation state or gastro-intestinal disorder can also reduce thiamine intake (Masumoto et. al., 2009; Matrana et. al., 2009; Ahmed et. al., 2011).

1.1.3.2 Increased metabolic consumption

Increased metabolic consumption of thiamine may occur because of pregnancy, hyperthyroidism, lactation, fever, severe infection and increased physical exercise. (Anderson et al, 1985; Braverman and Utiger, 1996).

1.1.3.3 Increased thiamine depletion

Increased thiamine depletion may occur in complications such as diarrhea, diuretic therapies, peritoneal dialysis, hemodialysis, hyperemesis gravidarum (Indraccolo et. al., 2005; Al-Attas et. Al., 2011).

1.1.3.4 Decreased absorption

The major factors responsible for the decreased thiamine absorption are chronic intestinal disease, alcoholism, malnutrition, gastric intestinal disorder, malabsorption syndrome; celiac and tropical sprue (Anderson et al, 1985).

1.1.3.5 Consequences of chronic alcoholism

The relation between alcohol intake and thiamine deficiency is well established and has been investigated in the past. Up to 80% of alcoholics in general population have a deficiency in thiamine (Morgan 1982), some of them will go on to develop WE and/or Korsakoff psychosis (WKS). However, the most debated issue regarding alcoholism in clinical setting is the occurrence of TD related brain damage with or without alcoholism (Charness 1993; Joyce1994; Butterworth 1995). In general, cerebral damage due to thiamine deficiency progresses with alcohol toxicity and vice versa. Therefore, alcoholism does not directly cause TD (Phillips et al. 1981), though it may induce such

deficiency because of its normal association with malnourishment and its related mechanism. Particularly, the low thiamine absorption rate at the mucosal level, the impaired hepatic function, and the raised alcohol-related thiamine metabolism may lead to the development of chronic thiamine deficiency (Harper 2009). Consequently, the effect of alcohol was investigated in metabolism (Ammon et al. 1965; Lindros 1982), oxidative stress, excitotoxicity (Lovinger 1993). Furthermore, ethanol and its more toxic oxidative metabolite acetaldehyde is involved directly or indirectly in brain damage, and it may be that high levels of acetaldehyde play a role in producing TD. Non-oxidative metabolites of alcohol metabolism particularly fatty acid ethyl esters are found increased in ethanol induced brain damage. Interactions between these conditions influence brain damage in alcoholism. Moreover, chronic alcohol administration accelerates the lesions of experimental TD, by up regulating NMDA receptor expression and excitotoxicity (Harper and Matsumoto 2005). Furthermore, the most frequently accessible target for the alcohol to trigger a detrimental effect in the brain, are the blood-brain barrier (BBB) and blood-CSF barrier (BCSFB) (Nixon et al. 2008). It is well established that the alcohol intoxication and thiamine deficient glucose metabolism increases the permeability of the blood brain barrier (BBB) leading to instability in the osmotic gradient (Calingasan et al. 1995; Nixon et al. 2008). This results in the swelling of intra- and extracellular spaces. Also, observed in the periventricular regions, the BBB is physiologically less tight and there is a high rate of thiamine-related glucose and oxidative metabolism. Among the neurological manifestations, ataxia was positively associated with alcoholism without any obvious cerebellar lesions, while infratentorial signal-intensity alterations were only observed in non alcoholics. Alternatively, choroid plexus (CP) is hypothesized as the

other target of alcohol intoxication and it is postulated that CP is the primary source of brain pathology in TD and alcohol toxicity (Nixon et al. 2009). Impairment of the BCSFB and BBB are not considered as a primary factor in the pathogenesis of WE or of ethanol intoxication, only for the reason that there has been insufficient assessment of the BCSFB in these conditions. Thus, it can be concluded that alcohol alone is not responsible for TD and its related brain damages, however, alcohol abuse does potentiate the neurological impairment in TD encephalopathy.

1.1.4 Major disorders of thiamine deficiency

TD progresses with a variety of clinical signs brought about by the presence of complicating factors, such as infections, or by the presence of symptoms from multiple deficiencies such as other B vitamins, vitamin C and minerals as well as the effects of stresses of many kinds, such as physical labour and pregnancy. However, vitamin B_1 deficiency manifests itself principally with changes involving the nervous system, the cardiovascular system, and also the gastrointestinal tract (Williams, 1961; Sebrell, 1962; Sauberlich, 1967).

1.1.4.1 Sub-clinical thiamine deficiency

The factors responsible for the occurrence of sub-clinical or mild thiamine deficiency are intake of high carbohydrate food and low thiamine intake, raised physiological or metabolic demand, primarily due to pregnancy and lactation, heavy physical exertion, inter-current illness (cancer, liver diseases, infections, hyperthyroidism), surgery, and wherever absorption is reduced by regular high blood alcohol levels, gastrointestinal disease; dysentery, diarrhoea, nausea/vomiting (Anderson et al, 1985).

Table 1 attempts to summarize some of the typical lesions seen in specific organ systems of the body as a result of thiamine deficiency.

Table 1. Organ Systems of the body affected in thiamine deficiency

Nervous system	WE and WKS; Polyneuritis (multifactorial); autonomic, sensory and motor nerves are affected; paraesthesia and hyperesthesia, loss of ankle and knee jerks with muscle wasting and paralysis -typically wrist- and foot-drop (symmetrical).
Eye	Nutritional amblyopia
Heart and blood vessels	Enlarged heart. Congestive heart failure which is one of the contributory causes of peripheral oedema and results in increase in circulating blood volume.
Gastrointestinal tract	Constipation (rarely diarrhea) with abdominal distension and colicky pains, anorexia, nausea, vomiting.

The symptoms of mild thiamine deficiency are elusive and can be attributed to other problems therefore diagnosis is often difficult. However, anorexia, which is one of the early symptoms of subclinical thiamine deficiency, is regarded to be a protective phenomenon since a high-carbohydrate diet is more dangerous in the presence of thiamine deficiency (Lonsdale et al, 1980).

1.1.4.2 Wernicke's Korsakov Syndrome (WKS)

WKS is characterized by symmetric hyperaemic brain lesions with glial proliferation, capillary dilatation, and perivascular haemorrhage. The syndrome is manifested by a confusional state, disorientation, ophthalmoplegia, nystagmus, diplopia, and gait ataxia (Wernicke's encephalopathy, WE), with severe loss of memory for recent events and confabulation (Korsakov's psychosis) occurring following recovery. It

appears that the disorder can have an autosomal recessive inheritance but is expressed as a clinical disease only in the event of TD.

Up to 80-90 % of patients with WE go on to develop the more debilitating chronic Korsakoff's psychosis. The first reports of the important role of TD in the etiology of WKS were described by Alexander and colleagues (1938) and Bowman et al (1939). DeWardner and Lennox (1947) later observed 52 malnourished prisoners of war and further established the important link between TD and WKS in humans. WKS is most commonly observed in alcoholics, the majority of them with liver disease, and is often precipitated abruptly by administration of glucose to patients severely deficient in thiamine. If untreated, death is common; even with treatment, 17% die within 3 weeks (Feldmann, 1988).

1.2 PATHOPHYSIOLOGY OF THIAMINE DEFICIENCY ENCEPHALOPATHY

1.2.1 Neuroanatomical damage in WE and TD

In TD, damage to the brain is focal in nature. Typically, vulnerable areas include the mammillary bodies, thalamus, inferior colliculus, brainstem, and cerebellum (Troncoso et al., 1981; Langlais et al., 1996). Although the thiamine content of the brain is almost uniform (13 μg/g dry weight) (Dreyfus et al., 1959; Cooper and Pincus, 1979), vulnerable areas at risk of damage show marked alterations in levels depending on the metabolic rate. Each organ or part of the nervous system appears to have its particular thiamine levels for depletion (Sharma and Quastel, 1965). Cerebral levels of thiamine are highest in the cerebellar vermis followed by caudate nucleus, brain stem, periaqueductal region, mamillary region and thalamus (Dreyfus et al., 1959). However, the precise

relationship between thiamine levels and subsequent damage during its depletion remains a mystery.

WE patients and TD animals characteristically display gross neuropathological changes that include brain atrophy, hemorrhages (Victor et al., 1989), edematous necrosis (Watanabe et al., 1981), white matter damage (Yamashita and Yamamoto, 1995; Langlais and Zhang, 1997), gliosis and significant neuronal loss (Witt, 1985; Todd et al., 1999; Mulholland, 2006). Typically, mammillary bodies as well as the medial, midline and intralaminar nuclei of the thalamus, inferior colliculus, periaqueductal area and floor of the fourth ventricle show severe damage. In addition, brainstem nuclei, cerebellum, cranial nerve nucleus, pretectal regions, and locus coeruleus are affected in WE (Torvik, 1987; Victor et al., 1989), with cerebellar damage being most prominent in the anterior superior vermis and more severe in alcoholics with WE (Phillips et al., 1990). Microscopically, loss of Purkinje cells and shrinkage of the molecular and granule cell layers have been identfied (Phillips et al., 1987), with marked reduction in Purkinje dendritic arborization commonly observed both in alcoholics and TD-dependent brain damage (Terasawa et al., 1999). Such occurrence of cerebellar degeneration in alcoholics is relatively common (Kril, 1996), and due to the similarity of this damage in both alcoholics and TD, it has been hypothesized that alcoholic cerebellar degeneration is nutritional in origin (Terasawa et al., 1999). Experimentally, the thalamus and, in particular, the medial dorsal nuclei, represents the area of brain most widely studied in TD (Langlais et al., 1992; Hazell et al., 1998a), with other affected areas including the anterior nuclear group, geniculate body, ventral posterior medial nucleus, dorsal nucleus, and lateral posterior nucleus. Moreover, the hypothalamus also displays abnormalities in

areas such as the suprachiasmatic nucleus, supraoptic nucleus, and medial preoptic nucleus. Evidence also indicates that regions of the brain such as the cerebral cortex, previously considered being unaffected, also sustaining damage in TD and WE.

Histological evidence of damage to the peripheral nervous system following metabolic and toxic insults has led many investigators to focus on the role of thiamine and the effects of alcohol on the progression of these diseases. Chronic TD and alcohol toxicity are often causes of peripheral neuropathy (Swank et al., 1940; Victor et al., 1989), with the most common being distal axonopathies, also termed "dying back" disease. Although the precise mechanism for this type of pathology had not yet been established, it was envisioned that damage to the axonal transport system and metabolic disturbances were the causative factor for the progression of this neuropathy (Schoental and Cavanagh, 1977). While 80% of the patients with WKS observed by Victor had evidence of a peripheral neuropathy, many of these cases exhibited alcohol-dependent TD, suggesting an involvement of the toxic effects of alcohol in the progression of peripheral neuropathy. To date, animal models of alcoholic neuropathy and its relationship to TD have yet to be established.

Many cases with TD have been shown to exhibit more subtle neurological signs and symptoms, including the abnormalities in brain regions such as, cerebellum, inflammation or degeneration of peripheral nerves (neuropathy) as well as changes in behavior and problems with learning, memory, and decision making (Torvik 1987). Autopsy studies have found that a region of the cerebellum known as the anterior superior cerebellar vermis most frequently exhibits TD–induced damage (Baker et al. 1999; Lavoie and Butterworth 1995; Victor et al. 1989). TD contributes to a reduction in

the number and size of a certain cerebellar cell type called Purkinje cells in parts of the cerebellar vermis (Philips et al. 1987). Cerebellum is involved primarily in muscle coordination and it is also recognized for its role in various aspects of cognitive and sensory functioning (Parks et al. 2003). Accordingly, cerebellar degeneration is associated with difficulties in movement coordination and involuntary eye movements, such as nystagmus. Cerebellar degeneration is found both in alcoholics with WKS and alcoholics alone, but because WKS patients typically have a higher degree of cerebellar atrophy, it appears likely that TD also is the predominant cause of cerebellar degeneration.

In addition to the cerebellum, numerous other brain regions and structures are damaged in people with WKS. Although animal studies have suggested that thiamine deficiency may contribute to damage to these structures, the exact role of TD and the level of sensitivity of these structures to thiamine deficiency have not yet been determined. Further studies are certainly needed in this area.

1.2.2 Effects of thiamine deficiency on enzyme activity

The pathophysiological mechanisms involved in TD are complex. Compromised brain energy metabolism is a major consequence of the disorder. Thiamine in the form of TPP is an important cofactor of three major enzyme systems: pyruvate dehydrogenase (EC 1.2.4.1) complex, an organized enzyme assembly that connects glycolysis with the TCA cycle, α-ketoglutarate dehydrogenase (EC 1.2.4.2) complex (KGDHC), a multicomponent enzyme complex associated with the TCA, and transketolase (EC 2.2.1.1), a key participant in the pentose phosphate shunt which is involved in nucleic acid and lipid biosynthesis. Chronic thiamine deprivation is accompanied by region-

selective reductions in levels of these thiamine-dependent enzymes in brain (Butterworth, 1986). However, decreased activity of KGDHC appears to be responsible for many of the reversible changes accompanying TD (Gibson et al., 1984; Butterworth and Heroux, 1989) (see Fig. 3). A fourth thiamine-dependent enzyme system, branched-chain α-ketoacid dehydrogenase complex, is associated with a rare inborn error of metabolism, maple syrup urine disease, involving an accumulation of the branched chain amino acids leucine, isoleucine, and valine (Wendel et al., 1983).

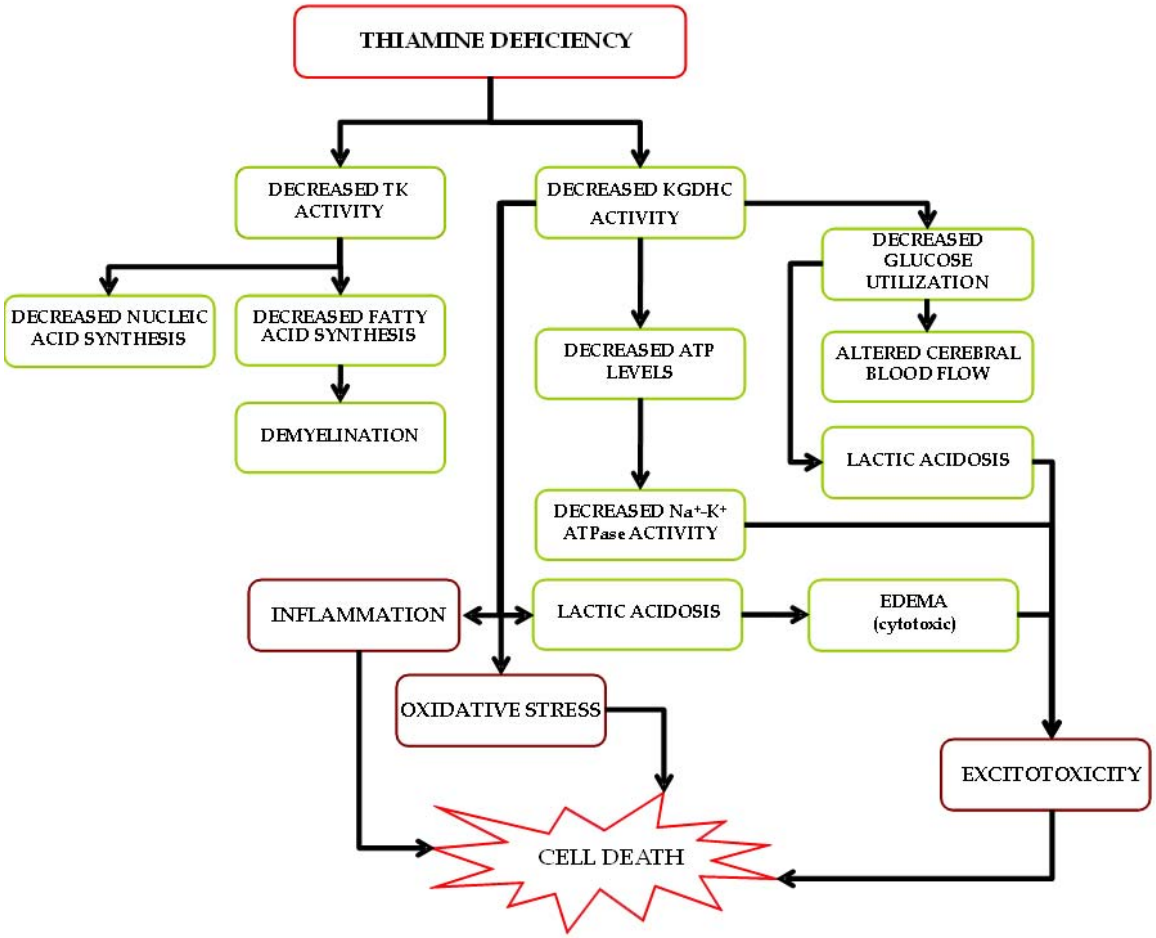

Figure 3. Schematic of pathophysiological mechanisms during TD

1.2.3 Lactic acid accumulation

During TD, oxidative decarboxylation of pyruvate and α-ketoglutarate is inhibited, resulting in decreased ATP production, pyruvate accumulation and lactate production (Aikawa et al., 1984; Navarro et al., 2005). This net synthesis of lactic acid within the brain of thiamine-deprived animals is a phenomenon that has been well recognized for many years (Kinnersley and Peters, 1930; Holowach et al., 1968; McCandless and Schenker, 1968). Lactic acidosis induced by TD is type B (i.e. acidosis in the presence of good tissue perfusion and oxygenation), with a pH of 7 and blood lactate below15 mmol/l and is typically unresponsive to alkalinization (Chadda et al., 2002). Unless thiamine is rapidly supplemented, refractory acidosis and death can occur within twenty four hours. Areas of increased lactic acidosis are localized to brain regions that subsequently develop histological lesions (McCandless, 1982; Hakim, 1984; Munujos et al., 1993), and likely play a significant role in the pathophysiology in TD animals.

1.2.4 Disruption of membrane potential

Thiamine and its derivatives are crucial in stabilizing the resting membrane potential (Itokawa and Cooper, 1970; Fox and Duppel, 1975), thereby maintaining ionic balance and conduction of the action potential (Cooper et al., 1963). Chronic deprivation of thiamine impairs this function and the electrophysiological characteristics of the cell, which can lead to severe pathological consequences.

1.2.5 Blood-brain barrier alterations in TD

Integrity of the BBB is crucial for normal CNS function. The first report of BBB damage in TD was proposed by Scholz (1949) and was described in more detail by

Pentschew and Garro (1966) in which they suggested blood vessels and, in particular, capillary endothelial cells, to be the primary site of damage, proposing the term "system-bound dysoric encephalopathy" for WE. Since then, considerable evidence for BBB damage has been described, with disturbances localized to brain regions vulnerable to TD (Calingasan et al., 1995b), and including the presence of hemorrhagic lesions (Torvik, 1985, Vortmeyer and Colmant, 1988). Such a process may also contribute to previous reports of brain edema identified in both TD and in cases of WE (see below).

Impairment in oxidative metabolism plays a significant role in BBB breakdown in TD in which oxidative stress mediated by eNOS is involved (Beauchesne et al., 2009a), and is likely also the case for several neurodegenerative disease states. In addition, inflammatory processes occur in TD (see below) which are known to disrupt the BBB (Guenther and Neu, 1984). CNS pathologies often involve BBB disturbances in which astrocyte-endothelial cell interaction is abnormal, and astrocytes secrete transforming growth factor-β, which downregulates brain capillary endothelial expression of the fibrinolytic enzyme tissue plasminogen activator and the anticoagulant thrombomodulin (Tran et al., 1999). Indeed, several chemical agents circulating in plasma or secreted from cells associated with the BBB are capable of increasing brain endothelial permeability and impairing its transport and metabolic function (Kis et al., 2001). A number of studies have evaluated BBB integrity, both spatially and temporally in experimental TD and acute WE. For example, findings have revealed disruption of BBB integrity adjacent to the third ventricle, cerebral aqueduct and fourth ventricle in acute WE (Schroth et al., 1991), consistent with the location of histological lesions. Increased BBB permeability was also reported in whole brain (Warnock and Burkhalter, 1968) and in vulnerable brain

regions in TD (Manz and Robertson, 1972), with a likely factor contributing to this susceptibility being the cerebral energy deficit. Interestingly, amyloid beta peptide (Aβ) has been reported to increase neuronal membrane fluidity and lipid peroxidation (Avdulov et al., 1997), which may contribute to the observed BBB changes in TD. Additionally, Aβ has been shown to increase endothelial cell permeability to albumin (Blanc et al., 1997), a finding which might explain the leakage of this protein into the brain parenchyma during TD (Harata and Iwasaki, 1995). Furthermore, this effect was also shown to be reversible following treatment with antioxidants (Blanc et al., 1997). Since Aβ may also play a role in excitotoxicity and is capable of inducing apoptosis (Forloni et al., 1993; Loo et al., 1993), a feature also reported in TD (Matsushima et al., 1997), altogether, these findings suggest this peptide plays a role in increased BBB permeability and possibly neuronal cell death in TD. Recent evidence also indicates that BBB tight junction proteins such as occludin and associated scaffolding proteins are decreased, concomitant with increased matrix metalloproteinase-9 levels in TD (Beauchesne et al., 2009b). The underlying basis for these changes, however, remains currently unresolved.

1.2.6 Oxidative stress

Maintenance of an optimal redox environment is the primary requirement for proper cellular functioning, and this environment is preserved by enzymes that maintain a reduced state through normal energy metabolism. Disturbances in this redox state can cause toxic effects through the production of net reactive oxygen species (ROS), particularly in the mitochondria (Lin and Beal, 2006), that can damage vital components of the cell, including proteins, lipids, and DNA. This imbalance in ROS metabolism, or

development of oxidative stress is an important factor in the pathogenesis of TD (Gibson and Blass, 2007). Increased ROS production in TD can trigger cell membrane damage, including lipoperoxidation (Valko et al., 2007) and alterations in the functional integrity of ion channels and transporters. Persistent net ROS formation in TD can initiate a cascade of cell death pathways via intracellular messengers, e.g. intracellular caspase-3-mediated apoptosis. Development of oxidative stress also leads to disturbances in brain function, including an inhibition of glutamate uptake due to transporter protein nitrosylation following peroxynitrite formation (Volterra et al., 1994; Trotti et al., 1996; Hazell, 2007). Under conditions of oxidative stress, levels of heme oxygenase-1, endothelial nitric oxide synthase (eNOS), the inducible form of NOS (iNOS), intracellular adhesion molecule-1, and microglial activation are increased (Calingasan et al., 1999,2000; Gibson et al., 2000). Thus, oxidative stress can lead to profound neuropathological consequences in TD.

1.2.7 Excitotoxicity

Excitotoxicity is the pathological process by which nerve cells are damaged and killed by excessive stimulation of receptors for the excitatory neurotransmitter glutamate. The normal levels of glutamate approach 10 mmol/kg during synaptic transmission, while extracellular concentrations remain approximately 25 nM (Schousboe, 1981; Herman and Jahr, 2007). These low extracellular levels of glutamate are essential to ensure appropriate signal to noise for excitatory signaling and to limit excessive activation of glutamate receptors that can cause excitotoxicity (Choi, 1992; Conti and Weinberg, 1999). The only process known to actively clear extracellular glutamate is mediated by a family of Na^+-dependent transporters also known as glutamate or excitatory amino acid

transporters (Schousboe, 1981; Danbolt, 2001). These transporters couple the movement of 3 Na^+ ions and 1 H^+ to the inward transport of glutamate, providing sufficient energy to maintain a transmembrane concentration gradient of up to one million-fold (Zerangue and Kavanaugh, 1996).

Excitotoxicity may be involved in spinal cord injury, stroke, traumatic brain injury, hearing loss (orototoxicity) and in neurodegenerative diseases such as, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, alcoholism or alcohol withdrawal, and Huntington's disease (Kim et. al., 2002; Hughes, 2009). Other common conditions that cause excessive glutamate concentrations around neurons are hypoglycemia (Camacho, 2006), status epilepticus (Fujikawa, 2005) and mitochondrial dysfunction (Jhala and Hazell, 2011). Consequences of excitotoxicity during TD are discussed in more detail in section 1.3

1.2.8 Inflammation

Cerebral inflammation is now recognized as a key component of the neurodegenerative process, and occurs e.g. in AD, PD, multiple sclerosis (Bojinov, 1971; Allen et al., 1981; Aisen and Davis, 1994), along with other neurological conditions such as stroke and brain trauma (Garcia, 1975; Mathew et al., 1994) Previous studies describing alterations in glial cell morphology in TD including evidence of swelling and the appearance of phagocytic vacuoles (Collins, 1967; Robertson et al., 1968) indicate that although neuronal damage is a feature of this disease process, glial elements are also profoundly affected. Evidence in support of the existence of an inflammatory process has been described in TD, including the early development of increased microglial reactivity (Todd and Butterworth, 1999), while production of pro-inflammatory cytokines in both

vulnerable and non-vulnerable regions of brain has been reported (Ke et al., 2006; Vemuganti et al., 2006; Karuppagounder et al., 2007). In a recent study, we demonstrated that in vulnerable brain regions in TD, inflammatory genes represent the largest functional group of transcripts upregulated (e.g. pro-inflammatory cytokines including IL-6, IL-18, TNF-α, AIF1, and osteopontin), interferons (IFNs), IFN-inducible proteins, and chemokines (Gro1, MCP-1, MIP-1α, and MIP-1β) (Vemuganti et al., 2006). Interestingly, many of these genes are also known to be expressed strongly in astrocytes during inflammation and may therefore be a contributing factor to astrocyte dysfunction in TD. In addition, many transcription factors known to control inflammatory gene expression such as Egr-1, c-EBP-β, c-EBP-δ, CPBP and Klf-4 are also upregulated following TD. During impaired oxidative metabolism, Egr-1 and c-EBP-β may play an important role in starting the inflammatory cascades. Furthermore, levels of these various inflammatory-related gene products in different brain regions may be an important determining factor for selective vulnerability in TD. Recent studies have also demonstrated that alcohol induces inflammatory responses in brain that include microglial activation and cytokine production (Qin et al., 2008; He and Crews, 2008). Such findings may also be relevant in the brains of cases of WE. Additional studies aimed at identifying how these expression changes are occurring in TD and in alcoholics are required to better understand the way in which the process of inflammation develops. Such investigations may also yield important details regarding the process of inflammation in neurodegenerative disease states, given the similarities of impaired oxidative metabolism between TD and these maladies.

1.2.9 Induction of immediate early genes

Immediate-early genes (IEGs) are induced rapidly and transiently in response to a wide variety of cellular stimuli. They represent a mechanism that is activated at the transcription level at a very early stage as a response to stimuli before any new proteins are synthesized. The earliest known and best characterized IEGs are c-fos, c-myc and c-jun, a group of genes homologous to retroviral oncogenes. Many of these transcription factors can bind to consensus sites on the promoter region of other genes, thus allowing regulation of downstream gene expression that may play a significant role in alterations in cell function that occur as a consequence of TD. Expression of IEGs has been found to be increased dramatically in association with cell death (Colotta et al. 1992; Dragunow et al. 1993; Estus et al. 1994), and in TD (Hazell et al., 1998c), and has provided additional support for an excitotoxic event, in which IEG induction is linked to membrane depolarization, L-type voltage-sensitive calcium channel activation, SCC activation, and subsequent loss of Ca^{2+} homeostasis (Morgan and Curran, 1986; Murphy et al., 1991), all features of this disorder.

1.3 GLUTAMATE-MEDIATED EXCITOTOXICITY

1.3.1 Cerebral vulnerability to glutamate mediated excitotoxicity

Glutamate is a principal excitatory neurotransmitter in the central nervous system and almost 90% of the synaptic connections in the brain are estimated to be glutamatergic. At chemical synapses, glutamate is stored in vesicles. Nerve impulses trigger release of glutamate from the pre-synaptic cell. In the opposing post-synaptic cell, glutamate receptors, such as the NMDA receptor, bind glutamate and are activated.

Because of its role in synaptic plasticity, glutamate is involved in cognitive functions like learning and memory in the brain. The form of plasticity known as long-term potentiation takes place at glutamatergic synapses in the hippocampus, neocortex, and other parts of the brain (Doble, 1999). Glutamate works not only as a point-to-point transmitter but also through spill-over synaptic crosstalk between synapses in which summation of glutamate released from a neighbouring synapse creates extrasynaptic signalling/volume transmission (Pellerin, 2005). Hence, it is the glutamate concentration in the surrounding extracellular fluid that determines the extent of receptor stimulation. Any disturbance in glutamate homeostasis may therefore have severe pathological consequences and may lead to glutamate excitotoxicity (Fig. 4). Thus, it appears that the cerebral vulnerability to glutamate excitotoxicity is associated with selective increase in extracellular glutamate concentration. Glutamate is constantly being released from cells and is continually being removed from the extracellular fluid by the action of high affinity Na+ dependent glutamate transporters or excitatory amino acid transporters. Five excitatory amino acid transporters cDNAs have been identified and cloned (EAAT1–5) thus far. The predominant glutamate transporters in the brain are GLAST and GLT-1, also identified in the rodent as glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1), respectively, which are primarily expressed in astrocytes, while EAAT3, EAAT4, and EAAT5 are found primarily in neurons. Downregulation of glutamate transporter followed by accumulation of glutamate in the extracellular fluid have been documented in chronic and debilitative neurological disorders of diverse etiology. Thus, any disturbance in the physiological processes implicated in the regulation of glutamate, namely glutamate release/transport/uptake and energy status, may increase the likelihood

of the brain to excitotoxic insult, i.e. making it vulnerable to glutamate mediated excitotoxic damage (Fig. 4).

1.3.2 Glutamate transport and release

Transport and release of excitatory neurotransmitter, glutamate, is an essential event in glutamate neurotransmission. Glutamate taken up by cells can be used either for metabolic purposes (protein synthesis, energy metabolism, ammonia fixation) or it can be reused as a transmitter. In nerve terminals, glutamate is transported into synaptic vesicles by vesicular glutamate transporters and subsequently released by exocytosis. It seems likely that glutamate is also released, to some extent, directly from cytosol (non-vesicularly) through plasma membrane proteins. In astrocytes, glutamate taken up from the extracellular fluid may be converted to glutamine which is released to the extracellular fluid, taken up by neurons and reconverted to glutamate inside neurons (Doble, 1999). Synaptic glutamate transmission requires two types of neurotransmitter transporters. First, vesicular neurotransmitter transporters remove transmitters from the cytosol and transport them into the lumen of secretory vesicles to allow exocytotic release (Moriyama and Omote, 2008). Four types of vesicular transporters have thus far been identified which include VAChT, VMATs, VGAT (or VIAAT) and vesicular glutamate transporters (VGLUTs). VGLUT1 and 2 are expressed in complementary subsets of glutamatergic neurons in the CNS. In contrast, the most recently identified isoform, VGLUT3, is co-expressed with VAChT and VMAT2 in a number of cholinergic and aminergic cell types (Fei et. al., 2008). Synaptic transporters then remove excess neurotransmitter from the synaptic cleft and transport them to the interior of the synaptic terminal for recycling. In addition to these transporters another essential group of proteins

involved in synaptic transmission are the complexions. They are small, cytosolic and highly charged molecules (molecular mass of approximately 15 kDa) that are localized in presynaptic nerve terminals. They bind to the neuronal SNARE (soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptors) complex and are involved in regulating calcium-dependent neurotransmitter release (Hazell and Wang 2011). Current evidence suggests that loss of VGLUTs and complexins may destabilize synaptic terminal release of glutamate, contributing to dysfunction of circuitry with possible pathological consequences. Alterations in the function and/or expression of these proteins is a complicating factor which can contribute to the excessive sustained release of glutamate which may lead to excitotoxicity.

1.3.3 Glutamate receptors and excitotoxicity

Excessive release of glutamate in the synaptic cleft leads to activation of several types of pre and post-synaptic glutamate receptors. The consequent rise in intracellular calcium concentration may lead to mitochondrial dysfunction, generation of reactive oxygen species, and the activation of proteases, phospholipases, and endonucleases, leading to cell death.

1.3.3.1 Ionotropic glutamate receptors

There are three known types of ionotropic glutamate receptors based on their pharmacological properties; the N-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor, and the kainate receptor. The NMDA and AMPA/kainate receptors are all glutamate gated ion channels (conducting only Na^+ or both Na^+ and Ca^{2+}). The AMPA receptors open readily upon glutamate exposure, but desensitize quickly and are of low affinity. In contrast, the

NMDA receptors have much higher affinities and are slowly inactivating. To be activated they need both glutamate binding and an already depolarized membrane. Additionally, NMDA receptors are highly permeable to calcium and distributed widely on CNS neurons and, are the major initiators of excitotoxicity (Fig. 4). Moreover, pretreatment with (+)-5-methyl-10, ll-dihydro- 5H-dibenzocyclohepten-5, lO-imine hydrogen maleate (MK-801), a noncompetitive antagonist of the NMDA type glutamate receptor, protects against TD-induced lesions and suggests that TD-induced neuronal loss, particularly within thalamus, may be mediated by glutamate excitotoxicity (Langlais and Zhang, 1993; Todd and Butterworth, 1998). However, activation of Ca^{2+} permeable AMPA or kainate receptors can also trigger neuronal cell death. Antagonists of these receptors display a higher protective efficacy than NMDA receptor antagonists in some experimental neurodegenerative conditions. Thus, neuroprotective influence exerted by NMDA receptor antagonists, as well as by AMPA and kainate receptor antagonists following brain injury in animal models, including TD, further supports the notion that excessive glutamate receptor stimulation contributes to damage via an excitotoxic process.

1.3.3.2 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors which produce their effects via signalling mechanisms involving phosphoinositide-dependent processes, cyclic AMP or protein kinase C. Recent studies have identified mGluRs as a way in which neural cells regulate the release of glutamate and its uptake. Three groups of mGluRs have been characterized to date. Group I mGluR agonists have been reported to cause a downregulation of the EAAT1 transporter, while the Group II

agonist DCG IV upregulates its expression. Group II mGluRs are found on both pre- and post-synaptic membranes as well as glial cells, are negatively coupled to cyclic AMP, and regulate glutamate release via presynaptic Group II autoreceptors. Group III mGluRs are also negatively coupled to cyclic AMP. TD results in decreased ATP levels, a source of cyclic AMP via activity of adenylate cyclase. Thus, it is conceivable that loss of glutamate transporter regulation occurs as a consequence of changes in activity of mGluRs due to the declining ATP status (Hazell, 2009).

1.3.4 Glutamate transporters

Of the five types of glutamate transporters cloned to date, considerable evidence indicates that GLT-1 contributes to excitotoxicity and neuronal death in a number of neurological disorders, including ischemic stroke, traumatic brain injury, and ALS. Previous studies indicate that extracellular glutamate concentration is increased in vulnerable brain regions in TD (Langlis and Zhang, 1993; Hazell et al., 1993). This effect of TD on glutamate transporter levels was also demonstrated in primary cultures of astrocytes (Hazell et al., 2003).

Table 2. Nomenclature and expression pattern of glutamate transporters

Glutamate transporter subtype	Human homolog	Cell type	Anatomic localization
GLAST	EAAT1	Astrocytes,	Cerebellum, cortex, spinal cord oligodendrocytes
GLT1	EAAT2	Astrocytes	Throughout brain and spinal cord
GLT1b	EAAT2b	Astrocytes and neurons	Throughout brain and spinal cord
EAAC1	EAAT3	Neurons	Hippocampus, cerebellum, striatum
EAAT4	EAAT4	Purkinje cells	Cerebellum
EAAT5	EAAT5	Photoreceptors and bipolar Cells	Retina

Furthermore, it has recently been demonstrated that loss of GLT-1 and GLAST also

occurs in the cerebral cortex of alcoholic cases of WE (Hazell et. al., 2010). Based on the evidence it is likely that downregulation of glutamate transporters exacerbate glutamate mediated brain damage or excitotoxicity in TD. Focal accumulation of lactic acid is a classical feature of TD and one of the major consequences is gliotoxicity (Navarro et. al., 2005). Astrocytes are particularly sensitive to lactate. Thus, exposing them for extended periods to lactic acidosis leads to an inability of these cells to maintain ATP production (Bender et. al., 1997). The resulting decrease in ATP levels then causes a collapse of the ionic gradients across the astrocyte cell membrane due to suppression of Na^+/K^+ ATPase activity, leading to movement of Na^+ into the cell along its concentration gradient. Elevated intracellular Na^+ concentration results in transport of glutamate from the astrocyte to the extracellular space. Moreover, astrocytic swelling due to glutamate uptake or excessive K^+ spatial buffering can also lead to the depolarization of these cells, leading to release of glutamate via transporter reversal (Chan et. al., 2004; Danbolt 2001). The major consequence of this transporter reversal is an increase in extracellular glutamate concentration which is a major causative factor implicated in excitotoxicity (Danbolt 2001).

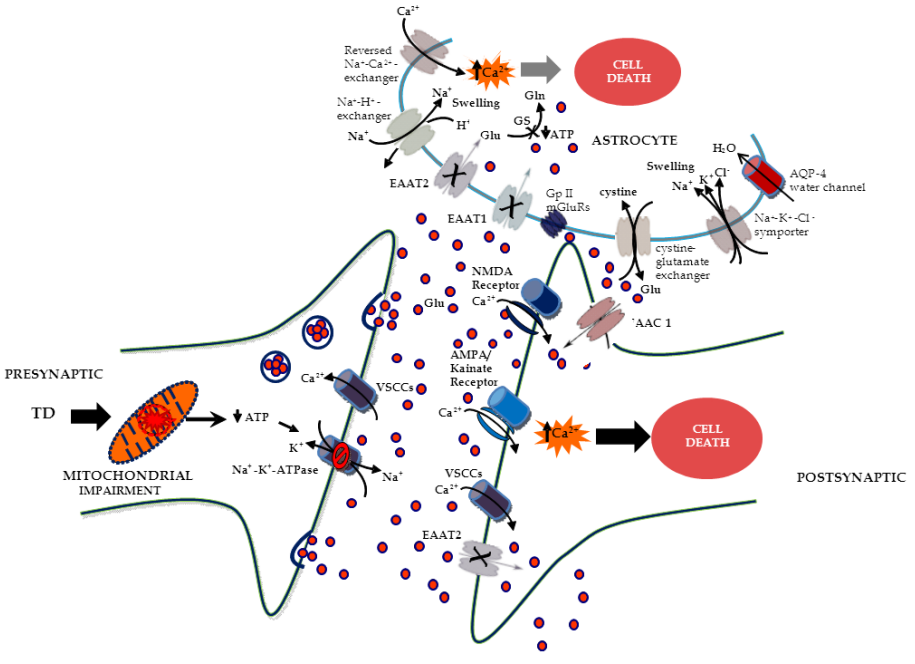

Figure 4. Glutamate trafficking in neurons and astrocytes.

1.4 REGULATION OF GLUTAMATE TRANSPORTER

Glutamate is the major neurotransmitter of the excitatory signaling pathway in the brain; in addition, it is involved in changes in the protein repertoire through the activation of signaling cascades, which regulate protein synthesis at transcriptional and translational levels. Activity-dependent differential gene expression by glutamate is related to the activation of ionotropic and metabotropic glutamate receptors and its subsequent removal from the extra-synaptic space by Na^+-dependent astrocytic glutamate transporter. Moreover, glutamate receptor stimulation is involved in processes of learning and memory as well as in other plastic changes in the CNS such as synapse induction and elimination during development. Excessive accumulation of extracellular glutamate and overactivation of glutamate receptors is associated with decreased expression and function of astrocyte glutamate transporters (Rothstein etal., 1996). In addition, a number of acute CNS diseases have been shown to be associated with astrocyte glutamate transporter dysfunction including CNS ischemia (Martin et al., 1997) and trauma (Yi and Hazell, 2006) as well as chronic neurodegenerative disorders such as Alzheimer's disease (AD) (Masliah et.al., 1996), and ALS (Lin et al., 1998), hepatic encephalopathy (Knecht et al., 1997), epilepsy (Mathern et al., 1999; Tanaka et al., 1997) and TD/WE (Jhala et.al., 2011; Hazell et.al., 2001, 2003, 2010). Glutamatergic synaptic transmission is involved in many important brain functions and elevated concentrations of extracellular glutamate can cause severe excitotoxic damage to the receiving neurons. It is therefore crucial to maintain efficient glutamate uptake. Recent studies involving targeted gene disruption confirmed significance of glutamate transporters in maintaining glutamate homeostasis (Tanaka et al. 1997). Based on these studies it is critical for our understanding to study

the regulation of glutamate transporter for its use as a pharmaceutical target in the treatment of neurodegenerative diseases. The mechanisms of glutamate transporter regulation are not well defined, and little is known about the factors that are responsible for regulating protein expression and activity. Regulation of transporter protein can occur at multiple levels, including DNA transcription and protein translation or posttranslational modification, consequently it may affect glutamate transporter activity, localization and protein targeting. Thus, it is likely that a combination of all of these mechanisms is important for the regulation of glutamate transporters.

1.4.1 Transcriptional regulation

1.4.1.1 Neuron derived factors

Studies over the past years have identified numerous chemical factors that are responsible for the transcriptional regulation of glutamate transporters. Among these factors most important are those that are derived from neurons. Upregulation of both types of astrocytic glutamate transporters, GLT-1/EAAT2 and GLAST/EAAT1 by neuron derived factors were reported previously (Drejer et al. 1983; Gegelashvili et al. 1997; Schlag et al. 1998). In the absence of neurons, astrocytes maintain polygonal shapes and express only the GLAST transporter. When co-cultured with neurons, astrocytes exhibit more complex morphologies and show increased expression for GLT-1 (Swanson et al. 1997), thus suggesting that there are neuronal soluble factors that increase the levels and expression of glutamate transporter protein and mRNA. Although the soluble factors present in neuron conditioned media (NCM) have not been yet identified, progress have been made in identifying their signal transduction pathways. Previously it has been proposed that upregulation of glutamate transporters depends on the activation of p42/44

MAP kinases via the tyrphostin sensitive receptor tyrosine kinase (RTK) signalling pathway (Swanson et al. 1997), and phosphatidylinositol 3-kinase (PI3K), tyrosine kinase (TKs), or nuclear transcription factor κB (NF-κB) inhibition almost completely blocked neuronal factors-induced glutamate transporters upregulation (Swanson et al. 1997; Zelenaia et al. 2000) (Fig. 5).

1.4.1.2 Growth factors

Other extracellular factors that regulate glial glutamate transporter expression are the epidermal growth factor receptor (EGFR) ligands, epidermal growth factor (EGF), and transforming growth factor α (TGFα) (Fig. 5) (Zelenaia et al., 2000). Interestingly, the expression levels of both EGF and TGFα growth factors increase after brain injury (Lisovoski et al., 1997), thus pointing to a crucial role of reactive brain processes in the restoration of glial glutamate uptake function. Additionally, it was proposed that EGF and TGF α induces upregulation of GLT-1 in cultured astrocytes, while platelet-derived growth factor (PDGF) showed no effect on the GLT-1 levels (Zelenaia et al. 2000). Similarly, basic fibroblast growth factor (bFGF), insulin, and nerve growth factor (NGF) showed no effect on GLT-1 expression in cultured astrocytes, while there was a significant upregulation of GLT-1 caused by dibutyryl-cAMP (dbcAMP) (Swanson et al. 1997; Zelenaia et al. 2000). The effects of EGF and dbcAMP were blocked by inhibitors of PI3K and NF-κB, similar to that observed for neuron conditioned media treated astrocytes (Zelenaia et al. 2000). However, inhibition of the EGF receptor during treatment with NCM did not block the increased expression of GLT-1 (Zelenaia et al. 2000). This suggests that there are independent signalling pathways responsible for the regulation of glutamate transporters in astrocytes.

1.4.1.3 Nuclear factor kappa B (NF-κB) signaling pathways

NF-κB is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors. The Rel/NF-κB families of transcription factors are involved mainly in stress-induced, immune, and inflammatory responses. More recently, NF-κB family members have been implicated in neoplastic progression and the formation of neuronal synapses. NF-κB is also an important regulator in cell fate decisions, such as programmed cell death and proliferation control, and is critical in tumorigenesis (Baldwin, 1996).

Upregulation of glutamate transporters expression by NCM or EGF, TGF-α, and dbcAMP, as well as bromo-cAMP is significantly blocked by NF-κB inhibition (Swanson et al. 1997; Zelenaia et al. 2000). On the other hand, NF-κB inhibition restored GLT-1 promoter activity during treatment with TNF-α. These findings were further confirmed when the GLT-1 promoter was cloned. The EAAT2 sequence revealed the presence of a consensus sequence for NF-κB binding within the 5′-untranslated region of the GLT-1/EAAT2 complementary DNA (cDNA) clones (Meyer et al. 1996), indicating the involvement of NF-κB mechanism in EAAT2 transcriptional regulation.

NF-κB can act both positively and negatively depending on the agent administered; however, the molecular basis of this phenomenon is still unclear. It has been proposed that EGF-induced NF-κB activation is independent of IκB degradation (Sitcheran et al., 2005), a common step in the signaling cascade upstream of NF-κB activation (Yamamoto and Gaynor 2004). Also, TNFα-mediated repression is dependent on the recruitment of N-myc, another transcription factor whose binding site is also present on the EAAT2 promoter sequence (Sitcheran et al. 2005).

1.4.1.4 Pituitary adenylate cyclase-activating polypeptide (PACAP)

PACAP is a neuron-derived factor that has been proposed to regulate the expression of GLT-1 and GLAST. Exposure of cortical glial cultures to PACAP increased glutamate uptake and protein expression of GLT-1 and GLAST as well as glutamine synthetase (Figiel and Engele 2000). This effect was significantly blocked by PACAP-inactivating antibodies or by PACAP receptor (PAC1) antagonists. The signalling pathways involved in the PACAP dependent regulation of GLT-1 expression are protein kinase A (PKA) and PKC pathways, while GLAST upregulation was only dependent on activation of PKA.

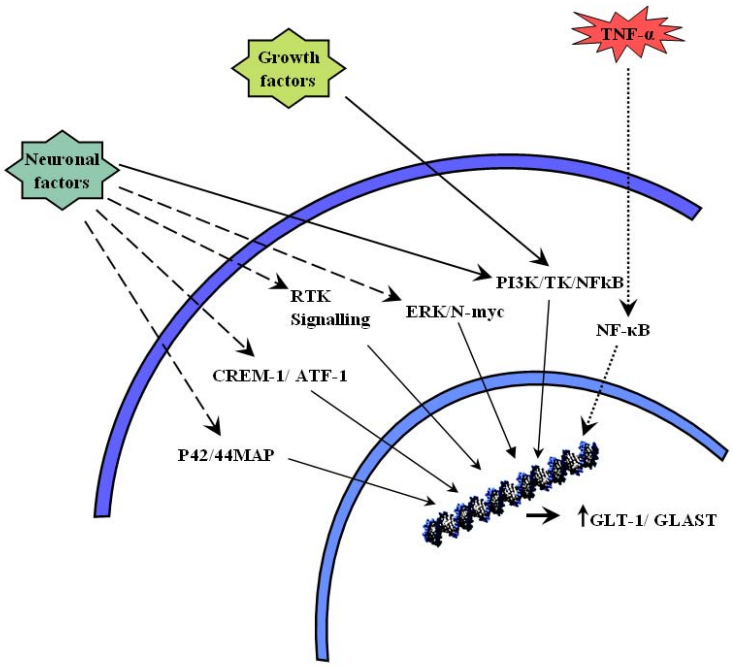

Figure 5. Suggested pathways effecting glutamate transporter expression.

Loss of PAC1 or knock-out of PAC1 showed a significant reduction in GLAST expression in the dentate gyrus, but not in the cortex, while expression of GLT-1 was found to be unaffected (Zink et al. 2004).

1.4.1.5 Glutamate

Most of the studies have identified soluble factors released by neurons that may promote GLT-1 expression; however, regulation of GLAST seems to be mediated through different signalling pathways. In a recent study L-glutamate as one soluble neuron-derived factor acting as a GLAST regulator (Gegelashvili et al. 1996) was identified. Glutamate induced an increase in glutamate uptake capacity in primary astrocyte culture and increased the levels of GLAST protein. Treatment with a specific inhibitor for glutamate receptors (specifically, AMPA/kainate) blocked this upregulation. The glutamate-mediated increase in GLAST protein levels was not accompanied by an increase in mRNA levels, suggesting regulation instead of transcriptional regulation. On the other hand, when cells were treated with dbcAMP, both mRNA and protein levels were significantly elevated compared to non-treated cultures (Gegelashvili et al. 1996; Swanson et al. 1997). This illustrates again that the regulation of glutamate transporters occurs at different levels and through different signaling pathways depending on the stimulating factor.

1.4.1.6 Steroids

Exposure of primary astroglial cells to estrogen was reported to cause a significant increase in GLAST (and GLT-1) mRNA levels as well as protein levels, which was accompanied by increased glutamate uptake (Pawlak et al. 2005). Similar observations were made with human cultured astrocytes derived from the cortex of AD patients (Liang et al. 2002). The authors suggested that upregulation of glutamate transporters may be one mechanism by which estrogens provide neuroprotection against excitotoxic glutamate overflow (Liang et al. 2002; Pawlak et al. 2005).

1.4.2 TRANSLATIONAL REGULATION

Past studies on molecular structure and membrane topology of glutamate transporters have provided the essential information on posttranslational regulation of glutamate transporters. Structurally, the transporter molecules are made of eight helical transmembrane domains (TMD) and two helical hairpin loops. Both, the N- and C-terminus are cytoplasmic with a large extracellular hydrophilic region between the third and fourth TMD (Slotboom et al. 1999). Using high-resolution spectroscopy of the bacterial glutamate transporter homolog GltPh, it has been suggested that the transporter is assembled as a trimer (Yernool et al. 2004). The potential regulatory mechanisms based on the structure homologue are discussed in the following sections.

1.4.2.1 Glycosylation and maturation

Glycosylation of proteins is a posttranslational modification that plays a role in molecular trafficking, protein folding, endocytosis, receptor activation, signal transduction, and cell adhesion (Ohtsubo and Marth, 2006). Two common forms of protein glycosylation include N-linked glycosylation and *O*-linked glycosylation. N-linked glycosylation is the covalent linkage of oligosaccharides to asparagines residues of proteins. N-glycosyl residues are processed as proteins are trafficked through the endoplasmic reticulum and golgi.

In addition to the glycosylated proteins which are subsequently transported to the plasma membrane, some proteins are retained in the ER and therefore develop into an immature, non-glycosylated protein. The mechanism for this ER retention involves an arginine based motif (RXR) in the nascent polypeptide that regulates ER retention

signals, and studies have reported an adjacent leucine based motif that function to suppress the ER retention signals by RXR (Kalandadze et al. 2004). The EAATs) are *N*-glycosylated proteins and they are regulated or dysregulated differentially by either of these glycosylation mechanisms, as discussed below.

1.4.2.1.1 EAAT1/GLAST

EAAT1 is variably expressed throughout the brain in astrocytes (Rothstein et al., 1994; Chaudhry et al., 1995; Lehre et al., 1995). Glycosylation sites were identified on GLAST in the extracellular loop of transmembrane helices 3 and 4 at Asn206 and Asn216 (Conradt et al. 1995). The two GLAST isoforms, 70-kDa and 64-kDa, differs only by the degree of N-glycosylation at Asn206 and Asn195 (Conradt et al., 1995). Glycosylation of this transporter may serve an important functional role because nonglycosylated GLAST does not form homomultimers, which are the native conformation of GLAST in vivo (Conradt et al., 1995). In addition, glycosylation of GLAST has been correlated with trafficking of GLAST to plasma membrane and increased glutamate uptake (Escartin et al., 2006).

1.4.2.1.2 EAAT2/GLT1

EAAT2 is an astrocytic transporter responsible for the majority of glutamate uptake in the cortex. Deglycosylation of the rodent isoforms of EAAT2 (GLT-1) resulted in a 10-15 kDa shift in molecular weight of the monomer band (Kalandadze et al., 2004). There is conflicting literature describing the functional effects of EAAT2 glycosylation. One group found that glycosylation-deficient GLT-1 (the rodent form of EAAT2) had a decreased rate of glutamate transport due to decreased expression in the plasma membrane (Trotti et al., 2001). This may be due to the retention of GLT-1 in the

endoplasmic reticulum, because mutant GLT-1 expressing an altered extracellular leucine-based motif is immaturely glycosylated and retained in the ER (Kalandadze et al., 2004). However, another group found no effect of N-glycosylation on the trafficking or transport activity of GLT-1 in transfected BHK cells, but increased stability at the plasma membrane, which may be critical for transporter localization in vivo (Raunser et al., 2005).

1.4.2.1.3 GLYCOSYLATION OF HUMAN EXCITATORY TRANSPORTER

Glycosylation of the EAATs in human brain has not been evaluated in detail, however, a recent study reported the identification of a splice variant of the human glutamate transporter EAAT1, EAAT1ex9skip (Vallejo-Illarramendi et al. 2005). This splice variant lacks the entire exon 9 of EAAT1, and its mRNA is translated into a truncated protein localized to the ER. Furthermore, when co-expressed with full-length EAAT1, EAAT1ex9skip acts as a negative regulator of EAAT1. This is quite similar to previous reports of alternate splice products of EAAT2 described by Lin et al. (1998). Interestingly, exon 9 includes the leucine-based motif and excludes the RXR motif. This confirms the repressor properties of the leucine-rich motif on RXR-induced ER retention and may explain the ER-restricted localization and functional inactivity of EAAT1ex9skip.

1.4.2.2 PROTEIN TARGETING AND STABILIZATION

1.4.2.2.1 Membrane domains

Efficient synaptic transmission requires a highly ordered arrangement of specialized membrane domains, including presynaptically localized vesicles, postsynaptic glutamate receptors and astrocytic or peri-synaptically localized glutamate transporters.

The close vicinity of all of these membrane domains is critical in shaping the amplitude of postsynaptic responses. (Jackson et al. 2001; Zhou and Sutherland 2004).

These specialized membrane domains include lipid rafts, which are lipid-protein microdomains of the plasma membrane that are enriched with cholesterol and glycosphingolipids (Simons and Toomre 2000). They participate in a number of cellular processes including regulation of trafficking and clustering of membrane-associated proteins and their intracellular signaling molecules (Becher et al. 2001). The role of these membrane domains in glutamate transporter regulation was first confirmed by Butchbach et al. (2004), they showed that depletion of membrane cholesterol by methyl-β-cyclodextrin reduced Na+-dependent glutamate uptake in primary cortical cultures. Biochemical analysis further confirmed the association of glutamate transporters with cholesterol-rich lipid raft microdomains of the plasma membrane (Butchbach et al. 2004). The reduced glutamate uptake in the absence of cholesterol was accompanied by a decrease of transporter protein at the cell surface, which was blocked by a non-specific inhibitor of receptor internalization. This suggests that endocytosis of the transporters may be increased when there is no membrane cholesterol present to stabilize the transporters at the cell surface, resulting in decreased glutamate uptake.

1.4.2.2.2 Excitatory transporters interacting proteins

Localization and stabilization of membrane proteins is often dependent on their interaction with intracellular anchoring proteins. This assembly of multiprotein complexes influences both specificity and efficiency of biological processes within the cell. In the brain, multiprotein assemblies of receptors, ion channels, and transporters have been studied extensively (Levitan, 2006; Muller et al., 2010). These complexes

execute critical biological functions, including stabilizing receptors near synapses, tethering kinases and phosphatases to limit nonspecific modification of proteins, and assembling enzymes to increase the efficiency of substrate transfer (Genda et al., 2011).

Glutamate transporter-mediated glutamate uptake is regulated by several excitatory transporters-interacting proteins that have been studied over the years (Danbolt, 2001; Jackson et al., 2001; Lin et al., 2001; Marie et al., 2002; Watanabe et al., 2003.). The excitatory transporter-interacting proteins utilize diverse mechanisms to modulate glutamate transport activity.

1.4.2.2.2.1 EAAT2/GLT1 interacting protein

The two GLT-1 interacting proteins Ajuba and G protein pathway suppressor-1 (GPS-1) were identified using yeast two-hybrid technique (Marie et al., 2002; Watanabe et al., 2003), where Ajuba interacts with the amino terminus of GLT-1 and may act as a scaffolding protein, linking GLT-1 with the cytoskeleton and various signaling pathways (Marie et al., 2002). GPS-1 is a subunit of COP9 signalosome, interacts with the C-terminus of GLT-1, and may be involved in the surface trafficking of GLT-1 via its leucine zipper-like motif. Coexpression of GPS-1 with GLT-1 in human embryonic kidney cells downregulates glutamate reuptake activity (Watanabe et al., 2004; 2003).

1.4.2.2.2.2 EAAT1/GLAST interacting protein

The consensus motif (last 8 residues) at C-terminus of GLAST consists of sequence homology to PDZ domain that might regulate the selective localization and function of GLAST in astrocytes. PDZ domains are a family of protein-protein interaction domains named after the first three proteins in which they were identified; post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1),

and zonula occludens-1 protein (zo-1). In a recent study two novel PDZ related scaffolding proteins, the Na^+/H^+ exchanger regulatory factors 1 and 2 (NHERF-1 and NHERF-2) were discovered. Studies using primary cortical astrocytes revealed that these cells are highly enriched in NHERF-2 relative to NHERF-1. Moreover, knockdown of NHERF-2 in astrocytes showed a significant reduction in GLAST activity, followed by corresponding decrease in expression of GLAST protein and reduced half-life, suggesting that NHERF-2 interaction with GLAST in astrocytes enhances GLAST stability and activity. However, this interacting at the C-terminus may also modulate a transporter's glutamate affinity. In one study (Marie and Attwell 1999), it has been suggested that disruption of an interaction between an intracellular protein and the last eight amino acids of the GLAST C-terminus, increases the glutamate affinity of GLAST. Thus, the interacting protein decreases the affinity of GLAST transporters. Consequently, this interaction may significantly slow the removal of low concentrations of glutamate from the extracellular space. Therefore, whether these protein interactions for GLAST motifs are acting as positive or negative modulators is still unknown and requires further investigation.

1.4.2.3 Glutamate transporter trafficking

Other mechanism for regulating neurotransmitter transport involves changes in membrane trafficking of the transporter. Trafficking of membrane proteins between the plasma membrane and intracellular stores that is mediated by an interplay of internalization and membrane insertion can occur in a very short period and therefore allows for very fast regulation of functional protein activity. Trafficking of other membrane proteins such as neurotransmitter receptors has been studied extensively,

however the regulation of glutamate transporter trafficking is yet to be studied. One of the major mechanisms for regulating membrane protein trafficking to and from the plasma membrane is PKC dependent direct phosphorylation of the membrane protein or its interacting scaffolding proteins. Protein kinase-C also (PKC) is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino-acid residues on these proteins. PKC enzymes in turn are activated by signals such as increases in the concentration of diacylglycerol (DAG) or calcium ions (Ca^{2+}). Hence PKC enzymes play important roles in several signal transduction pathways. Protein kinase C directly phosphorylate GLAST and reduces the transport activity up to 25% with no change in cell surface expression (Conradt and Stoffel, 1997). Elimination of protein kinase C consensus sites does not eliminate the effect, suggesting that the phosphorylation occurs at a novel site. GLT1 was originally reported to be stimulated by protein kinase C mediated phosphorylation of serine-113 when expressed in HeLa cells (Casado et al., 1993). However, protein kinase C activation in MDCK cells with stable expression of human GLT1 leads to a reduced GLT1 cell surface expression (Carrick and Dunlop, 1999). Thus, the phosphorylation varies between different cell types because of the different intracellular machinery which respond differently to the phosphorylation mechanism (Carrick and Dunlop, 1999).

1.4.3 GLUTAMATE TRANSPORTER MODIFICATION

1.4.3.1 Arachidonic acid

Arachidonic acid (AA) is a polyunsaturated fatty acid present in the phospholipids and is a precursor for the cellular synthesis of prostaglandins, prostacyclins, thromboxanes and leukotrienes. AA is released from neurons and glial cells during synaptic activity and can modulate synaptic transmission (Dumuis et al., 1988; Stella et al., 1994) upon activation of glutamate receptors. In addition, AA has been reported to inhibit the glutamate uptake capacity in neuronal synaptic terminals and astrocytes (Dorandeu et al. 1998; Lundy and McBean 1995).

1.4.3.2 Sulfhydryl based redox mechanisms

Glutamate transporters are further modified and regulated by sulfhydryl based redox mechanisms (extensively reviewed by Trotti et al. 1998). Exposure of cortical astrocytes to H_2O_2 or xanthine/ xanthine oxidase decreases glutamate uptake dramatically, and the effect was partly reversible by DTT (Albrecht et al., 1993) or free radical scavenger enzymes (Volterra et al. 1994). The inhibitory effect produced by sulfhydryl based redox mechanism is attributed to the cysteine residues of transporters polypeptide. Mutation of cysteine residues in GLAST (canine origin) showed oligomer formation, plasmamembrane localization, and the transport kinetics similar to wildtype GLAST (Tamahara et al. 2002). In addition, it has been suggested that cysteine residues are not critical for the functional expression of GLAST. While oxidative processes play an important role during pathological conditions and could explain decreased functional activity of glutamate transporters in disease, the physiological role of these oxidative processes in regulation of glutamate transporter function is still unclear.

1.4.4 Summary of glutamate transporter regulation

The most common dysfunction of glutamate transport in neurodegenerative disease seems to be caused by a decrease in transporter protein level. The mechanisms for loss of protein (transcriptional/translational) are generally not known. Protein downregulation is found in acute neurodegenerative diseases, such as ischemia/hypoxia (Chen et al. 2005; Fukamachi et al. 2001; Inage et al. 1998; Martin et al. 1997) and a number of chronic neurodegenerative disorders, including HD (Behrens et al. 2002), PD (Ginsberg et al. 1995; Levy et al. 1995), AD (Masliah et al. 2000), and ALS (Rothstein et al. 1995). The downregulation of transporter protein was not always paralleled by decreases in mRNA levels. For example, our thiamine deficiency model showed decreased glutamate uptake and protein levels for GLAST (Hazell et. al., 2003) and GLT-1a/b, but had no effect on the mRNA expression for GLT-1a/b (Jhala et. al., 2011). Similar findings were observed in mice expressing mutant amyloid precursor protein (Masliah et al. 2000) and from a postmortem analysis of frontal cortex of AD patients. A possible explanation for the loss in transporter protein without a loss in mRNA could be a dysregulation of transporter protein degradation processes. Irrespective of the mechanism for loss of transporter protein following different neurological insults, overcoming this loss is therapeutically significant. Few studies in the past have made significant progress using pharmacologically intervening agents against the loss of transporter protein in different diseases. In one study, transgenic mice overexpressing GLT-1 (1.5- to 5-fold over wildtype mice) (Sutherland et al. 2001) were crossed with transgenic mice overexpressing a mutant form of SOD1 (an animal model for ALS), the animals showed a delayed onset of motor neuron degeneration and increased survival, suggesting that

increasing the total number of GLT-1 molecules protects against neurodegeneration. In another study (Rothstein et al., 2005) discovered that β-lactam antibiotics increase protein expression of GLT-1, both in vitro and in vivo. Moreover, treatment SOD1 mutant mice with ceftriaxone significantly delayed disease onset as well as increases neuron survival, confirming a neuroprotective effect of β-lactam antibiotics through upregulation of glutamate transporter protein. Similar findings were obtained with ceftriaxone, our TD animal model and astrocyte cultures treated with TD (Jhala et. al., 2011).

Thus, glutamate transporters can be regulated via multiple mechanisms, earlier at the level of gene transcription to modulations of the transporter protein through posttranslational modifications. The understanding of these processes has provided us with an alternative to identify the signaling pathways involved in a transporter dysfunction under different pathological conditions. More importantly, the identification of these pathways has further advanced the development of new strategies for pharmacological intervention and therapy in diseases associated with a dysfunction of glutamate transporters.

1.5 THE GLUTAMATE HYPOTHESIS OF TD

In TD, considerable evidence now exists for excitotoxic-mediated cell death localized in areas of focal vulnerability. Over the years, a number of studies have identified glutamate as being a major factor in the pathophysiology of TD. Early reports of glutamate levels being reduced in whole brain of TD animals (Gubler et al., 1974) were consistent with decreases in the conversion of [^{14}C]glucose to glutamate in TD rats (Gaitonde et al., 1975) and reduced Ca^{2+}-dependent release of glutamate in hippocampal slices from symptomatic TD animals (Lê et al., 1991), suggesting a role for glutamate in this disorder. Studies also reported similarity in the appearance and development of thalamic lesions in TD to that of damage observed following excitotoxic conditions (Armstrong-James et al., 1988; Zhang et al., 1995). In addition, treatment with the noncompetitive NMDA receptor antagonist MK-801 was shown to lead to a reduction in the extent of neuronal damage in rats with TD (Langlais and Mair, 1990; Todd and Butterworth, 1998), providing further support for excitotoxic-mediated pathology in this disorder.

The first direct evidence for existence of a glutamate-mediated excitotoxic event was reported when Hazell and colleagues (1993) and others demonstrated that extracellular glutamate concentration was increased in vulnerable brain regions during the symptomatic stage of TD following the appearance of neurological dysfunction (Hazell et al., 1993). Figures 3 and 5 show some of the major pathophysiological consequences of TD, including the development of excitotoxicity. At the same time, L-type voltage-sensitive calcium channels (VSCCs) are activated (Hazell et al., 1998b), synonymous with sustained depolarization, suggesting the entry of Ca^{2+} into cells, and

which together with the rise in interstitial glutamate levels strongly indicates the likelihood of an excitotoxic event in affected regions of the brain in TD.

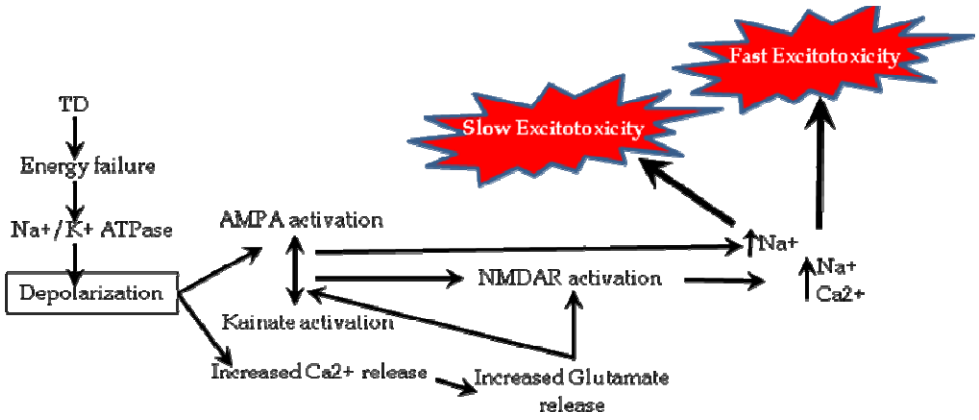

Figure 6. Excitotoxic mechanism in thiamine deficiency

Previous studies have demonstrated that such an imbalance in Ca^{2+} homeostasis is one of the most significant factors involved in excitotoxic cell death (Olney, 1971; Schanne et al., 1979; Arundine and Tymianski, 2003). Similarly, In TD the focal brain regions experiencing these changes in glutamate levels and depolarization also sustain a large downregulation of the astrocytic glutamate transporters GLT-1 and GLAST (Hazell et al., 2001). This was also confirmed in cultured astrocytes made thiamine-deficient, in which a decrease in the V_{max} for glutamate uptake and downregulation of EAAT1 were a consequence (Hazell et al., 2003). Such findings were also consistent with other evidence suggesting the extracellular glutamate rise observed in the thalamus in TD rats is due to a Ca^{2+}-independent processs (Hazell and Hakim, 1994).

OBJECTIVE AND HYPOTHESIS

Hypothesis: *Loss of the astrocytic glutamate transporter splice-variant GLT-1b is involved in brain lesions resulting from thiamine deficiency.*

The specific goals are to investigate i) the effects of TD on glutamate transporter GLT-1b expression and protein levels, and ii) the mechanism(s) involved in altered regulation of GLT-1b during TD using the pyrithiamine-induced rodent model of TD and in our *in vitro* model of TD involving primary cultures of astrocytes.

CHAPTER 2

Loss of the glutamate transporter splice-variant GLT-1b in inferior colliculus and its prevention by ceftriaxone in thiamine deficiency

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LOSS OF THE GLUTAMATE TRANSPORTER SPLICE-VARIANT GLT-1B IN INFERIOR COLLICULUS AND ITS PREVENTION BY CEFTRIAXONE IN THIAMINE DEFICIENCY

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Abstract

Downregulation of astrocytic glutamate transporters is a feature of thiamine deficiency (TD), the underlying cause of Wernicke's encephalopathy, and plays a major role in its pathophysiology. Recent investigations suggest that ceftriaxone, a β-lactam antibiotic, stimulates GLT-1 expression and confers neuroprotection against ischemic and motor neuron degeneration. Thus, ceftriaxone treatment may be a protective strategy against excitotoxic conditions. In the present study, we examined the effects of ceftriaxone on the glutamate transporter splice-variant GLT-1b in rats with TD and in cultured astrocytes under TD conditions. Our results indicate that ceftriaxone protects against loss of GLT-1b levels in the inferior colliculus of rats during TD, but with no significant effect in the thalamus and frontal cortex by immunoblotting and immunohistochemistry. Ceftriaxone also normalized the loss of GLT-1b in astrocyte cultures under conditions of TD. These results suggest that ceftriaxone has the ability to increase GLT-1b levels in astrocytes during TD, and may be an important pharmacological strategy for the treatment of excitotoxicity in this disorder.

1. Introduction

Excitatory amino acid transporters (EAATs) are considered to be a potential target for neuroprotection against excitotoxicity, normally removing extracellular glutamate by an efficient uptake process (Anderson and Swanson, 2000; Danbolt, 2001; Sattler and Rothstein, 2006). Several glutamate transporter proteins have been cloned and sequenced, including EAAT1 (Storck et al., 1992), EAAT2 (Pines et al., 1992), EAAT3 (Kanai and Hediger, 1992), EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997). Of three major glutamate transporters identified in the rodent forebrain, glutamate transporter 1 (GLT-1) (or EAAT2) and glutamate/aspartate transporter (GLAST) (or EAAT1) are expressed mainly in astrocytes (Danbolt et al., 1992; Rothstein et al., 1994; Lehre et al., 1995), while excitatory amino acid carrier 1 (or EAAC1) is expressed in neurons. GLT-1 is considered the major glutamate transporter responsible for clearance of glutamate from the extracellular space (Lehre and Danbolt,1998; Rothstein et al., 1996; Selkirk et al., 2005). In addition, considerable evidence indicates that GLT-1 contributes to excitotoxicity and neuronal death in a number of neurological disorders, including ischemic stroke, traumatic brain injury, and amyotrophic lateral sclerosis (Rao et al., 2001; Allen et al., 2004; Maragakis and Rothstein, 2004).

Previous studies indicate that extracellular glutamate concentration is increased in vulnerable brain regions in thiamine deficiency (TD) (Hazell et al., 1993; Langlais and Zhang, 1993), the cause of Wernicke's encephalopathy (WE), and in which loss of GLT-1 and GLAST also occurs (Hazell et al., 2001). This effect of TD on glutamate transporter levels was also demonstrated in primary cultures of astrocytes (Hazell et al., 2003). Furthermore, it has recently been demonstrated that loss of GLT-1 and GLAST also occurs in the cerebral cortex of alcoholic

cases of WE (Hazell et al., 2010), consistent with evidence that TD produces an impairment of glutamate uptake in this area of the brain (Carvalho et al., 2006).

Recently, studies have reported an upregulation of GLT-1 by β-lactam antibiotics following downregulation of this transporter in in vitro models of ischemic brain injury and motor neuron degeneration (Rothstein et al., 2005). The β–lactam antibiotic ceftriaxone alters glutamate transport and upregulates GLT-1 levels via an NFκB-mediated activation of the GLT-1 promotor (Lee et al., 2008). Thus, administration of ceftriaxone may have therapeutic potential against excitotoxicity in various neurodegenerative conditions. Ceftriaxone also confers protection against glutamate toxicity by inducing the glutamate/cystine-antiporter system, independent of glutamate transporter regulation (Lewerenz et al., 2009).

This study examines the effect of ceftriaxone on excitotoxicity associated with TD. Our results indicate that ceftriaxone completely blocks loss of the splice-variant GLT-1b in the inferior colliculus but is ineffective in the medial thalamus, another vulnerable brain region in this disorder.

2. Materials and methods

2.1. Rat model of TD

Male Sprague–Dawley rats (Charles River, St-Constant, QC, Canada) were used for all experiments. Animals were housed individually under constant conditions of temperature, humidity and 12 h light/dark cycles. Rats were acclimatized for at least 2 days prior to the initiation of treatment, and neurological assessment of the animals (ataxia, opisthotonus, loss of righting reflexes, convulsions, nystagmus) were made on a daily basis. All experimental procedures were approved by the Animal Ethics Committee of Saint-Luc Hospital (CHUM) and

the University of Montreal. Rats in the TD group (n = 9) were fed a thiamine-deficient diet (ICN Nutritional Biochemicals, Cleveland, OH, USA) and administered daily pyrithiamine hydrobromide (Sigma–Aldrich, St Louis, MO, USA) (0.5 mg/kg body weight, i.p.). Control rats (n = 9) were pair-fed to TD animals using the same thiamine5 deficient diet and supplemented with daily i.p. injections of thiamine (0.1 mg per kg body weight). Treatments were continued until the symptomatic stage (loss of righting reflex). Any animals exhibiting spontaneous seizures were eliminated from the study. Separate groups of rats were treated with ceftriaxone (200 mg/kg, i.p. for 5 days commencing at day 7 of TD.

2.2. Cell culture preparation

Primary astrocyte cultures from newborn rats were prepared as previously described (Hazell et al., 1997). Briefly, cerebral cortices were removed and the tissue was dissociated, passed through sterile nylon sieves, and then suspended in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Burlington, Ontario, Canada) containing 10% fetal calf serum. Cells were then seeded in 35 mm culture dishes, which were maintained in an incubator at 37°C provided with a mixture of 5% CO2 and 95% air. After 2 weeks, cells attained confluency, at which point the fetal calf serum was replaced by horse serum. Cultures were grown for 3-5 weeks, during which the medium was changed twice a week, and supplemented with dibutyryl cyclic AMP after 2 weeks. At least 95% of cells were determined to be astrocytes, based on GFAP immunohistochemistry.

To induce TD, cells were exposed to a custom-designed DMEM media for 10 days lacking in thiamine (Invitrogen Canada, Burlington, ON, Canada) and containing 5% horse serum, in the presence of pyrithiamine (20 μM). Control astrocytes were treated with TD media in which normal levels of thiamine (10 μM) had been added.

2.3. Immunoblotting studies

At the appropriate time, animals were sacrificed by decapitation, the brains were removed and flash frozen in isopentane on dry ice and stored at -800C. The frontal cortex, medial thalamus and inferior colliculus were then dissected out according to the rat brain atlas of Paxinos and Watson (1998). Samples of rat brain tissue were homogenized in buffer containing 50 mM Tris, 150 mM NaCl, 0.1% 6 sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate (pH 8.0) and protease inhibitor cocktail, and centrifuged at 10,000g for 10 min at 4oC. Preliminary studies carried out on the pellet and supernatant indicated that GLT-1b was completely soluble in this buffer under our conditions. Thus, the supernatant was retained and used for study. Astrocyte cultures were harvested in the same buffer. Protein content of all samples was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Sample buffer was added to aliquots of the tissue (30 μg) or cultures (50 μg) and the samples boiled for 5 min. Aliquots were subjected to (SDS)-polyacrylamide gel electrophoresis (8% polyacrylamide) and the proteins subsequently transferred to polyvinylidene difluoride membranes by wet transfer at 20 V over 24 h. The transfer buffer consisted of 48 mM Tris (pH 8.3), 39 mM glycine, 0.037% SDS, and 20% methanol. Membranes were subsequently incubated in blocking buffer (10 mM Tris, 100 mM NaCl, 5% non fat dried milk) followed by incubations with rabbit polyclonal antisera directed against GLT-1b (B1b558; Ab #358) (0.05 μg/ml). Reblocking was followed by incubation with HRP-coupled antirabbit IgG (0.01 μg/mL) secondary antiserum. Each incubation step was of 1 h duration following which blots were washed several times with buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20). For the detection of specific antibody binding, the membranes were treated in accordance with the ECL-kit instructions and developed on X-OMAT film. Signal intensities were subsequently measured by densitometry using Image J software (NIMH, USA).

Linearity of the relationship between optical density and protein concentration was verified using appropriate standard curves. Blots were reversibly stained with Ponceau-S to monitor protein transfer efficiency and equal loading.

2.4. Immunohistochemistry and histology

At the appropriate time, animals were deeply anesthetized with pentobarbital (60 mg/kg, i.p.) 7 and perfused transcardially as described previously (Hazell et al., 2001). Brains were removed and postfixed overnight in neutral-buffered formalin containing 4% formaldehyde, 0.5% sodium phosphate buffer, and 1.5% methanol, pH.7.0. Coronal sections (23.8 to 25.8 mm relative to bregma) of 40-μm thickness were cut using a vibrotome according to the rat brain atlas of Paxinos and Watson (1998). Immunohistochemistry was performed according to Hazell et al. (2001). Briefly, rat brain sections were incubated for 10 min in phosphate-buffered saline (PBS) containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were washed in PBS (10 min), blocked for 20 min in 0.5% Triton X-100 and 5% donkey serum, and incubated with or without 0.5% Triton X-100, along with 5% donkey serum and primary rabbit antisera directed against GLT-1b (B1b558; Ab #358) (0.02 μg/ml) at room temperature or 4oC overnight. Sections were then washed (10 min) and incubated in PBS containing biotinylated donkey anti-rabbit/mouse IgG (1:100). Sections were then incubated for 1 h in streptavidin-HRP conjugate (1:100) followed by washing (10 min), and then incubation with DAB (0.05%) in PBS containing in some cases 25 mg/mL nickel ammonium sulfate for signal enhancement and in the presence of H2O2 (0.03%) for 2-10 min. Sections were then mounted on poly L-lysine coated slide (Fisher Scientific, Ottawa, ON, Canada), dehydrated in graded alcohols, cleared in xylene, and coverslipped with Permount. Negative controls consisted of omission of primary or secondary antibody, resulting in loss of immunoreactivity. Sections of rat brain were stained with cresyl

violet for histological evaluation. Neuronal cell numbers were counted in four adjacent boxes (0.06 mm2 each) at a magnification of 400X using an Olympus BX51 microscope and attached Spot RT digital camera (Diagnostics Instruments, Inc., Sterling Heights, MI, USA).

2.5. Real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from rat brains (frontal cortex, posterior medial thalamus and 8 inferior colliculus) using the Trizol reagent (Invitrogen Canada Inc., Burlington, ON, Canada), according to the manufacturer's instructions. Expression levels were assessed by real-time PCR in which cDNA was synthesized using a thermoscript RT-PCR system (Invitrogen). One microgram of RNA was reverse transcripted in a RotorGene 3000t Real time DNA detection system (Corbett Life Science, Sydney, Australia) using the QuantiTect SYBRGreen I PCR kit (Qiagen, Valencia, CA, USA). The PCR program was 95°C 15 min, followed by 35 or 45 cycles (94°C 15 sec, 55°C 30 sec and 72°C 30 sec). Oligonucleotide primer sequences were designed using the Primer 3 software (Rozen and Skaletsky, 2000) based on the following GenBank accession numbers: NM_017215 (GLT-1a), NM_001035233 (GLT-1b), and NM_031144 (β-actin). The specificity of the oligonucleotide primers was verified using the program BLASTN from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). The forward and reverse primers employed were: GLT-1a, 5'-gaccaagacgcagtccattt-3', and 5'-ggctgagaatcgggtcatta-3'; GLT-1b, 5'-aggaatcatgtcacccaagc-3', and 5'-atcttggcccaaagagtgaa-3'; β-actin, 5'-gtcgtaccactggcattgtg-3', and 5'-ctctcagctgtggtggtgaa-3'. Expression levels were normalized to the housekeeping gene β-actin.

2.6. Statistical analysis

Statistical analysis was performed using one way ANOVA with *post-hoc* Friedman's test. A probability of $p < 0.05$ was chosen to establish significance between groups. Data were analyzed using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. General observations

TD in the rat led to neurological changes at the symptomatic stage that included ataxia, opisthotonic episodes and nystagmus, culminating in a loss of righting reflexes. PFC animals at 9 equivalent time points did not show any such neurological changes. Histological evaluation of rats with TD revealed a major loss of neurons in the inferior colliculus at the symptomatic stage (66 ±1%) (Fig. 1). As reported in previous studies from our laboratory, the medial thalamus also showed significant neuronal loss (not shown). In contrast, the frontal cortex showed no obvious changes in neuronal cell numbers. Primary astrocyte cultures exposed to TD showed no apparent changes in cell morphology.

3.2. Effect of ceftriaxone on the expression of GLT-1 splice-variants in TD

Real-time PCR analysis of GLT-1a and GLT-1b mRNA revealed that treatment with ceftriaxone during TD did not increase the expression of either splice-variant in the inferior colliculus, medial thalamus and frontal cortex (data not shown). Interestingly, levels of GLT-1a were in general higher in the frontal cortex than the thalamus and inferior colliculus (Fig. 2).

3.3. Effect of ceftriaxone on protein levels of GLT-1b in TD

Treatment with TD resulted in decreased levels of GLT-1b in the thalamus and inferior colliculus of 58.3 % and 65.8 % respectively ($p < 0.05$), with no change in levels of the splice-variant in the frontal cortex, as determined by immunoblot analysis (Fig. 3). Ceftriaxone co-treatment almost completely prevented the decrease in GLT-1b in the inferior colliculus, restoring transporter levels to 96 % of the normal value (Fig. 3A,B). However, no significant improvement was observed in the thalamus and frontal cortex (Fig. 3C-F), compared to the PFC group. To further confirm the effect of the drug in TD, primary cultures of astrocytes were first treated with

pyrithiamine (20 μM) to induce TD for 10 days followed by co-treatment with ceftriaxone (300 μM) for 5 days. Immunoblotting showed a 33.1 % decrease in GLT-1b in astrocytes with TD ($p < 0.05$), with ceftriaxone treatment completely preventing this loss of the GLT-1b splice-variant (Fig. 4).

Immunohistochemical staining of the TD rat brain showed a decrease in GLT-1b 10 immunoreactivity in thalamus and inferior colliculus (Fig. 5). However, the effect was reduced in the ceftriaxone-treated TD group, consistent with a ceftriaxone-dependent regulation of this splice-variant.

4. Discussion

Ceftriaxone belongs to the family of β-lactam antibiotics that has been described as a potent positive regulator of the glutamate transporter GLT-1 in amyotropic lateral sclerosis (Rothestein et al., 2005), Huntington disease (Miller et al., 2008), hypobaric hypoxia (Hota et al., 2008), and stroke (Lipski et al., 2007). In the present study, we investigated the effect of ceftriaxone in TD. Previous studies indicate that excitotoxicity is a major pathophysiological event in TD characterized by increased extracellular glutamate concentration (Hazell et al., 1993), increased depolarization (Hazell et al., 1998), and profound loss of astrocytic glutamate transporters in both TD (Hazell et al., 2001) and Wernicke's encephalopathy (Hazell et al., 2010).

Our findings indicate that ceftriaxone upregulates GLT-1 protein levels in the inferior colliculus in TD rats and in an *in vitro* astrocyte model of the disorder. The reason for the selective effect on the inferior colliculus is unclear but previous studies have reported differences in the pathology of the thalamus and inferior colliculus due to TD (Vortmeyer and Colmant, 1988). Thus, the focal response of this brain region may be related to differences in the underlying

pathophysiologic mechanisms between the colliculus and thalamus. Since ceftriaxone is reported to enhance GLT-1b levels via NFkB-linked stimulation of the GLT-1 promotor, such regional differences in the response to this antibiotic may be mediated via this mechanism. We are currently investigating this possibility in more detail.

While ceftriaxone treatment upregulated GLT-1b transporter protein levels in the inferor 11 colliculus, no changes in mRNA expression of either GLT-1a or GLT-1b were observed due to TD conditions. Since TD produces a downregulation of GLT-1 in vulnerable brain regions, this suggests that post-transcriptional changes are involved in this process, and are selectively responsive to ceftriaxone in the inferior colliculus.

Co-treatment of astrocyte cultures with ceftriaxone completely protected against the decrease in GLT-1b protein occurring due to TD. Loss of the GLAST transporter in astrocytes exposed to TD conditions was earlier reported by us (Hazell et al., 2003); this finding of decreased levels of a GLT-1 splice-variant in TD is to our knowledge the first to be described. The ability of ceftriax one to prevent loss of GLT-1b in TD under *in vitro* conditions should provide a means by which to further investigate the mechanism(s) underlying this process in the future. Interestingly, ceftriaxone also upregulates the catalytic subunit of the cystine-glutamate exchanger (Knackstedt et al., 2010). Cystine, a dimeric amino acid formed by the oxidation of two cysteine residues, is a provider of cysteine in the cell which is required for synthesis of glutathione, an important naturally occurring endogenous antioxidant in astrocytes, and upregulation of the cystine-glutamate exchanger is likely to result in increased entry of cystine, and ultimately increased protection against oxidative stress for these cells (Shih et al., 2006). Importantly, decreased cystine levels may lead to glutathione depletion and cell death (Lewerenz et al*.,* 2009). Recently, we demonstrated that co-treatment of rats with TD and *N*-acetylcysteine, a well-established

antioxidant, protected against loss of GLT-1 and neuronal cell death in the vulnerable medial thalamus (Hazell et al., 2010). Thus, ceftriaxone may positively regulate GLT-1b in the inferior colliculus and in cultured astrocytes via an oxidative-stress-related mechanism, although why this effect is not apparent in the thalamus remains unclear, suggesting a combination of pathophysiologic processes may be involved.

In conclusion, the findings of this study indicate that the β-lactam antibiotic ceftriaxone 12 exerts a protective influence on GLT-1 protein levels under conditions of TD. Excitotoxicity is a major consequence of TD, and the present results suggest that ceftriaxone treatment may be beneficial in minimising the associated pathology.

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REFERENCES

Allen, N.J., Káradóttir, R., Attwell, D., 2004. Reversal or reduction of glutamate and GABA transport in CNS pathology and therapy. Pflugers Arch. 449, 132-142.

Anderson, C.M., Swanson, R.A., 2000. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32, 1-14.

Arriza, J.L., Eliasof, S., Kavanaugh, M.P., Amara, S.G., 1997. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc. Natl. Acad. Sci. 94, 4155-4160.

Carvalho, F.M., Pereira, S.R., Pires, R.G., Ferraz, V.P., Romano-Silva, M.A., Oliveira-Silva, I.F., Ribeiro, A.M., 2006. Thiamine deficiency decreases glutamate uptake in the prefrontal cortex and impairs spatial memory performance in a water maze test. Pharmacol. Biochem. Behav. 83, 481-489.

Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1-105.

Danbolt, N.C., Storm-Mathisen, J., Kanner, B.I., 1992. An [Na+ + K+]coupled L-glutamate transporter purified from rat brain is located in glial cell processes. Neuroscience 51, 295-310.

Fairman, W.A., Vandenberg, R.J., Arriza, J.L., Kavanaugh, M.P., Amara, S.G., 1995. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. 375, 599-603.

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, 1155-1158.

Hazell, A.S., Itzhak, Y., Liu, H., Norenberg, M.D., 1997. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) decreases glutamate uptake in cultured astrocytes. J. Neurochem. 68, 2216-2219.

Hazell, A.S., Hakim, A.M., Senterman, M.K., Hogan, M.J., 1998. Regional activation of L-type voltage-sensitive calcium channels in experimental thiamine deficiency. J. Neurosci. Res. 52, 742-749.

Hazell, A.S., Rao, K.V., Danbolt, N.C., Pow, D.V., Butterworth, R.F., 2001. Selective downregulation of the astrocyte glutamate transporters GLT-1 and GLAST within the medial thalamus in experimental Wernicke's encephalopathy. J. Neurochem. 78, 560-568.

Hazell, A.S., Pannunzio, P., Rama Rao, K.V., Pow, D.V., Rambaldi, A., 2003. Thiamine deficiencyresults in downregulation of the GLAST glutamate transporter in cultured astrocytes. Glia 43, 175-184.

Hazell, A.S., Sheedy, D., Oanea, R., Aghourian, M., Sun, S., Jung, J.Y., Wang, D., Wang, C., 2010. Loss of astrocytic glutamate transporters in Wernicke encephalopathy. Glia 58, 148-156.

Héroux, M, Raghavendra Rao, V.L., Lavoie, J., Richardson, J.S., Butterworth, R.F., 1996. Alterations of thiamine phosphorylation and of thiamine-dependent enzymes in Alzheimer's disease. Metab. Brain Dis. 11, 81-88.

Hota, S.K., Barhwal, K., Ray, K., Singh, S.B., Ilavazhagan, G., 2008. Ceftriaxone rescues hippocampal neurons from excitotoxicity and enhances memory retrieval in chronic hypobaric hypoxia. Neurobiol. Learn. Mem. 89522-89532.

Kanai, Y., Hediger, M.A., 1992. Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360, 467-471.

Knackstedt, L.A., Melendez, R.I., Kalivas, P.W., 2010. Ceftriaxone restores glutamate homeostasis and prevents relapse to cocaine seeking. Biol. Psychiatry 67, 81-84.

Langlais, P.J., Zhang, S.X., 1993. Extracellular glutamate is increased in thalamus during thiamine deficiency-induced lesions and is blocked by MK-801. J. Neurochem. 61, 2175-2182.

Lee, S.G., Su, Z.Z., Emdad, L., Gupta, P., Sarkar, D., Borjabad, A., Volsky, D.J., Fisher, P.B., 2008.Mechanism of ceftriaxone induction of excitatory amino acid transporter-2 expression and glutamate uptake in primary human astrocytes. J. Biol. Chem. 283, 13116-13123.

Lehre, K.P., Danbolt, N.C., 1998. The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. J. Neurosci. 18, 8751-8757.

Lehre, K.P., Levy, L.M., Ottersen, O.P., Storm-Mathisen, J., Danbolt, N.C., 1995. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J. Neurosci. 15, 1835-1853.

Lewerenz, J., Albrecht, P., Tien, M.L., Henke, N., Karumbayaram, S., Kornblum, H.I., Wiedau- Pazos, M., Schubert, D., Maher, P., Methner, A., 2009. Induction of Nrf2 and xCT are involved in the action of the neuroprotective antibiotic ceftriaxone in vitro. J. Neurochem. 111, 332-343.

Lipski, J., Wan, C.K., Bai, J.Z., Pi, R., Li, D., Donnelly, D., 2007. Neuroprotective potential of ceftriaxone in in vitro models of stroke. Neuroscience 146, 617-629.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Maragakis, N.J., Rothstein, J.D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol Dis. 15, 461-473.

Miller, B.R., Dorner, J.L., Shou, M., Sari, Y., Barton, S.J., Sengelaub, D.R., Kennedy, R.T., Rebec, G.V., 2008. Up-regulation of GLT1 expression increases glutamate uptake and attenuates the Huntington's disease phenotype in the R6/2 mouse. Neuroscience 153, 329-337.

Olney, J.W., Collins, R.C., Sloviter, R.S., 1986. Excitotoxic mechanisms of epileptic brain damage. Adv. Neurol. 44, 857-877.

Palmer, A.M., Marion, D.W., Botscheller, M.L., Swedlow, P.E., Styren, S.D., DeKosky, S.T., 1993. Traumatic brain injury-induced excitotoxicity assessed in a controlled cortical impact model. J. Neurochem. 61, 2015-2024.

Paxinos, G., Watson, C., 1998. The Rat Brain in Stereotaxic Coordinates, fourth ed. Academic Press, New York.

Pines, G., Danbolt, N.C., Bjørås, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E., Kanner, B.I., 1992. Cloning and expression of a rat brain L-glutamate transporter. Nature 360, 464-467.

Rao, V.L., Bowen, K.K., Dempsey, R.J., 2001. Transient focal cerebral ischemia down-regulates glutamate transporters GLT-1 and EAAC1 expression in rat brain. Neurochem. Res. 26, 497-502.

Rothstein, J.D., 1996. Excitotoxicity hypothesis. Neurology 47, 19-25.

Rothstein, J.D., Martin, L., Levey, A.I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N., Kuncl, RW., 1994. Localization of neuronal and glial glutamate transporters. Neuron. 13, 713-725.

Rothstein, J.D., Patel, S., Regan, M.R., Haenggeli, C., Huang, Y.H., Bergles, D.E., Jin, L., Dykes Hoberg, M., Vidensky, S., Chung, D.S., Toan, S.V., Bruijn, L.I., Su, Z.Z., Gupta, P., Fisher, P.B., 2005. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433, 73-77.

Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A.,Wang, Y., Schielke, J.P.,Welty, D.F., 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16, 675–686.

Sattler, R., Rothstein, J.D., 2006. Regulation and dysregulation of glutamate transporters. Handb. Exp. Pharmacol. 175, 277-303.

Selkirk, J.V., Stiefel, T.H., Stone, I.M., Naeve, G.S., Foster, A.C., Poulsen, D.J., 2005. Overexpression of the human EAAT2 glutamate transporter within neurons of mouse organotypic hippocampal slice cultures leads to increased vulnerability of CA1 pyramidal cells. Eur. J. Neurosci. 21, 2291-2296.

Shih, A.Y., Erb, H., Sun, X., Toda, S., Kalivas, P.W., Murphy, T.H., 2006. Cystine/glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation. J. Neurosci. 26, 10514-10523.

Storck, T., Schulte, S., Hofmann, K., Stoffel, W., 1992. Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain. Proc. Natl. Acad. Sci. 89, 10955-10959.

Vortmeyer AO, Colmant H.J. 1988. Differentiation between brain lesions in experimental thiamine deficiency. Virchows Arch A Pathol Anat Histopathol. 414(1):61-7.

Younes-Mhenni, S., Derex, L., Berruyer, M., Nighoghossian, N., Philippeau, F., Salzmann, M., Trouillas, P., 2004. Large-artery stroke in a young patient with Crohn's disease. Role of vitamin B6 deficiency-induced hyperhomocysteinemia. J. Neurol. Sci. 221, 113-115.

FIGURE LEGENDS

Figure 1. Histological analysis of the inferior colliculus from pair-fed control (A, C) and acute symptomatic thiamine-deficient (TD) rats (B, D). Photomicrographs show cresyl violet staining of brain sections. Panels C and D display higher magnification images of the boxed areas shown in A and B respectively. Scale bars: 400 μm (A,B); 200 μm (C,D).

Figure 2. Real-time PCR analysis of the splice-variant glutamate transporter GLT-1a in the frontal cortex (FC), medial thalamus (MT), and inferior colliculus (IC) of PFC, TD, and TD rats co-treated with ceftriaxone (C). *$p < 0.05$ compared to PFC group (one-way ANOVA with *posthoc* Friedman's test for multiple comparisons).

Figure 3. Effect of ceftriaxone on GLT-1b in the rat brain in thiamine deficiency. The inferior colliculus, medial thalamus and frontal cortex were examined in pair-fed controls (PFC), acute symptomatic TD and ceftriaxone-treated TD rats (TD-CEF) for protein levels of the glutamate transporters GLT-1b by immunoblotting. Results show representative blots of each brain region and their quantitative analysis. Levels of GLT-1b were decreased in the inferior colliculus (A,C) and thalamus (B,D) but not in the frontal cortex of TD animals. Co-treatment with ceftriaxone completely prevented the loss of GLT-1b in inferior colliculus but not in the medial thalamus. *$p < 0.05$ compared to PFC group (one-way ANOVA with *post-hoc* Friedman's test for multiple comparisons).

Figure 4. Effect of ceftriaxone on GLT 1b protein levels in astrocyte cultures treated with TD.

A) Representative immunoblot for control, thiamine deficient (TD) and ceftriaxone-treated TD astrocyte culture (TD+CEF). B) Immunoblot analysis shows that TD resulted in a downregulation of GLT-1b that was prevented by ceftriaxone. *$p < 0.05$ compared to PFC group (one-way ANOVA with *post-hoc* Friedman's test for multiple comparisons).

Figure 5. Immunohistochemical staining of the glutamate transporter splice-variant GLT-1b in the inferior colliculus of representative PFC (A,B), TD rats (C,D), and ceftriaxone-treated TD rats (E,F). Photomicrographs show decreased GLT-1b immunoreactivity in TD animals compared to PFC and an amelioration of this downregulation in ceftriaxone-treated animals. Panels B,D,F show higher power images of sections shown in A,C,E respectively. Scale bars: 300 μm (A,C,E); 150 μm (B,D,F)

Figure 1

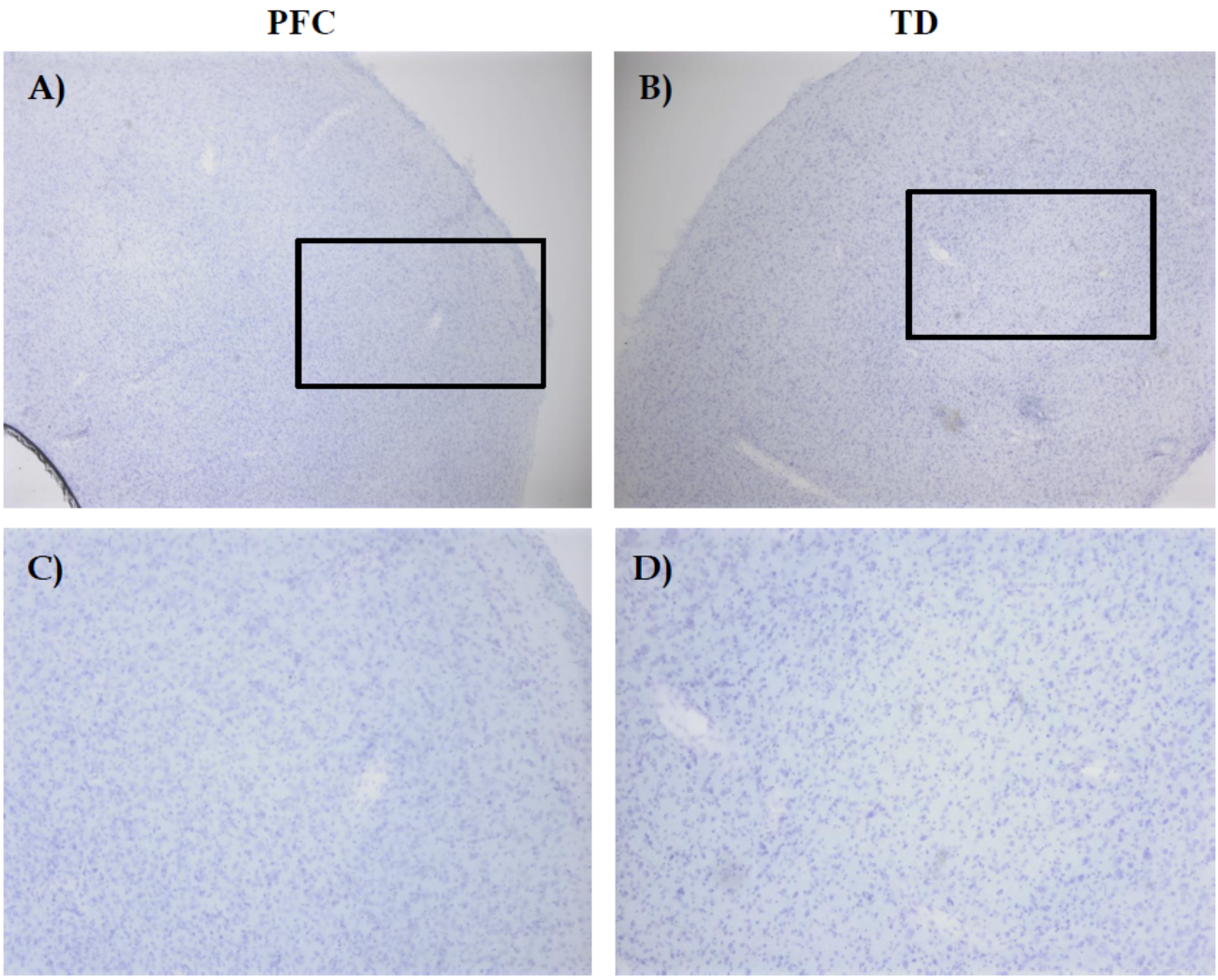

Figure 1. Jhala et.al., 2011

Figure 2

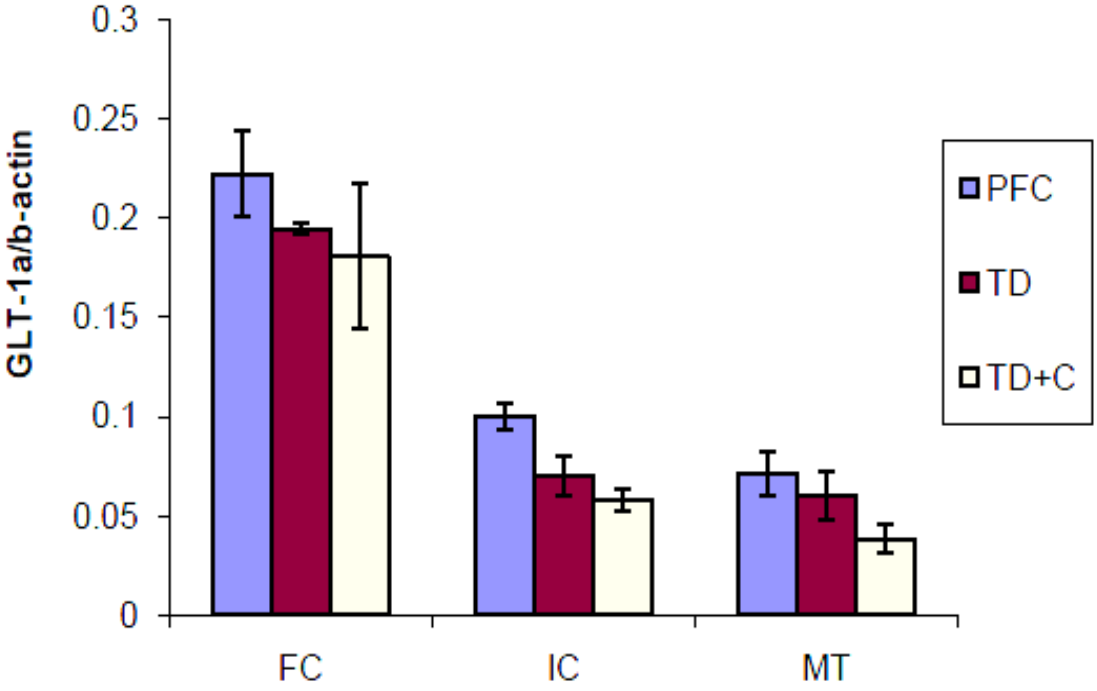

Figure 2 Jhala et.al., 2011

Figure 3(i)

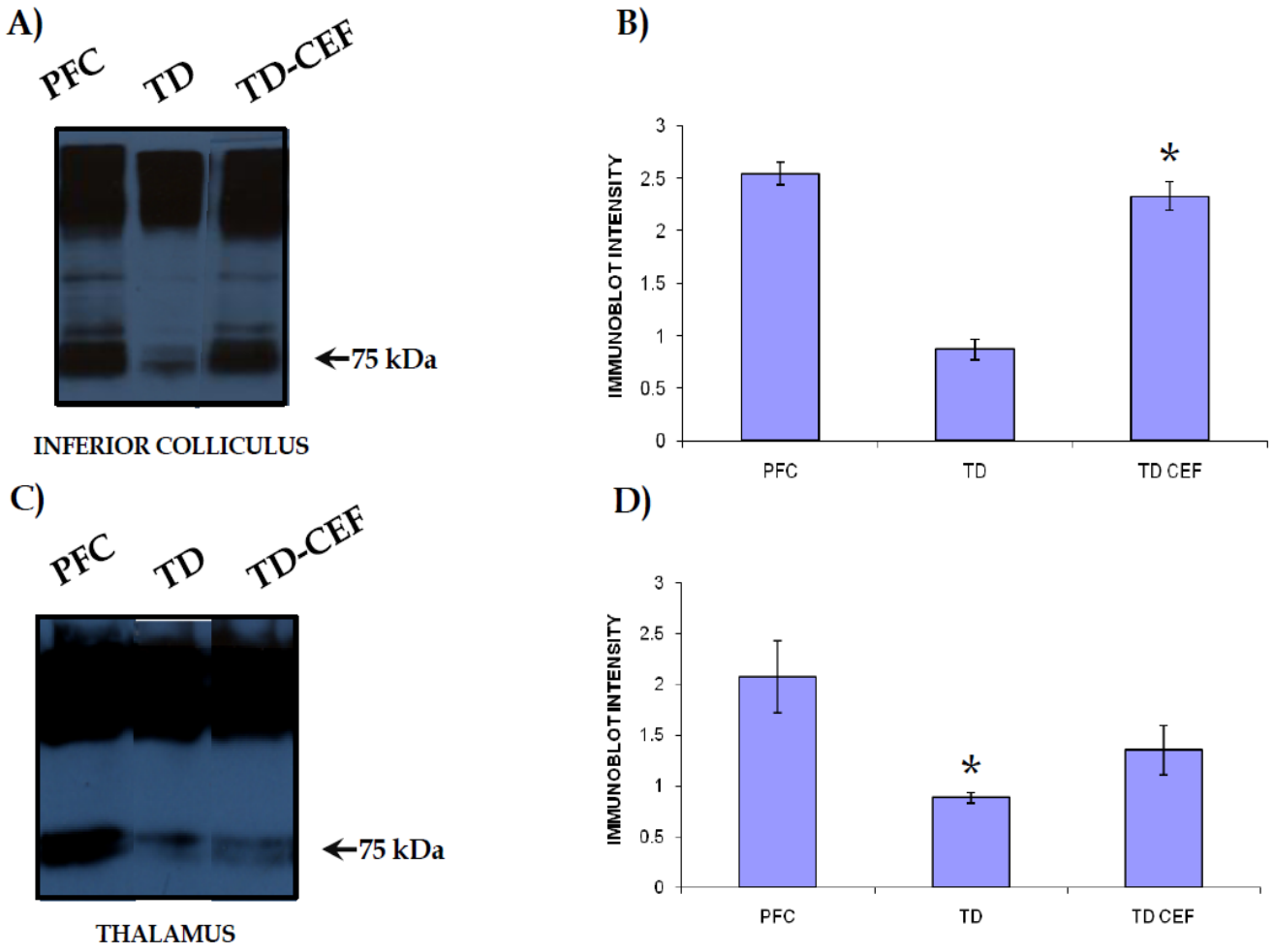

Figure 3(i) Jhala et.al., 2011

Figure 3(ii)

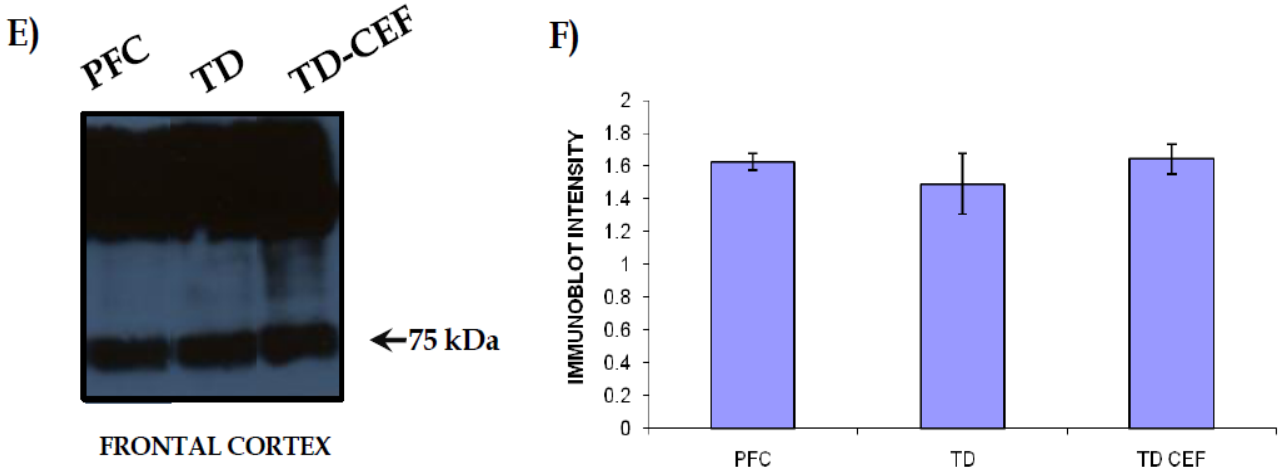

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Figure 4

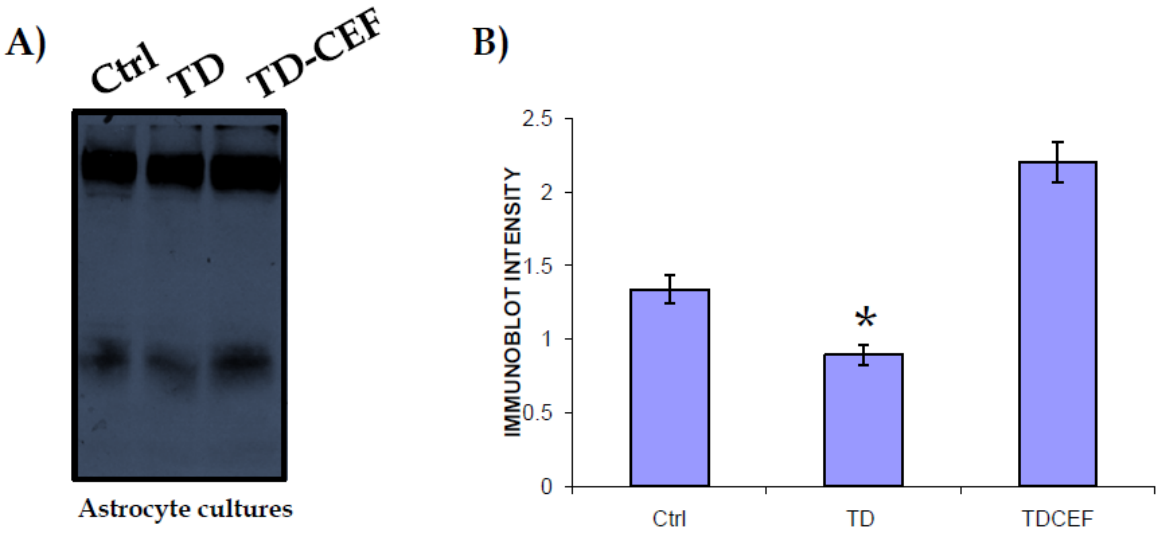

Figure 4 Jhala et.al., 2011

Figure 5

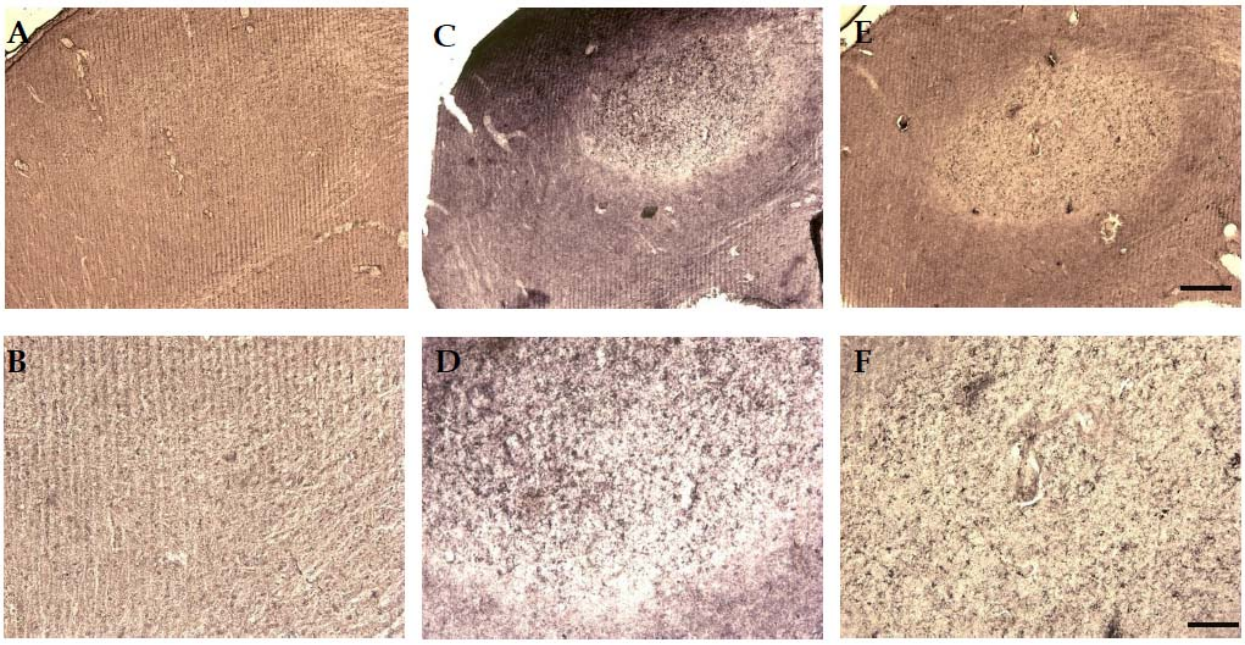

Figure 5 Jhala et.al., 2011

CHAPTER 3

Astrocytic PARP-1 regulates glutamate transporter levels via NFκB in thiamine deficiency

Glia: Submitted (2013)

ASTROCYTIC PARP-1 REGULATES GLUTAMATE TRANSPORTER LEVELS VIA NFκB IN THIAMINE DEFICIENCY

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ABSTRACT

Downregulation of the astrocytic glutamate transporters GLT-1 and GLAST and ensuing excitotoxic damage are important features of the pathophysiology of thiamine deficiency (TD), the cause of Wernicke's encephalopathy. Exactly how the levels of these transporters are altered in this disorder, however, is unknown. Poly (ADP) ribose polymerase-1 (PARP-1), an important enzyme of poly (ADP)-ribosylation with a key role in the regulation of transcription and the development of excitotoxicity is associated with the pathogenesis of various disorders of the CNS. In addition, nuclear factor (NF)-kB has previously been linked to the regulation of GLT-1, and PARP-1 is required and sufficient for specific transcriptional activation of NF-kB. To better understand the relationship between PARP-1 and the regulation of glutamate transporters in TD, we treated primary cultures of astrocytes with TD. Using a combination of immunoblotting, flow cytometry, and immunocytochemistry, we show that cells treated with TD displayed a profound increase in the 50 kDa cleaved fragment of PARP-1 and IkB phosphorylation suggestive of NF-kB activation and nuclear translocation. These changes occurred in association with a decrease in levels of the splice-variant GLT-1b and were also apparent in a well-established rat model of TD. Using specific PARP-1 and NF-kB inhibitors, we demonstrate a strong likelihood that excessive PARP activation due to impaired energy status or oxidative insult as a consequence of TD promotes NF-κB-dependent downregulation of GLT-1b. Our findings indicate that PARP-1 activation and NF-kB play an important role in the loss of astrocytic glutamate transporters in TD.

1. INTRODUCTION

Thiamine is an essential cofactor for the enzymes involved in cellular energy metabolism and thus mitochondrial dysfunction and its associated cerebral energy depletion are major consequences of thiamine deficiency (TD) (Pannunzio *et al.,* 2000; Jhala and Hazell, 2011). Bioenergetic depletion, excessive glutamate accumulation, astrocytic dysfunction and downregulation of glutamate transporters as a consequence of TD have previously been reported (Aikawa et al., 1984; Hazell et al., 1993, 2003; Jhala *et. al.,* 2011), and are key features of this disorder.

In recent studies, poly (ADP) ribose polymerase-1 (PARP-1), an important enzyme of poly(ADP) -ribosylation has been linked to the pathogenesis of various disorders of the CNS, including excitotoxicity and ischemic injury (Meng *et. al.,* 2000; Ying *et. al.,* 2001; Tang *et. al.,* 2010). It is a nuclear chromatin-associated protein which catalyzes the synthesis of large branched polymers of ADP-ribose units by using β-nicotinamide adenine dinucleotide (NAD^+) as substrate (Hassa and Hottiger, 1999). Reduction in neuronal necrosis, improvement in neurological status, protection against white-matter damage and AIF translocation has been observed following pharmacological inhibition of PARP-1 enzyme and in PARP-1 deficient animal models of stroke, traumatic brain injury and Parkinson disease. (Eliasson, 1997; Zingarelli *et. al.,* 1998; Virag, and Szabo, 2002).

An important physiological role of PARP is its ability to regulate transcription. For example, PARP-1 is required and sufficient for specific transcriptional activation of NF-kB in response to pro-inflammatory stimuli and genotoxic stress (Hassa and Hottiger, 1999; Ha et. al., 2002). Thus, expression of several inflammatory response genes such as TNF-α, IL-6, IFNy, VCAM, ICAM, P-Selectin and iNOS were shown to be drastically reduced (60-90%) in PARP-1 deficient mice after treatment with inflammatory stimuli such as LPS or streptozotocin (Shall and Murcia, 2000). It is

therefore possible that PARP-1 along with other inflammatory precursors can synergistically co-activate NF-kB in response to any oxidative injury. However, poly (ADP) ribosylation affects neither the DNA binding activity of NF-kB nor required for NF-kB dependent gene expression (Hassa and Hottiger, 1999; 2002). In brain, poly (ADP) ribosylation and its associated PARP-1 enzyme activation and/or cleavage has been implicated in neural cell loss under *in vitro* and *in vivo* conditions (Eliasson, 1997; Meng *et. al.,* 2000; Ha *et. al.,* 2002).

The cause of PARP activation in brain is associated with metabolic stress and mitochondrial dysfunction, important features of TD, and it has been suggested that PARP activation under these conditions can exacerbate the extent of damage associated with the disorders (Trushina and McMurray, 2007). Previous literature indicates that astrocytes are more vulnerable to ribosylation or PARP activation than neurons (Ha *et. al.,* 2002; Tang *et. al.,* 2010). Given that astrocytes appear to be targeted in TD (Hazell, 2009; Hazell et al., 2010), in the present study we investigated a potential linkage between glutamate signalling and NF-kB activation in relation to poly (ADP) ribosylation in both astrocyte cultures exposed to TD and in rats treated with TD.

2. Experimental procedure

2.1. Rat model of TD

All the experimental procedures were approved by the Animal Ethics Committee of Saint-Luc Hospital (CHUM) and the University of Montreal. Male Sprague–Dawley rats (Charles River, St-Constant, QC) were used for all experiments. Animals were housed individually under constant conditions of temperature, humidity and 12 h light/dark cycles. Thiamine deficiency was produced by administering pyrithiamine hydrobromide as previously described (Hazell *et al.,* 1998a). Briefly, rats were acclimatized for at least 2 days before the treatments and behavioural assessment of the

animals preceding neurological signs of TD (ataxia, opisthotonus, loss of righting reflexes, convulsions, nystagmus) were made on a daily basis. Rat in the TD group (n = 9) were fed a thiamine-deficient diet (ICN Nutritional Biochemicals, Cleveland, OH) and administered daily pyrithiamine (0.5 mg/kg body weight) with or without PDTC (20 mg/kg body weight) (Sigma-Aldrich, St Louis, MO) intraperitoneally. Control rats (n = 9) were pair-fed to equal food consumption with the thiamine deficient rat using the same thiamine-deficient diet and supplemented with daily i.p. injections of thiamine (0.1 mg per kg body weight). Treatment was continued until the symptomatic stage (loss of righting reflex). Any rat exhibiting spontaneous seizures were removed from the study.

2.2. Cell Culture Preparation

Primary astrocyte cultures from newborn rats were prepared using a modification of the method of Booher and Sensenbrenner (1972). Briefly, cerebral cortices were removed and the tissue was dissociated, passed through sterile nylon sieves, and then suspended in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Burlington, ON) containing 10% fetal calf serum. Cells were then seeded in 35 and 100 mm culture dishes, which were maintained in an incubator at 37°C provided with a mixture of 5% CO2 and 95% air. Cultures were grown for 3–5 weeks, during which the medium was changed twice a week. To induce TD, cells were exposed to a custom-designed DMEM media lacking in thiamine (Invitrogen, Burlington, ON) and containing 5% horse serum, in the presence of pyrithiamine. Control astrocytes were treated with TD media in which normal levels of thiamine (11 μM) had been added. Astrocyte cultures treated with TD were administered inhibitors against PARP (DPQ, 25uM) and NF-kB (PDTC, 50uM) for 3 days. Growth media was changed regularly every 3-4 days, depending on treatment conditions. All culture plates for each

experiment were harvested on the same day.

2.3. Flow cytometry

Astrocytes were harvested in papain solution (Worthington Biochemical Corp., Lakewood, NJ) for 15-20 min and resuspended in flow cytometry buffer, consisting of 1X PBS, pH 7.2, 1% fetal bovine serum (Invitrogen, Burlington, ON). Cells were counted and diluted to a density of $1x10^6$ cells per milliliter of buffer. Aliquotes of 1×10^5 cell were incubated with polyclonal antisera directed against PARP-1 (Cell Signalling Technology, Inc., Danvers, MA), and GFAP (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), or GLT-1b (1:500) and phospho-IkB (1:1000, Cell Signalling Technology, Inc., Danvers, MA) for 30 min on ice. Cells were washed in buffer, and then specific Alexa Fluor-592 conjugated IgG and Alexa Fluor-488 conjugated IgG secondary fluorescent-conjugated antibodies (Invitrogen, Burlington, ON) added at the appropriate dilution (1:100) and incubated on ice for 15 minutes. Viable cells were gated by light scatter and were analyzed by FACScan (BD LSRII) (BD Biosciences, Mississauga, ON) using a Cell Quest program (BD Biosciences, Mississauga, ON). Fluorescence background was measured using unlabeled cells and cells labeled with secondary antibody alone; these set gating parameters between positive and negative cell populations. Cell aggregates and small debris were excluded from analysis or isolation on the basis of side scatter (measuring cell granularity) and forward scatter (measuring cell size). Fluorescent intensities for cells in the population were plotted as quadrants and/or histograms using CellQuest software (BD Biosciences, Mississauga, ON). All statistical calculations were done using GraphPad Prism (GraphPad, software, Inc. La Jolla, CA).

2.4. Glutamate uptake

Glutamate uptake study was done as described according to Hazell et al. (1997) with some modifications. Briefly, cells were incubated in DMEM media containing the glutamate analogue D-aspartate and 0.2 μCi/ml of [^{3}H]-D-aspartate in 5% CO_2 /95% air at 37°C, incubation time for all experiments was 2 min. Uptake was stopped by aspiration of the media and rapid washing of cells three times with ice-cold PBS. Cells were then harvested with 0.5 ml of 1 M NaOH. Sample aliquots were measured for protein content (Lowry et al., 1951) and the radioactivity for calculation of the uptake rate was measured by liquid scintillation counting.

2.5. Immunocytochemistry

Media was aspirated and cells were washed for three times in PBS. Cells were then fixed for 10 min with 10% neutral buffered formalin. After 10 min incubation cells were again washed in PBS (3 x 10 min), blocked for 20 min with 5% donkey serum in PBS, and then incubated with 5% donkey serum and polyclonal mouse antisera directed against GFAP (1: 250, Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal rabbit antisera directed against PARP-1 (1:250, Cell Signalling Technology, Inc., Danvers, MA) for overnight at 4°C. Cells were then washed (3 x 10 min) and incubated for 1 h with Alexa Fluor-488 (green) and Alexa Fluor-592 (red) secondary antibodies (1:200), then mounted in Prolong Gold AntiFade reagent and examined using an Olympus BX51 microscope and attached Spot RT digital camera. Negative controls consisted of omission of primary or secondary antibody, resulting in loss of immunoreactivity. Images were processed using Image-Pro Plus 6.2 image analysis software (Media Cybernetics, Inc., Bethesda, MD, USA).

2.6. Immunoblotting

Cells were harvested in RIPA buffer containing 50 mM Tris,150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate (pH 8.0), and protease inhibitor cocktail and centrifuged at 10,000 g for 10 min at 4°C. Protein content of all samples was determined by methods of Lowry and colleagues (Lowry et al., 1951) using bovine serum albumin as the standard. Aliquots were subjected to (SDS)-polyacrylamide gel electrophoresis (8% polyacrylamide) and the proteins subsequently transferred to PVDF membranes by wet transfer at 20V over 24 h. The transfer buffer consisted of 48 mM Tris (pH 8.3), 39 mM glycine, 0.037% SDS, and 20% methanol. Membranes were subsequently incubated in blocking buffer (10 mM Tris, 100 mM NaCl, 5% non fat dried milk) followed by incubations with rabbit polyclonal antisera directed against GLT-1b (1:1000), Phospho-IkB (1:1000), PARP-1 (1:1000) overnight at 4°C. Reblocking was followed by incubation with HRP-coupled anti-rabbit IgG (0.01 μg/mL) secondary antiserum. Each incubation step was of 1h duration following which blots were washed several times with buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20). For the detection of specific antibody binding, the membranes were treated in accordance with the ECL-kit instructions and developed on a photosensitive X-OMAT film. Signal intensities were subsequently measured by densitometry using an Image J (Image processing and analysis system, NIHM, USA). Linearity of the relationship between optical density and protein concentration was verified using appropriate standard curves. Blots were reversibly stained with Ponceau-S to monitor protein transfer efficiency and equal loading.

2.7. Statistical analysis

Statistical analysis was performed using Man-Whitney test for two group comparison with

one-way ANOVA being used for multiple comparisons. A probability of $p < 0.05$ was chosen to establish significance between groups. Data were analyzed using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean ± S.E.M. values.

3. RESULTS

3.1. Increased PARP-1 in TD

PARP-1 is specifically proteolysed by caspases to a 24 kDa DNA-binding domain and to a 89 kDa catalytic fragment during execution of the apoptotic program. Significant DNA damage associated with severe energy depletion may initiate a necrosis with a cleavage at 50 kDa, which is believed to prevent execution of the apoptotic program (Eguchi *et al.,* 1997; Leist *et al.,* 1997). Cultured astrocytes exposed to TD displayed an increased PARP-1 immunoreactivity (Fig. 1). This pattern of cleavage of PARP-1 was also observed using immunoblot analysis, indicating atypical cleavage with a band intensity at approximately 50 kDa (Fig. 2A). Astrocytes under TD conditions treated with the PARP inhibitor DPQ (25μM) or exogenous NAD^+ (1mM) blocked this PARP-1 activation and cleavage (Fig. 2A). Treatment of rats with TD resulted in large increases in the 24 kD fragment of PARP-1 in the vulnerable thalamus and inferior colliculus (Fig. 2B). In contrast, no significant change was observed in the non-vulnerable frontal cortex compared to control animals. Co-treatment of TD animals with PDTC resulted in no significant improvement in PARP-1 levels relative to PFC animals.

Figure 3 shows flow cytometry analysis of PARP-1 in TD astrocyte cultures. Treatment of astrocytes with TD conditions produced an almost 4-fold increase in PARP-1 levels compared to control astrocytes. In the presence of DPQ and NAD^+, increased PARP-1 levels were attenuated by 61% and 30%, respectively. No significant change was observed in the levels of PARP-1 after

PDTC administration.

3.2. Regulation of NF-κB by PARP-1 in cultured astrocytes with TD

PARP is involved in the regulation of transcription of several genes by interaction with specific transcription factors (Qu *et. al.,* 1994; Meisterernst *et. al.,* 1997; Rawling *et. al.,* 1997; JM *et. al.,* 1997). To investigate the relationship between PARP and NF-κB during TD, we examined NF-κB activation in TD astrocyte cultures treated with or without the PARP-1 specific inhibitor DPQ. Transcription factor- NF-κB is located in the cytosol as an inactive complex p50/p65(Rel A)/IκB. The NF-κB complex is activated by dissociation of IκB through several signaling pathways and it involves phophorylation of IκB (Ghosh *et. al.,* 1998). Using immunoblotting and flow cytometry we analysed the levels of phospho-IκB in astrocytes treated with TD. Immunoblotting showed that treatment with TD conditions dramatically induced IκB levels in these cells (Fig. 4), while co-treatment with the PARP-1 inhibitor DPQ (25 μM) completely blocked this effect, indicating that NF-κB activation is responsive to PARP-1. In contrast, NAD^+ treatment showed no effect on the levels of phospho-IκB.

3.3. Regulation of GLT-1 by NF-κB in TD

Downregulation of astrocytic glutamate transporters during TD has been reported in our lab previously (Hazell *et. al.,* 2003, 2010). However, the mechanism(s) of this downregulation remains unknown. Studies using pharmacological and genetic inhibitors suggest that these effects can depend on the signaling pathways through PI3K and NF-κB mechanisms (Ghosh *et. al.,* 2011). Figure 5A,B shows flow cytometry analysis of GLT-1b in TD astrocyte cultures. Cells treated with TD showed a reduction in GLT-1b levels of 54.7% compared to the control group. This downregulation of GLT-1b protein was blocked in the presence of (DPQ, 25μM) and/or NF-κB inhibitors (PDTC: 50 μM)

and NAD^+(1mM), suggesting that GLT-1b levels are dependent on both NF-κB and PARP-1. In addition, increased levels of phospho-IκB were observed under conditions of TD which were partially blocked in the presence of DPQ and PDTC (Fig. 5A,C). However, NAD^+ treatment showed no effect on the increased levels of phospho-IkB in these TD treated cells..

3.4. Increased PARP activation inhibits glutamate uptake in astrocytes with TD

Measurement of glutamate transport in astrocytes co-treated with TD and pharmacological specific inhibitors for PARP and NF-κB indicated an impairment of glutamate uptake. Treatment with DPQ restored the glutamate uptake capacity of TD astrocytes and this effect was also observed after NF-κB inhibition. (Fig. 7).

4. DISCUSSION

TD results in mitochondrial dysfunction and oxidative stress with major impairment in the production of high energy phosphates and nucleotides (Jhala and Hazell, 2011). This increase in oxidative stress and onset of an excitotoxic environment can lead to activation of PARP-1 which catalyses the synthesis of polyADP-ribose attached to protein acceptors both in neurons and astrocytes. The use of PARP inhibitors has therefore been proposed as a protective therapy in decreasing excitotoxic neuronal cell death (Eliasson *et al.,* 1994; Zhang *et al.,* 1994; Ha and Snyder, 2000). In the present study using pharmacological specific inhibitors for NF-κB and PARP; we have investigated the relationship between poly (ADP) ribosylation and NF-κB and its role in the regulation of glutamate transporters, specifically the GLT-1b splice-variant under conditions of TD.

Our results suggest that TD produces increased activation of PARP, resulting in the activation of NF-κB. The relationship between excessive PARP activation and IκB phosphorylation

as a function of NF-κB activation was studied using PARP inhibitor (DPQ) treatment. Our findings show that this PARP inhibitor treatment completely blocked the activation of PARP and inhibited NF-κB activation in astrocyte cultures. This decreased NF-κB activity under conditions of TD in astrocytes also promoted GLT-1b protein expression and increased the overall glutamate uptake capacity. In addition, both PARP and NF-κB inhibition were found to promote glutamate transporter activity and expression of GLT-1b. Overall, our findings suggest that excessive PARP activity induces NF-κB activation leading to its translocation to the nucleus, and this activated NF-κB complex then leads to the downregulation of glutamate transporters during TD.

NF-κB is primarily a transcription activator (Baldwin, 1996) and has been shown to promote the transcription of many genes including glutamate transporters (Sitcheran *et al.,* 2005; Ghosh *et. al.,* 2011). However, there are specific examples where it can also act as a transcriptional repressor (Gires *et al.,* 2001; Ke *et al.,* 2001). Thus, our current findings suggest that under conditions of TD, PARP-1 overactivation regulates NF-κB-dependent modulation of glutamate transporter GLT-1b in astrocytes.

PARP-1 was one of the first identified substrates of caspases and thus most of the studies on PARP-1 and cell death were mostly focused on its possible role in apoptosis (Alnemri *et. al.,* 1996; Schreiber *et. al.,* 2006). In apoptosis, caspase 7 and caspase 3 cleave PARP1 into two fragments: 89kDa and 24kDa. The cleavage of PARP1 separates its DNA binding domain from its catalytic domain, which inactivates the enzyme. Interestingly, PARP-1 cleavage occurs differently in necrosis from that observed during apoptosis, generating major fragments at 89kDa and 50 kDa and minor fragments at 40kDa and 35 kDa (Shah *et. al.,* 1996). However, PARP-1 cleavage is more appropriately considered as a marker, rather than an executor of apoptosis (Herceg and Wang, 1999).

It is well known that NF-κB-related protein family is located in cytosol as an inactive

complex (p50/p65(Rel A)/IκB) (Ghosh *et al.,* 1998) and the complex is activated by dissociation of IκB through several signalling pathways which involve IκB phosphorylation, and then the activated NF-κB complex is targeted to the nucleus (Gilmore, 2006; Perkins, 2007). Activated NF-κB molecule regulates transcription of several genes primarily as a transcriptional activator (Baldwin, 1996). However, under condition of stress in certain pathological conditions NF-κB can also act as a transcriptional repressor (Gires *et al.,* 2001; Ke *et al.,* 2001). A very recent report indicate that PARP inhibitors, DHIQ and 3-AB, possess free radical scavenging properties (Czapski et al., 2004); since oxidative stress has been implicated in TD and in PARP-1 activation, it is possible that PARP inhibitors with antioxidative potency contribute indirectly and non-specifically to decreased NF-κB dependent transcriptional activity by reduction of reactive oxygen species. Thus, poly (ADP) ribosylation is not directly required for repression or stimulation of the transcriptional activity of NF-κB under physiological conditions, rather it is involved in indirect regulation of NF-κB transcription.

In conclusion, the findings of this study provide evidence that excessive PARP activation due to impaired energy status or oxidative insult as a consequence of TD, promotes NF-κB-dependent regulation of the astrocytic glutamate transporter GLT-1b and may be a major contributor to excitotoxicity in this disorder. Excitotoxicity is a major pathophysiological event in several neurodegenerative diseases, and the present results in TD may provide new insight into mechanisms of glutamate transporter dysfunction in these conditions.

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REFERENCES

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

Butler AJ, Ordahl CP. (1999). Poly (ADP-ribose) polymerase binds with transcription enhancer factor 1 to MCAT1 elements to regulate muscle-specific transcription. *Mol Cell Biol.* 19:296-306.

Eguchi, Y., Shimizu, S. and Tsujimoto, Y. (1997). Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res*., 57, 1835- 1840.

Leist, M., Single, B., Castoldi, A. F., Kuhnle, S. and Nicotera, P. (1997). Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J. Exp. Med.* 185, 1481-1486.

Ghosh S, May MJ, Kopp EB.(1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*. 16:225-60.

Qu Z, Fujimoto S, Taniguchi T. (1994). Enhancement of interferon-gamma-induced major histocompatibility complex class II gene expression by expressing an antisense RNA of poly(ADP-ribose) synthetase. *J Biol Chem*. 25;269(8):5543-7.

Meisterernst M, Stelzer G, Roeder RG. (1997). Poly(ADP-ribose) polymerase enhances activator-dependent transcription in vitro. *Proc Natl Acad Sci U S A*. 18;94(6):2261-5.

JM, Alvarez-Gonzalez R. (1997). TFIIF, a basal eukaryotic transcription factor, is a substrate for poly(ADP-ribosyl)ation. *Biochem J.* 15;324 (Pt 1):249-53.

Butler AJ, Ordahl CP. (1999). Poly (ADP-ribose) polymerase binds with transcription enhancer factor 1 to MCAT1 elements to regulate muscle-specific transcription. *Mol Cell Biol.* 19:296-306.

Ghosh S, May MJ, Kopp EB.(1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*. 16:225-60.

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. Y. (1996) Human Ice/Ced-3 protease nomenclature. Cell 87, 171.

Baldwin AS, Jr. (1996) The NF-kappa B and I kappa B proteins: New discoveries and insights. Annu Rev Immunol 14:649–683.

Booher J, Sensenbrenner M., (1972) Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and human brain in flask cultures. Neurobiology 2, 97–105.

Czapski GA, Cakala M, Kopczuk D , Strosznajder JB (2004) Effect of poly(ADP-ribose) polymerase

inhibitors on oxidative stress evoked hydroxyl radical level and macromolecules oxidation in cell free system of rat brain cortex. Neurosci Lett 356(1):45-48.

Eguchi, Y., Shimizu, S. and Tsujimoto, Y. (1997). Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res., 57, 1835- 1840.

Eliasson MJ, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang ZQ, Dawson TM, Snyder SH, Dawson VL. (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. Nature Med. 3, 1089–1095.

Ghosh, S., May, M. J. and Kopp, E. B. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16, 225-260.

Gires O, Kieu C, Fix P, Schmitt B, Munz M, Wollenberg B, Zeidler R. (2001) Tumor necrosis factor alpha negatively regulates the expression of the carcinoma-associated antigen epithelial cell adhesion molecule. Cancer 92:620–628.

Gilmore TD. (2006) Introduction to NF-kappaB: players, pathways, perspectives. Oncogene. 25, 6680-4.

Ha HC, Hester LD, Snyder SH. (2002) Poly(ADP-ribose) polymerase-1 dependence of stress-induced transcription factors and associated gene expression in glia. Proc Natl Acad Sci U S A. 5;99(5):3270-5.

Ha, H. C. and Snyder, S. H. (2000) Poly(ADP-ribose) polymerase-1 in the nervous system. Neurobiol. Dis. 7, 225–239.

Hassa PO, Hottiger MO. (1999) A role of poly (ADP-ribose) polymerase in NF-kappaB transcriptional activation. Biol Chem. 380(7-8):953-959.

Hassa PO, Hottiger MO. (2002) The functional role of poly(ADP-ribose)polymerase 1 as novel coactivator of NF-kappaB in inflammatory disorders. Cell Mol Life Sci. 59(9):1534-53.

Hazell AS, Sheedy D, Oanea R, Aghourian M, Sun S, Jung JY, Wang D, Wang C. (2010) Loss of astrocytic glutamate transporters in Wernicke encephalopathy. Glia. 15;58(2):148-56.

Hazell AS, Pannunzio P, Rama Rao KV, Pow DV, Rambaldi A. (2003) Thiamine deficiency results in downregulation of the GLAST glutamate transporter in cultured astrocytes. Glia.43(2):175-84.

Hazell AS, Rao KV, Danbolt NC, Pow DV, Butterworth RF. (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):560-8.

Hazell AS, Itzhak Y, Liu H, Norenberg MD., (1997) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) decreases glutamate uptake in cultured astrocytes. J Neurochem 68, 2216 –2219.

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, 1155-1158.

Herceg, Z., and Wang, Z. Q., (1999) Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis. Mol. Cell. Biol. 19, 5124–5133.

Jagtap P, Szabó C. (2005) Poly(ADP-ribose) polymerase and the therapeutic effects of its inhibitors. Nat Rev Drug Discov. 4(5):421-40.

Jhala SS, Wang D, Hazell AS. (2011) Loss of the glutamate transporter splice-variant GLT-1b in inferior colliculus and its prevention by ceftriaxone in thiamine deficiency. Neurochem Int. 58(5):558-63.

Jhala SS, Hazell AS. (2011) Modeling neurodegenerative disease pathophysiology in thiamine deficiency: Consequences of impaired oxidative metabolism. Neurochem Int. 58(3):248-60.

Ke S, Rabson AB, Germino JF, Gallo MA, Tian Y. (2001) Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor-alpha and lipopolysaccharide. J Biol Chem 276:39638–39644.

Leist, M., Single, B., Castoldi, A. F., Kuhnle, S. and Nicotera, P. (1997). Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J. Exp.

Med. 185, 1481-1486.

Li LB, Toan SV, Zelenaia O, Watson DJ, Wolfe JH, Rothstein JD, Robinson MB., (2006) Regulation of astrocytic glutamate transporter expression by Akt: Evidence for a selective transcriptional effect on the GLT-1/EAAT2 subtype. J Neurochem. 97, 759–771.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ., (1951) Protein measurement with the folin phenol reagent. J Biol Chem 19, 263–275

Meng SZ, Itoh M, Obonai T, Takashima S. (2000) Roles of glutamate transporter and receptors, poly (ADPribose) polymerase, and transforming growth factor-beta1 in pontosubicular neuron necrosis. J Child Neurol.15(6):362-9.

Meisterernst M, Stelzer G, Roeder RG. (1997). Poly(ADP-ribose) polymerase enhances activator-dependent transcription in vitro. Proc Natl Acad Sci U S A. 18;94(6):2261-5.

Pannunzio P, Hazell AS, Pannunzio M, Rao KV, Butterworth RF. (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. 15;62(2):286-92.

Perkins ND. (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol. 1, 49-62.

Qu Z, Fujimoto S, Taniguchi T. (1994). Enhancement of interferon-gamma-induced major histocompatibility complex class II gene expression by expressing an antisense RNA of poly(ADP-ribose) synthetase. J Biol Chem. 25;269(8):5543-7.

Rawling JM, Alvarez-Gonzalez R. (1997). TFIIF, a basal eukaryotic transcription factor, is a substrate for poly(ADP-ribosyl)ation. Biochem J. 15;324 (Pt 1):249-53.

Shin BH, Choi SH, Cho EY, Shin MJ, Hwang KC, Cho HK, Chung JH, Jang Y. (2004) Thiamine attenuates hypoxia-induced cell death in cultured neonatal rat cardiomyocytes. Mol Cells. 31;18(2):133-40.

Schreiber V, Dantzer F, Ame JC, de Murcia G. (2006) Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol. 7(7):517-28.

Shah, G. M., Shah, R. G., and Poirier, G. G. (1996). Different cleavage pattern for poly(ADP-ribose) polymerase during necrosis and apoptosis in HL-60 cells. Biochem. Biophys. Res. Commun. 229, 838–844.

Shall S, de Murcia G. (2000) Poly(ADP-ribose) polymerase-l: what have we learned from the deficient mouse model? Mutat Res. 460(1): 1-15.

Su ZZ, Leszczyniecka M, Kang DC, Sarkar D, ChaoW, Volsky DJ, Fisher PB. (2003) Insights into glutamate transport regulation in human astrocytes: Cloning of the promoter for excitatory amino

acid transporter 2 (EAAT2). Proc Natl Acad Sci USA. 100, 1955–1960.

Tang KS, Suh SW, Alano CC, Shao Z, Hunt WT, Swanson RA, Anderson CM. (2010) Astrocytic poly(ADP-ribose) polymerase-1 activation leads to bioenergetic depletion and inhibition of glutamate uptake capacity. Glia. 58(4):446-57.

Trushina E, McMurray CT. (2007) Oxidative stress and mitochondrial dysfunction in neurodegenerative diseases. Neuroscience. 14;145(4):1233-48.

Ying W, Sevigny MB, Chen Y, Swanson RA., (2001) Poly(ADP-ribose) glycohydrolase mediates oxidative and excitotoxic neuronal death. Proc Natl Acad Sci U S A. 98, 12227-32.

Virág, L., and Szabó, C., (2002) The therapeutic potential of poly (ADP-ribose) polymerase inhibitors. Pharmacol. Rev. 54, 375–429.

Zhang, J., Dawson, V. L., Dawson, T. M. and Snyder, S. H., (1994) Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. Science. 263, 687–689.

Zelenaia O, Schlag BD, Gochenauer GE, Ganel R, Song W, Beesley JS, Grinspan JB, Rothstein JD, Robinson MB., (2000) Epidermal growth factor receptor agonists increase expression of glutamate transporter GLT-1 in astrocytes through pathways dependent on phosphatidylinositol 3-kinase and transcription factorNF-kappaB. Mol Pharmacol. 57, 667–678.

Zingarelli, B., Salzman, A. L. and Szabó, C., (1998) Genetic disruption of poly (ADP-ribose) synthetase inhibits the expression of P-selectin and intercellular adhesion molecule-1 in myocardial ischemia/reperfusion injury. Circ. Res. 83, 85–94.

FIGURE LEGENDS

Figure 1. Photomicrographs of representative astrocyte cultures with or without exposure to TD, showing increased PARP-1 immunoreactivity. Bar, 100μM

Figure 2. Immunoblot analysis of PARP-1 cleavage patterns in cultured astrocytes and rats exposed to TD. A) Astrocytes exposed to TD showed increased levels of the cleaved 50kDa fragment of PARP-1. PARP-1 cleavage was almost completely blocked by the PARP-1 specific inhibitor, 3,4-dihydro-5[4-(1-piperindinyl) butoxy]-1(2H)-isoquinoline DPQ (25μM), and after exogenous NAD (1mM) exposure of astrocytes to TD. B) Rats with TD showed an increase in levels of the PARP-1 cleaved 24kDa fragment in the vulnerable thalamus and inferior colliculus compared to PFCs. In comparison, the non-vulnerable frontal cortex did not display any changes in the levels of this cleavage fragment in any of the three regions examined. Co-treatment of rats with the NF-κB inhibitor PDTC did not prevent these changes when compared to animals exposed to TD alone. Graphs show relative band intensities of the various treatments. Values are mean ± SEM.

Figure 3. Flow cytometry analysis of PARP-1 in thiamine deficient astrocyte cultures. Astrocytes were cultured for two weeks prior to the experiments. TD in astrocytes resulted in an almost 4-fold increase in PARP-1 levels compared to control astrocytes. In the presence of DPQ and NAD, PARP-1 levels were reduced by 61% and 30%, respectively. No significant change was observed in the levels of PARP-1 after PDTC administration. The top panel (A) shows percent immunoreactivity for GFAP/ PARP-1 in total cell population. The values in each quadarant represent the percentage of $GFAP^+$ and $PARP\text{-}1^+$ cells. Numbers indicate percentages of main population events within each

quadrant for this experiment only. The bottom panel (B) showing the histogram plotted with the values obtained as a percent mean of three individual experiments. Values are mean ± SEM.

Figure 4. Immunoblot analysis of phospho-IκB in cultured astrocytes during TD. Cells exposed to TD conditions showed a large induction of phospho-IκB which was blocked using the PARP-1 inhibitor DPQ. However, TD treated astrocytes exposed to exogenous NAD^+ (1mM) showed no significant change in phospho-IkB levels. Values are mean ± SEM.

Figure 5. Flow cytometry analysis of phopho-IkB and GLT-1b in astrocyte cultures treated with TD. Exposure of cells to TD resulted in a downregulation of GLT-1b that was blocked following PARP-1 (DPQ) and NF-κB (PDTC, NAD^+) inhibition (A,B). Increased levels of phospho-IkB were observed during TD and were blocked following PARP-1 (DPQ) and NF-κB (PDTC, NAD^+) inhibition (A,C). Panel A shows percentage of total immunoreactivity for GLT-1b and IκB following different treatments (TD, DPQ, PDTC, NAD^+). The values in each quadrant represent the percentage of GLT-$1b^+$ and phosphor-IκB-1^+ cells. Numbers indicate percentages of main population events within each quadrant for this experiment only. B and C shows the histogram plotted with the values obtained as a percent mean of three individual experiments for GLT-1b and IκB. Values are mean ± SEM.

Figure 6. Effect of PARP-1 or NF-κB inhibition on GLT-1b in astrocytes treated with TD.Cells exposed to TD conditions showed a reduction in GLT-1b levels which was ameliorated following co-treatment with either the PARP-1 inhibitor DPQ or NF-κB inhibitors PDTC or NAD. Values are mean ± SEM.

Figure 7. Effect of PARP-1 or NF-κB inhibition on glutamate uptake in astrocytes treated with TD. Decreased uptake observed with exposure to TD conditions was ameliorated following co-treatment with either the PARP-1 inhibitor DPQ or NF-κB inhibitor PDTC. Values are mean ± SEM. Glutamate uptake was measured using the non-metabolized glutamate analogue [^{3}H]-D-aspartate (nmol/mg protein/min). Values are mean ± SEM.

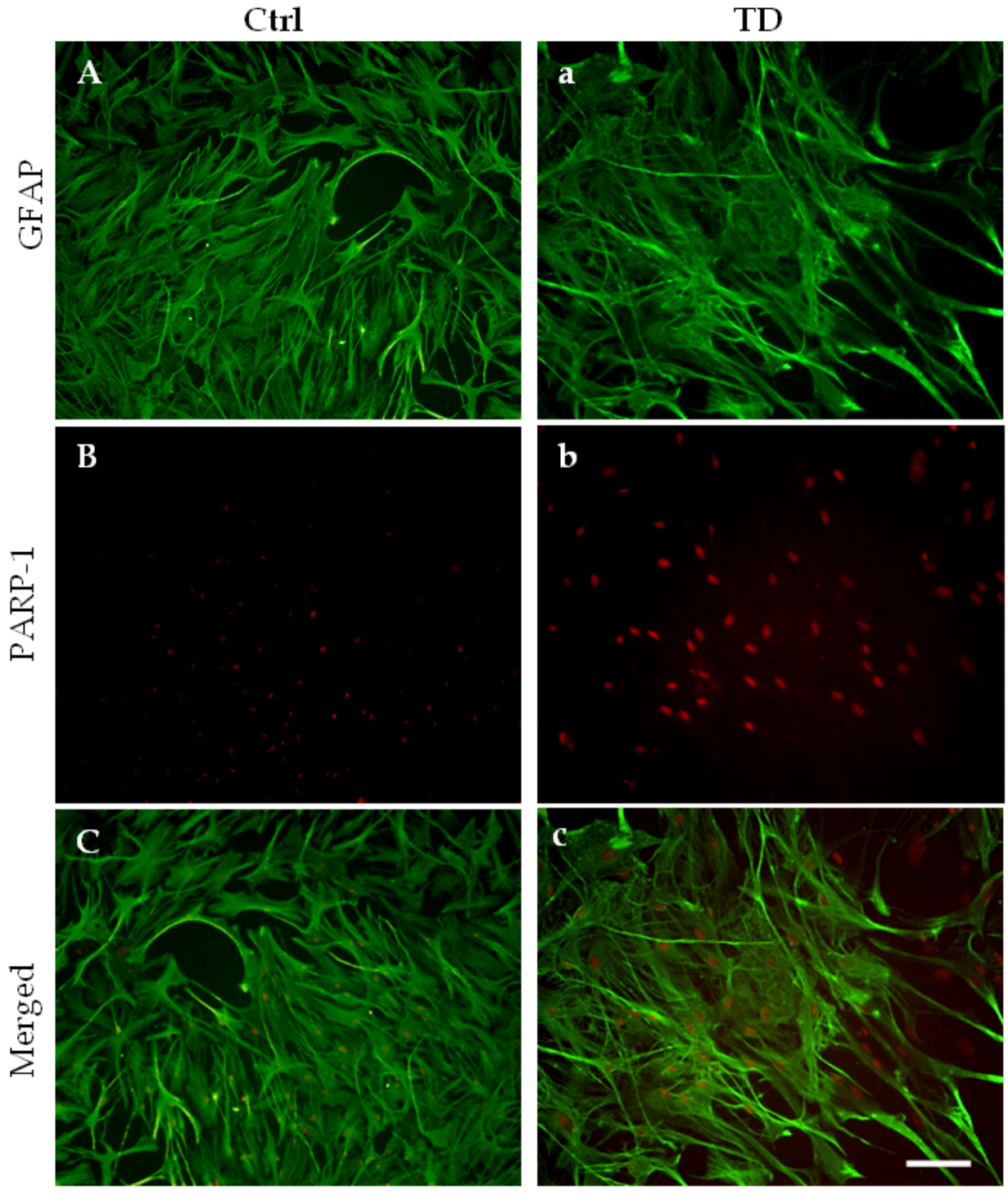

FIGURE 1

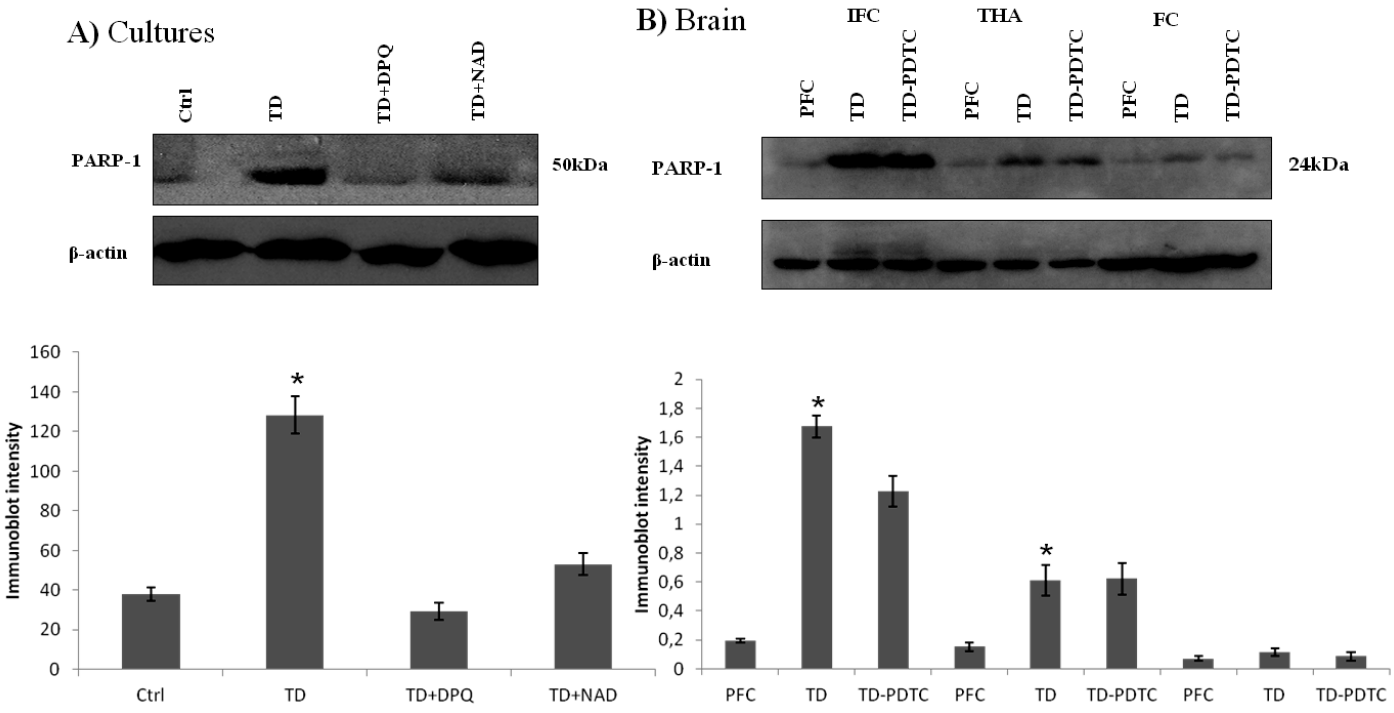
A) Cultures
Ctrl
TD
TD+DPQ
TD+NAD
PARP-1
50kDa
β-actin
B) Brain
IFC
THA
FC
PFC
TD
TD-PDTC
PARP-1
24kDa
β-actin
Immunoblot intensity
160
140
120
100
80
60
40
20
0
Ctrl
TD
TD+DPQ
TD+NAD
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
PFC
TD
TD-PDTC

FIGURE 2

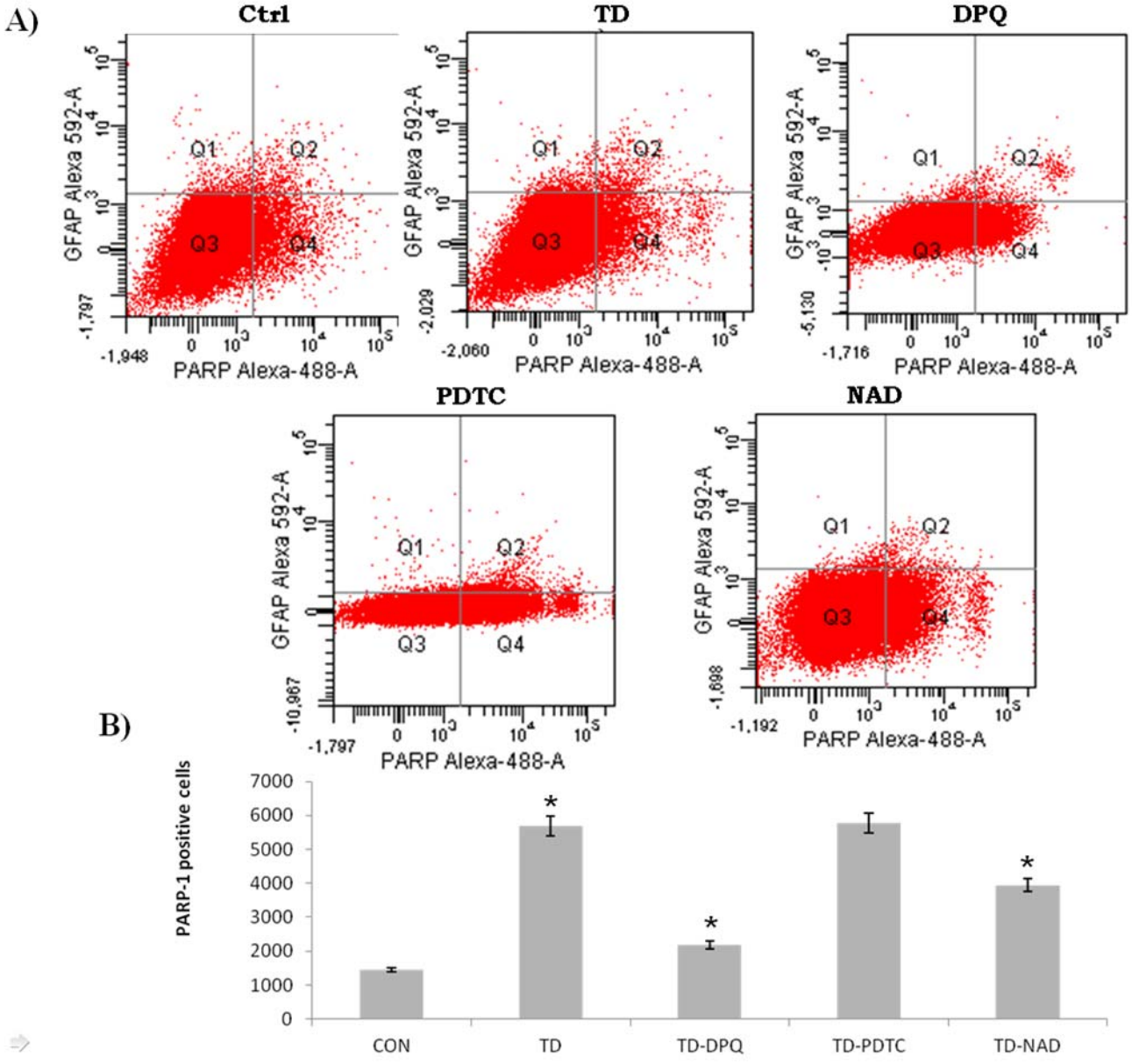
A)
Ctrl
TD
DPQ
PDTC
NAD
GFAP Alexa 592-A
PARP Alexa-488-A
Q1
Q2
Q3
Q4
B)
PARP-1 positive cells
7000
6000
5000
4000
3000
2000
1000
0
CON
TD
TD-DPQ
TD-PDTC
TD-NAD

FIGURE 3

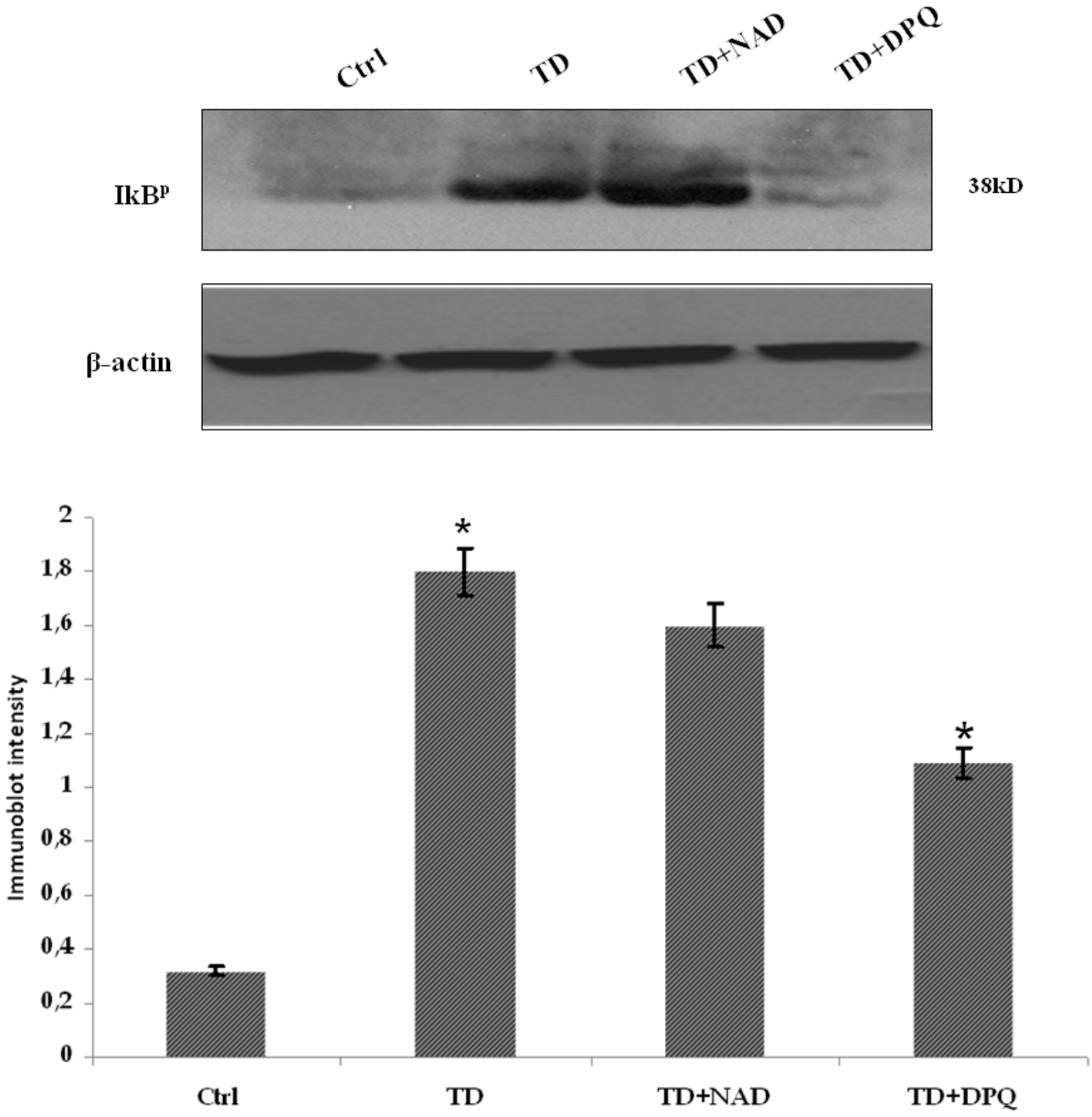
Ctrl
TD
TD+NAD
TD+DPQ
IkBp
38kD
β-actin
Immunoblot intensity
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
Ctrl
TD
TD+NAD
TD+DPQ

FIGURE 4

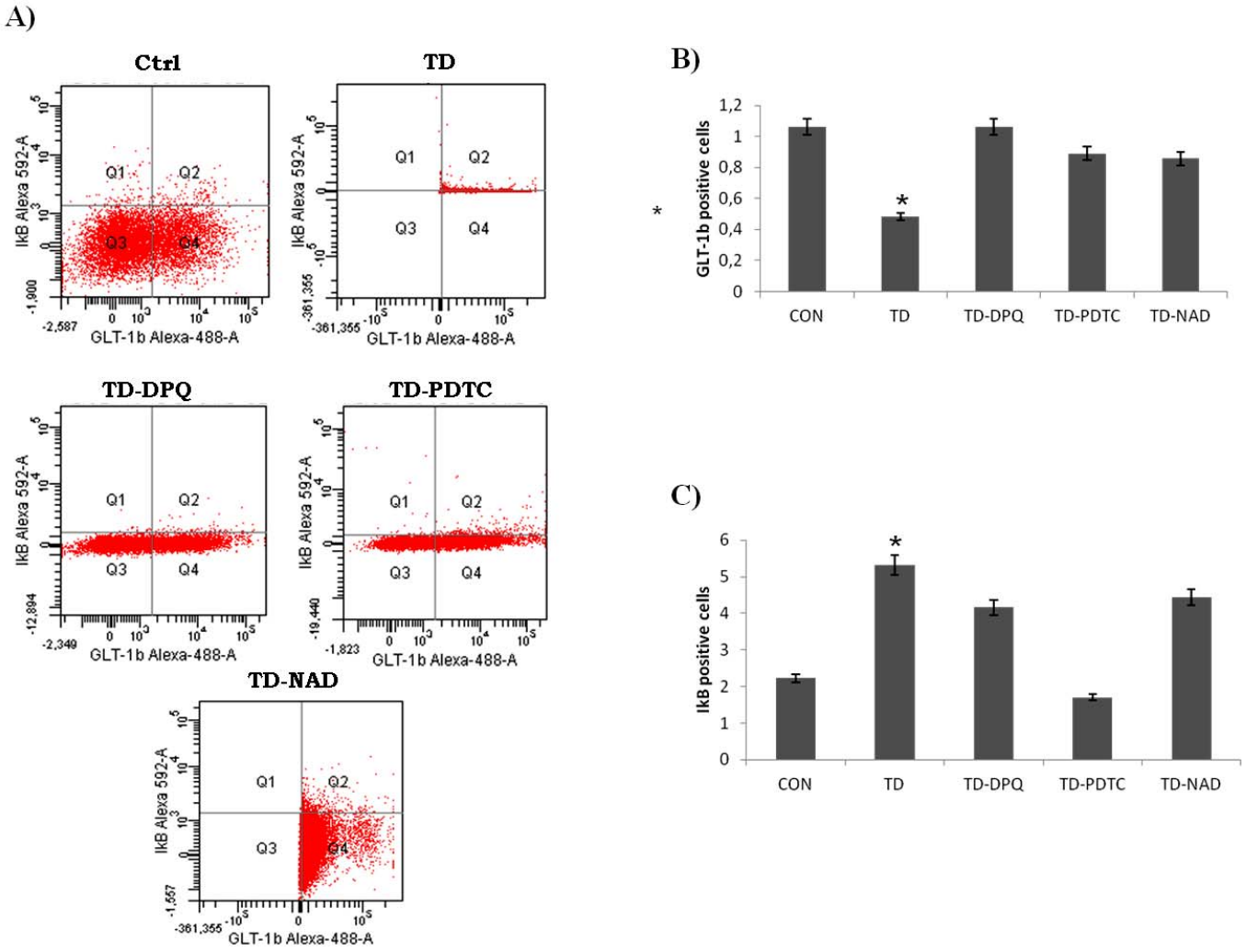
A)
Ctrl
TD
TD-DPQ
TD-PDTC
TD-NAD
IkB Alexa 592-A
GLT-1b Alexa-488-A
Q1
Q2
Q3
Q4
B)
GLT-1b positive cells
CON
TD
TD-DPQ
TD-PDTC
TD-NAD
C)
IkB positive cells

FIGURE 5

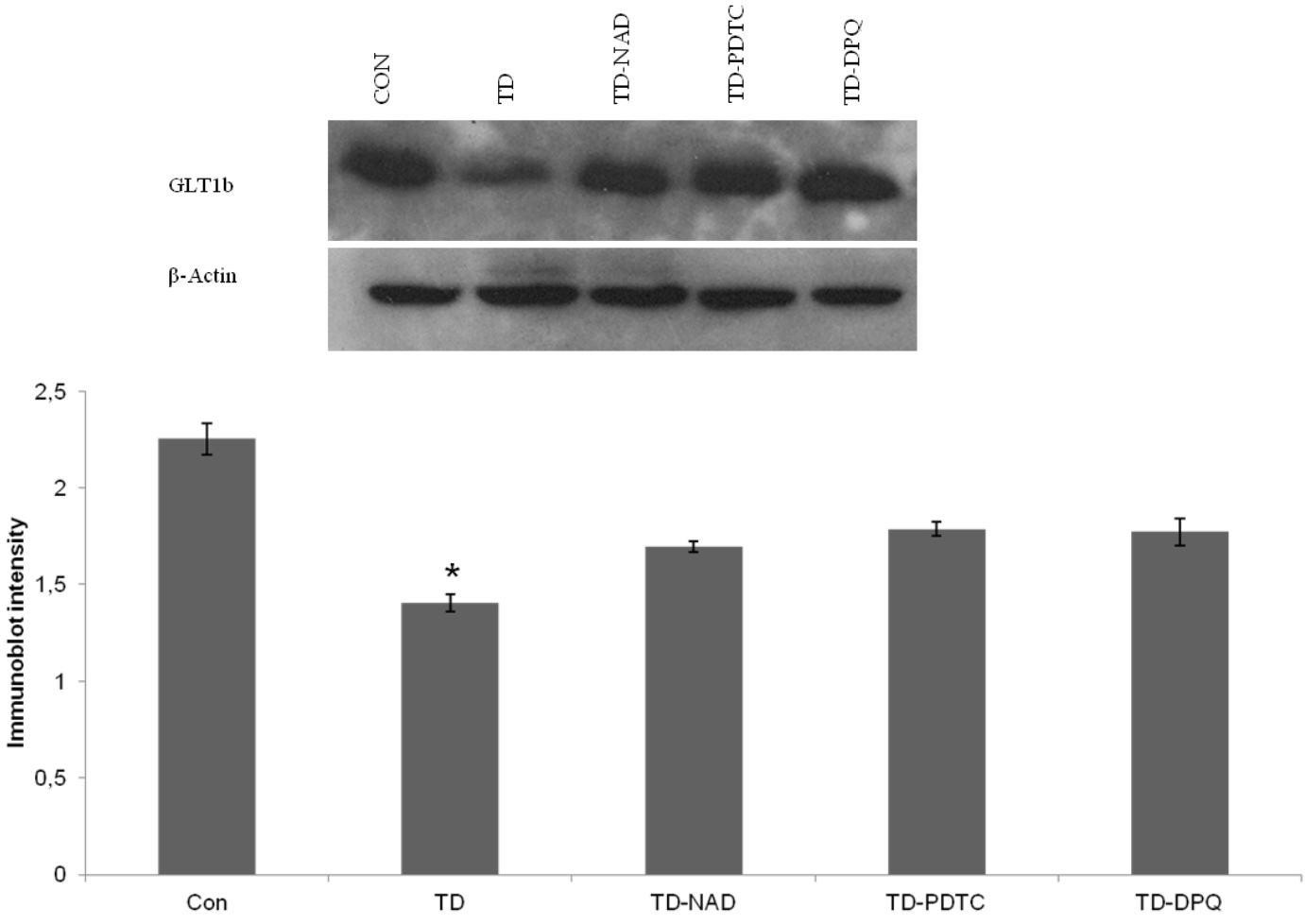
CON
TD
TD-NAD
TD-PDTC
TD-DPQ
GLT1b
β-Actin
Immunoblot intensity
2,5
2
1,5
1
0,5
0
*
Con
TD
TD-NAD
TD-PDTC
TD-DPQ

FIGURE 6

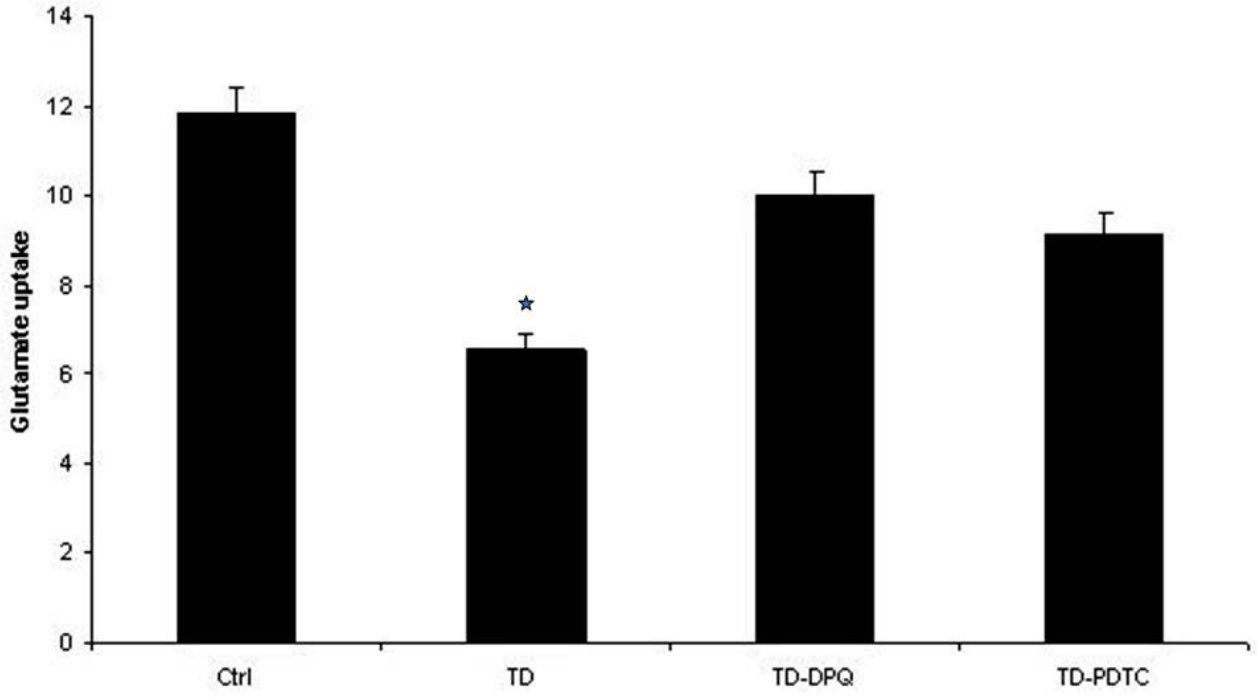

FIGURE 7

CHAPTER 4

Astrocytic soluble factors disrupt mitochondrial membrane potential and downregulate GLT-1 in thiamine deficiency

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ASTROCYTIC SOLUBLE FACTORS DISRUPT MITOCHONDRIAL MEMBRANE POTENTIAL AND DOWNREGULATE GLT-1 IN THIAMINE DEFICIENCY

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ABSTRACT

Loss of astrocytic glutamate transporters is a major feature of both TD and human cases of Wernicke's encephalopathy, in which cerebral energy metabolism is significantly affected. However, the underlying basis of this process remains unclear presently. Previous studies have reported evidence for the involvement of soluble neuronal factors that can regulate the levels of these glutamate transporters. In the present study we have investigated the possibility of release of astrocytic soluble factors that might be involved in the regulation of the glutamate transporter GLT-1 on these cells. Treatment of naïve astrocytes with conditioned media from astrocytes exposed toTD conditions resulted in a progressive decrease in glutamate uptake over 24h, along with increased release of glutamate. Immunoblotting and flow cytometry measurements indicated this was accompanied by a 20-40% loss of GLT-1 in these cells. Lactic acid, produced in astrocytes as a consequence of TD, was found to decrease glutamate uptake as with TD conditioned media but exerted no effect on the downregulation of GLT-1. Determination of the mitochondrial membrane potential ($\Delta\psi m$) in naïve astrocytes using the membrane potential-dependent aggregate-forming lipophilic cation JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanide iodide) indicated that treatment of astrocytes with either TD or TD conditioned media showed increased disruption of $\Delta\psi m$ compared to control cells.Treatment of astrocytes with TD resulted in an increase in the pro-inflammatory cytokine TNF-α and elevated levels of the phospho-IκB fragment, indicative of increased activation of NFκB. Inhibition of TNF-α activity with the use of a neutralizing antibody blocked the increased NFκB activation, while inhibition of NFκB ameliorated the decrease in GLT-1 and reversed the decrease in glutamate uptake occurring with TD treatment. Interestingly, inhibition of either TNF-α activity or NFκB activation failed to prevent the loss in glutamate uptake following treatment of astrocytes with TD conditioned media, suggesting that

TNF-α and NFκB contribute to loss of glutamate transporter function in astrocytes with TD but that these factors are not effective in, or sufficient for, reducing transporter function in normal astrocytes exposed to TD-conditioned media. Together, these findings indicate that astrocytes exposed to TD conditions show a number of responses suggesting that soluble factors released by these cells under conditions of TD play a regulatory role in terms of glutamate transport function and mitochondrial integrity.

INTRODUCTION

Thiamine deficiency (TD), the cause of Wernicke's encephalopathy (WE), is implicated in the impairment of mitochondrial energy metabolism in both neurons and astrocytes. These two cell types have fundamental differences in their responses in various CNS pathologies (Pekny and Nilsson, 2005.). Astrocytes are highly abundant cells in the brain and play an important role in normal synaptic transmission (Araque et al., 2001; Muyderman et al., 2001; Fields and Stevens-Graham, 2002). Many functions of astrocytes are likely to be important in determining the tissue response to TD or other CNS pathology, including their role in the control of fluid movements between the intracellular and extracellular space, the ability to take up glutamate and reduce excitotoxicity, and their involvement in spatial buffering (Anderson et al., 2003).

TD is a metabolic disorder that occurs due to inhibition of oxidative decarboxylation of pyruvate and α-ketoglutarate, leading to decreased ATP production, pyruvate accumulation and increased lactate production (Aikawa et al., 1984; Navarro et al., 2005). During TD, both neuronal and astrocytic intermediary metabolism is impaired and protective astrocytic functions, such as glutamate uptake, K^+ buffering, or elimination of reactive oxygen species (ROS) become compromised (Jhala et. al., 2011; 2010; Hazell et al., 2003; Hazell et al., 1998).

Treatment with pyrithiamine, a thiamine antagonist, results in downregulation of the astrocytic glutamate transporters GLT-1 and GLAST (Jhala et. al., 2011; Hazell et. al., 2001; 2003), a process also occuring in human cases of WE (Hazell et. al., 2010). Although the mechanism of this transporter dysfunction is still unclear, a few studies have reported the role of extrinsic factors in the regulation of glutamate transporter activity. Exogenous lactate administration to cultured astrocytes inhibit glutamate uptake capacity by its effects on astrocyte cell swelling and acidification (Bender et al., 1997). Lactic acid acidosis may inhibit glutamate uptake directly by its effect on the

transporters, possibly by affecting charged groups on the glutamate carrier proteins thereby altering the tertiary structure of the transporter (Barbour et. al., 1988). Thus, decreased glutamate uptake could also occur due to ionic imbalance. In addition, increased release of ROS along with increased TNF-α, are known to induce glutamate neurotoxicity by inhibition of glutamate uptake (Zou and Crews, 2005).

Treatment of astrocytes with lactate leads to a reduction in glutamate uptake (Bender et al., 1997). In addition, increased pro-inflammatory cytokine production has been shown to inhibit glutamate uptake capacity and has been linked to the pathogenesis of excitotoxicity (Zou and Crews, 2005). In the present study, we therefore investigated whether TD-induced loss of glutamate transporter activity is due to accumulation of lactate or an inflammatory-based process.

2. EXPERIMENTAL PROCEDURE

2.1 Cell culture preparation

Astrocyte cultures from newborn rats were prepared using a method described by Booher and Sensenbrenner (1972) with slight modifications. Briefly, cerebral cortices were removed and the tissue was dissociated mechanically and passed through sterile nylon sieves, and then suspended in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Burlington, ON) containing 10% fetal calf serum. Cells were then seeded in 35 mm culture plates, which were maintained in an incubator at 37°C provided with a mixture of 5% CO2 and 95% air. Cultures were grown for 3–5 weeks, during which the medium was changed twice a week. To induce TD, cells were exposed to a custom-designed DMEM media lacking in thiamine (Invitrogen, Burlington, ON) and containing 5% horse serum, in the presence of the thiamine antagonist pyrithiamine (10 μM). Control astrocytes were treated with TD media in which normal levels of thiamine (4 mg/L) had been added. Media

was changed every 3-4 days.

2.2 Production of GLT-1b antibody

A polyclonal antibody against the synthetic peptide ECKVPFPFLDI ETCI corresponding to the last 15 amino acids of (amino acid 548–562) of the GLT-1b splice-variant and conjugated to keyhole limpet hemocyanin (Sheldon Biotechnology Centre, Montreal, QC) was generated in rabbits using a standard protocol (Comparative Medicine & Animal Resources Centre, Mcgill University). The GLT-1b sequence that was chosen represents a unique carboxyl terminal region and has no similarity with other sequences in the standard gene database (Chen et. al., 2002; Bassan et. al., 2008). The specificity of the antiserum was initially evaluated by immunoblotting as previously described (Hazell et. al., 2003). Immunodetection was performed using the primary antiserum at a range of dilution (0.01- 0.1 μg/mL).

2.3 Immunoblotting studies

Cell culture plates were harvested in buffer containing 50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate (pH 8.0) and protease inhibitor cocktail, and centrifuged at 10,000g for 10 min at 4^{o}C. Preliminary studies carried out on the pellet and supernatant indicated that GLT-1 was completely soluble in this buffer under our conditions. Thus, the supernatant was retained and used for study. Protein content of all samples was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard. Sample buffer was added to aliquots of the cells (30 μg) and the samples boiled for 5 min. Aliquots were subjected to (SDS)-polyacrylamide gel electrophoresis (8% polyacrylamide) and the proteins subsequently transferred to PVDF membranes by wet transfer at 20 V over 24 h. The transfer buffer consisted of 48 mM Tris (pH 8.3), 39 mM glycine, 0.037% SDS, and 20% methanol.

Membranes were subsequently incubated in blocking buffer (10 mM Tris, 100 mM NaCl, 5% non fat dried milk) followed by incubations with rabbit polyclonal antisera directed against GLT-1b, phosphor IκB (Cell Signaling Technology, Inc., Danvers, MA). Reblocking was followed by incubation with HRP-coupled anti-rabbit IgG (0.01 lg/mL) secondary antiserum. Each incubation step was of 1h duration following which blots were washed several times with buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20). For the detection of specific antibody binding, the membranes were treated in accordance with the ECL-kit instructions and developed on a photosensitive X-OMAT film. Signal intensities were subsequently measured by densitometry using ImageJ software (National Institutes of Health , Bethesda, ML). Linearity of the relationship between optical density and protein concentration was verified using appropriate standard curves. Blots were reversibly stained with Ponceau-S to monitor protein transfer efficiency and equal loading.

2.4 Immunocytochemistry

Immunocytochemistry was perfomed as previously described, Hazell et al. (2001). Briefly, astrocytes were washed in PBS and fixed for 10 min with 10% neutral buffered formalin. After washing cells were blocked for 30 min with 1% donkey serum in PBS, and then incubated with 1% donkey serum and polyclonal goat antisera directed against GFAP (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). Sections were then washed (10 min) and incubated for 1 h with Alexa Fluor-488 (green) secondary antibody (1:200), then mounted in Prolong Gold AntiFade reagent and examined using an Olympus BX51 microscope and attached Spot RT digital camera. Negative controls consisted of omission of primary or secondary antibody, resulting in loss of immunoreactivity. Images were processed using Image-Pro Plus 6.2 image analysis software (Media Cybernetics, Inc., Bethesda, MD).

2.5 Changes in Mitochondrial Membrane Potential

Cultured astrocytes were analyzed for changes in the mitochondrial membrane potential ($\Delta\psi m$) using the potential-dependent aggregate-forming lipophilic cation JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanide iodide) (Invitrogen, Burlington, ON). Cells were assayed according to the manufacturer's instructions. Changes in astrocytic $\Delta\psi m$ in TD and TD media treated astrocytes were quantified by FACScan flow cytometry. Images were captured using an Olympus BX51 microscope (original magnification ×100) and merged using Image J software (Wayne Rasband, NIH).

2.6 Flow cytometry

Astrocytes were harvested in papain solution (Worthington Biochemical Corp., Lakewood, NJ) for 15-20 min and resuspended in flow cytometry buffer, consisting of 1X PBS, pH 7.2, 1% fetal bovine serum (Invitrogen, Burlington, ON). Cells were counted and diluted to a density of 1x106 cells per milliliter of buffer. Aliquotes of 1 x 105 cell were incubated with polyclonal antisera directed against GLT-1b (Cell Signalling Technology, Inc., Danvers, MA) and polyclonal goat antisera directed against GFAP (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), for 30 min on ice. Cells were washed in buffer, and then specific secondary fluorescent-conjugated antibodies, Alexa fluor-592 conjugated anti-mouse IgG and Alexa fluor-488 conjugated anti-rabbit IgG (Invitrogen, Burlington, ON) was added at the appropriate dilution (1:100) and incubated on ice for 15 minutes. Viable cells were gated by light scatter and were analyzed by FACScan (BD LSRII) (BD Biosciences, Mississauga, ON) using a Cell Quest program (BD Biosciences, Mississauga, ON). Fluorescence background was measured using unlabeled cells and cells labeled with secondary antibody alone; these set gating parameters between positive and negative cell populations. Cell

aggregates and small debris were excluded from analysis or isolation on the basis of side scatter (measuring cell granularity) and forward scatter (measuring cell size). Fluorescent intensities for cells in the population were plotted as quadrants and/or histograms using CellQuest software (BD Biosciences, Mississauga, ON). All statistical calculations were done using GraphPad Prism (GraphPad, software, Inc. La Jolla, CA).

2.7 Glutamate uptake

Glutamate uptake study was performed as described according to Hazell et al (2003) with some modifications. Briefly, cells were incubated in DMEM media containing the glutamate analogue D-aspartate and 0.2 μCi/ml of [^{3}H]-D-aspartate in 5% CO_2 /95% air at 37°C, the incubation time for all experiments being of 2 min duration. Uptake was stopped by aspiration of the media and rapid washing of cells three times with ice-cold PBS. Cells were then harvested with 0.5 ml of 1 M NaOH. Sample aliquots were measured for protein content (Lowry et al., 1951) and the radioactivity for determination of the uptake rate was measured by liquid scintillation counting.

2.8 Glutamate release

Glutamate release was performed using D-[^{3}H] aspartate, a non-metabolizable analog of glutamate. D-Asp is very commonly used to study L-Glu release, although the release rates may differ slightly for L-Glu and D-Asp. Briefly, cells were incubated for 15 min with 1 ml of D-Asp (1.0 μCi/ml) at 37^{o}C to radiolabel the cells. After washing, release was measured by incubating the cells with 1 ml of DMEM media, which was replaced every 5 min (Bender et. al., 1997). The radioactivity was measured for the collected medium (media collected every 5 min for 30 min) by liquid scintillation counting.

2.9 Lactate assay

L(+)-Lactate was assayed in medium collected from the TD astrocyte cultures at 0, 4, 7 10 days, using a lactate assay kit (K627-100, BioVision Inc., Milpitas, CA) according to the manufacturer's instructions. The assay was performed after the medium collected and mixed with the appropriate reagents as provided with the kit. The optical density of the samples was measure at 450nm in a microplate reader. The absorbances were initially converted to nmol/µl using standard curves prepared at different concentration supplied with the kit.

2.10 Measurement of TNF-α using ELISA and treatment of astrocytes with anti-TNF-α neutralizing antibody

Protein levels of TNF-α was measured in astrocyte cultures using a commercial ELISA kit (R&D Systems, Minneapolis, MN; Invitrogen, Burlington, ON) according to the manufacturer's instructions. The plates were read at 450 nm and the absorbances were converted to pg/mL using standard curves prepared with recombinant cytokines. Astrocytes were treated with anti-TNF-α neutralizing antibody (R&D systems, Minneapolis, MN) at a concentration of 0.5µg/ml according to the manufacturer's guidelines.

2.11 Statistical analysis

Data are expressed as mean ± S.E.M. values. Statistical analysis was performed using one way ANOVA for multiple comparisons. A probability of $p < 0.05$ was chosen to establish significance between groups. Data were analyzed by using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).

3. RESULTS

3.1 General observations

When astrocytes were stimulated with dBcAMP (0.5 mM), polygonal astrocytes differentiated into process bearing, stellate cells. Treatment with TD for 10 days produced alterations in cell morphology (Fig. 1). TD resulted in a reduction of astrocytic processes, towards a less fibrous shape compared to controls. However, naïve astrocytes treated for 24h with conditioned media from TD treated cells showed no change in morphology from that observed in control astrocytes.

3.2 Treatment with TD conditioned media

To examine if astrocyes respond to conditioned media from TD treated cells, we studied its effect on glutamate uptake and release in naïve astrocytes exposed to conditioned media for 24h. Figure 2A shows a progressive decrease in glutamate uptake with time with uptake reduced by over 70% after 24h. Treatment with conditioned media resulted a 2-fold increase in glutamate release (Fig. 2B). Next, we assessed whether these alterations in glutamate transport were caused by a change in the regulation of GLT-1 using immunoblotting. Immunoblot analysis of conditioned media-treated astrocytes indicated a 40% loss of GLT-1 compared to controls (Fig. 3A,C). To further confirm and quantify this finding we used flow cytometric analysis, with 24h treatment with TD conditioned media showing a 20% decrease in the level of GLT-1, consistent with the immunoblotting results given its limitations (Fig. 3B,C). Together, these findings indicate that soluble factors released from astrocytes exposed to TD conditions downregulate glutamate transporter levels.

3.4 Involvement of lactate in TD-induced glutamate transporter dysfunction

To specifically address the involvement of lactate accumulation in TD-induced loss of glutamate transporter function, we measured lactate concentration in the culture medium at 0, 4, 7, 10 d after exposure of the cells to TD. The extracellular lactate concentration on day 4 of TD was 0.4 ± 0.0 nmol/µl compared to that in the media from control cells showing 0.3 ± 0.0 nmol/µl (Fig. 4A). TD did not increase bulk lactate concentration in the medium at this time point, but increased lactate concentration was observed after 7 and 10 days (0.6 ± 0.0 and 0.5 ± 0.0 nmol/µl respectively) compared to control plates. To determine if extracellular lactate accumulation under conditions of TD might be responsible for glutamate transporter dysfunction, we treated naïve astrocytes with 25 mM exogenous lactate. Lactate treatment decreased glutamate uptake after 24h treatment to a similar extent as did conditioned media (Fig. 4B), suggesting an involvement of extracellular lactate accumulation in glutamate transporter dysfunction in TD. However, immunoblot analysis of cultured astrocytes exposed to lactate showed no change in protein levels of GLT-1 (Fig. 4C).

3.5 Effect of TD conditioned media on mitochondrial function

We used JC-1 to probe mitochondrial function by imaging the monomeric form (green) and the aggregate form (red) of this fluorescent probe. A decrease in red fluorescence and an increase in green fluorescence in TD astrocytes were observed, indicating $\Delta\psi m$ collapse (Fig. 5I). CCCP (1 µM/ml) treatment for 10 min was used as a positive control in these experiments. TD astrocytes showed abrupt change in $\Delta\psi m$ values and, similar to TD, treatment with TD conditioned media (24h) showed increased mitochondrial depolarization in naïve astrocyte cultures. Next, to quantitatively assay JC-1 signals, we used flow cytometry to compare the ratio of the monomeric form to that of the aggregate form (Fig. 5II). Accumulative fluorescence intensity for JC-1 monomer

representing the extent of Δψm depolarization was significantly higher in TD (~46%) and TD conditioned media treated astrocytes (~27%) relative to control astrocytes (~4%). The data obtained from flow cytometry confirmed the findings of cellular imaging with the results indicating that soluble factors released from TD treated astrocytes produce mitochondrial dysfunction in naïve astrocytes.

3.6 Influence of oxidative stress on the effects of TD conditioned media on glutamate transport function

To examine if oxidative stress may play a role in the effects of TD conditioned media on glutamate transporters in TD, astrocytes treated with TD conditioned media were also exposed to the antioxidants DTT and glutathione. Treatment with DTT and reduced glutathione ameliorated the increase in release of glutamate but had no effect on decreased glutamate uptake compared to conditioned media treated naïve astrocytes (Fig. 6A,B, respectively).

3.7 Involvement of TNF-α dependent NFκB activation in glutamate transporter dysfunction

To investigate the role of TNF-α and its associated NFκB activation in the loss of glutamate transporters under conditions of TD, we used ELISA and immunoblotting respectively to specifically measure the relative levels of TNF-α in astrocytes exposed to TD conditions. TD treatment resulted in increased production of TNF-α (Fig. 7A) and elevated levels of the phospho-IκB fragment, indicative of increased activation of NFκB (Fig. 7B). Inhibition of TNF-α activity in astrocytes with TD by treatment with a specific anti-TNF-α neutralizing antibody prevented the increase in levels of phospho-IκB fragment (Fig. 7B). In addition, exposure to the specific NFκB inhibitor PDTC (100μM) produced an amelioration of the loss of GLT-1 in astrocytes with TD (Fig.

7C) and restored glutamate uptake capacity (Fig. 7D).

To determine if accumulation of TNF-α in the medium from TD-treated astrocytes contributes to the glutamate transporter downregulation observed in astrocytes exposed to this conditioned media, naïve cells exposed to conditioned media were co-treated with either anti-TNF-α neutralizing antibody or PDTC. Figure 8 shows that inhibition of either TNF-α activity or NFκB activation in astrocytes failed to prevent the decrease in glutamate uptake observed with conditioned media treatment.

4. DISCUSSION

Excitatory amino acid transporters located on astrocytes play a crucial role in maintaining normal levels of extracellular glutamate. Loss of these transporters in TD has been reported from our lab previously Hazell et. al., 2001,2003; Jhala et. al., 2011). However, the mechanism of the loss of transporters under conditions of TD remains unresolved. Previous studies have identified that soluble factors released by neurons can influence levels of astrocytic glutamate transporters (Gegelashvili et al., 1997). Under conditions of impaired energy metabolism, astrocytes may produce chemical factors that not only can regulate glutamate transporter function from inside the cell, but these factors may also exit the cells and can potentially be measured and identified by their influence(s) on normal cells. In the present study, using our *in vitro* TD astrocyte culture model we investigated the effects of TD conditioned media on glutamate transport function. Our results indicate that naïve astrocytes treated with TD astrocyte culture media show a number of responses suggesting that soluble factors released by these cells under conditions of TD play a regulatory role in terms of glutamate transport function and mitochondrial integrity.

Treatment of normal astrocytes with TD conditioned media resulted in a loss of glutamate

uptake and increased glutamate release from these cells. This indicates that certain factors in the media previously exposed to astrocytes with TD exert a regulatory influence on glutamate transporters in these cells. Stimulation of glutamate release may contribute to glutamate uptake inhibition. For example, under depolarizing conditions such as during increased neuronal activity, high extracellular K^+ concentration can produce reversal of glutamate transporters and a resulting increased release of glutamate, with the overall effect being a decrease in glutamate uptake (Kimelberg et al., 2005). Our findings that the decreased uptake with TD conditioned media is associated with a decrease in the levels of GLT-1 in untreated astrocytes indicates that soluble factors are able to alter the protein expression of this transporter. Exactly how this regulation occurs is unclear but it can occur via a direct effect on gene expression or via an indirect effect involving other influences.

An example of the latter possibility is lactate accumulation due to the resulting metabolic impairment associated with TD producing dysregulation of glutamate transport capacity. Increased accumulation of lactate under conditions of TD has been described in vulnerable areas of brain (Hakim, 1984; Navarro et. al., 2005) and C6-glioma cells, neuroblastoma cell lines and endothelial cells (Ham and Karska-Wysocki, 2005; Schwartz et. al., 1975). Previous studies have demomstrated that lactate production inhibits glutamate uptake (Bender et al., 1997) although the exact mechanism for this is still unclear. In the present study, we showed that lactate accumulates in the media during exposure of astrocytes to TD conditions, and that exogenous application of lactate to cells decreases glutamate uptake similar to the conditioned media. However, lactate did not decrease GLT-1 levels, suggesting the influence of lactate is likely restricted to alterations in activity of the transporter rather than a change in gene expression. Thus other factors are also likely to be involved in downregulation of GLT-1 in astrocytes exposed to TD conditioned media.

To further examine the nature of these soluble factors, an evaluation of the influence of these factors on mitochondrial functional integrity was performed. Impaired energy metabolism and mitochondrial dysfunction have been implicated in the pathogenesis of TD (Aikawa et al., 1984; Pannunzio et. al., 2000). We addressed this issue using conditioned media from TD astrocytes to see whether TD extracellular factors released by the astrocytes have the ability to induce mitochondrial dysfunction using the $\Delta\psi m$ -dependent aggregate-forming lipophilic cation JC-1. Our findings indicate that factors present in the TD conditioned media are able to produce depolarization of $\Delta\psi m$ within 24 hr following exposure of the media to cells. Therefore, these soluble factors rapidly exert an effect on mitochondrial integrity, likely leading to loss of GLT-1 due to the consequential negative effects on ATP production.

Oxidative stress is a major contributing factor in the pathophysiology of TD (Jhala and Hazell, 2010) and increased production of ROS has been reported in TD animals (Langlais et al., 1997). Treatment of the conditioned media with antioxidants did not alter the decrease in glutamate uptake, but reduced the increase in glutamate release in naïve astrocytes. This suggests an influence of oxidative stress on certain aspects of glutamate transport but not others, which indicates the action of ROS likely present in the media is limited in its effects on the transporter protein under these conditions.

The proinflammatory cytokine tumor necrosis factor-a (TNF-α) is a 17-kDa peptide and forms multimers that binds to TNF-α receptors which is expressed in both neurons and glia cells (Benveniste et. al., 1995). TNF-α can be synthesized and released in the brain by astrocytes, microglial, and some neurons and the increased expression and release of TNF-α have been reported in various pathological conditions, such as trauma, ischemia, and inflammatory diseases (Allan and Rothwell, 2001; Liu et. al., 1994; Wang et. al., 1994). Clinical studies have found increased

expression of TNF-α in cerebrospinal fluid (CSF), plasma, or post mortem brain tissue in patients following stroke, brain injury, HIV dementia, alcoholism, and Alzheimer's disease (Allan and Rothwell, 2001; Khoruts et. al., 1991). In addition, it has been suggested that increased level of TNF-α in the brain can also leads to the activation of nuclear transcription factor NFκB. Moreover, TNF-α dependent NFkB activation is also implicated in the pathogenesis of glutamate neurotoxicity (Zou and Crews, 2005). Furthermore, increased levels of TNF-α has been shown to inhibit glutamate transport activity in the organotypic hippocampal slice culture. In the present study, we showed that TNF-α dependent activation of NFκB transcription factor plays a major role in the regulation of glutamate transporter function in astrocytes under conditions of TD. Our findings suggest that TNF-α and NFκB contribute to loss of glutamate transporter function in astrocytes with TD but that these factors are not effective in, or sufficient for, reducing transporter function in normal astrocytes exposed to TD-conditioned media. Further studies are required to investigate the ramifications of NFκB activation and the detailed basis of TNF-α dependent regulation of glutamate transporters during TD.

Another potential mechanism by which lactic acid in TD conditioned media decreases glutamate uptake is due to lactic acid-induced swelling. Astrocyte swelling has been shown to inhibit glutamate uptake and increase glutamate release in the culture astrocytes (Kimelberg, et. al., 1995). However, blocking astrocytic swelling by treatment with D-mannitol had no effect on lactic acid mediated decrease in glutamate uptake, suggesting swelling does not contribute to the mechanism of lactic acid-induced inhibition of glutamate uptake (Bender et. al., 1997).

Excitotoxicity is an important event in the pathogenesis of TD. Thus it is essential for our understanding to study the signaling pathways that might be involved in the regulation of glutamate transporters. In previous studies we have reported increased extracellular glutamate concentration in

focal areas of damage in TD animals, and loss of glutamate transporters and their function under both *in vivo* and *in vitro* conditions. Findings from the present study thus provide an alternative mechanism of release of soluble factors from astrocytes during TD that might contribute to glutamate transporter regulation under these conditions.

In conclusion, major findings of this study indicate that the release of lactic acid, TNF-α and other soluble factors may contribute to decreased glutamate transport capacity during TD. Additional studies are required to further investigate the nature of these factors released by metabolically impaired astrocytes and their precise role in glutamate transporter loss in TD.

ACKNOWLEDGEMENTS

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REFERENCES

A. Khoruts., L. Stahnke., C.J. McClain., G. Logan, J.I. Allen., (1991) Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients, Hepatology 13 267– 276.

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

Anderson CM., Swanson RA., (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32:1–14.

Araque A., Carmignoto G., Haydon PG., (2001) Dynamic signalling between astrocytes and neurons. Annu Rev Physiol. 63: 795–813.

Barbour, B., Brew, H. and Attwell, D., (1988) Electrogenic glutamate uptake in glial cells is activated by intracellular potassium, Nature, 335, 433–435.

Bassan et al., (2008) Interaction between the glutamate transporter GLT1b and the synaptic PDZ domain protein PICK1. Eur J Neurosci. 27, 66-82.

Bender AS, Young LP, Norenberg MD. (1997) Effect of lactic acid on L-glutamate uptake in cultured astrocytes: mechanistic considerations. Brain Res. 750:59-66.

Booher J, Sensenbrenner M. (1972) Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and human brain in flask cultures. Neurobiology. 2: 97-105.

Chen W., Aoki C., Mahadomrongkul V., Gruber CE., Wang GJ., Blitzblau R., Irwin N., Rosenberg PA., (2002) Expression of a variant form of the glutamate transporter GLT1 in neuronal cultures and in neurons and astrocytes in the rat brain. J Neurosci. 22, 2142-52.

E.N. Benveniste., D.J. Benos., (1995) TNF-alpha- and IFN-gamma-mediated signal transduction pathways: effects on glial cell gene expression and function, FASEB J. 9 1577– 1584.

Fernandez-Fernandez S, Almeida A, Bolaños JP. (2012) Antioxidant and bioenergetic coupling between neurons and astrocytes. Biochem J. 443: 3-11.

Fields RD., Stevens-Graham B., (2002) New insights into neuron-glia coupling. Science 298:556–562.

Frantseva MV, Kokarovtseva L, Perez-Valazquez JL. (2002) Ischemia induced brain damage depends on specific gap-junctional coupling. J Cereb Blood Flow Metab 22:453–462.

Gegelashvili G, Danbolt NC, Schousboe A. (1997) Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. J Neurochem. 69, 2612-5.

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

Ham D, Karska-Wysocki B. (2005) Energy and glucose pathways in thiamine deficient primary rat brain microvascular endothelial cells. Gen Physiol Biophys. 24: 467-74.

Hazell AS, Sheedy D, Oanea R, Aghourian M, Sun S, Jung JY, Wang D, Wang C. (2010) Loss of astrocytic glutamate transporters in Wernicke encephalopathy. Glia. 58: 148-56.

Hazell, A. S., Pannunzio, P., Rama Rao, K. V., Pow, D. V. and Rambaldi, A. (2003) Thiamine deficiency results in downregulation of the GLAST glutamate transporter in cultured astrocytes. Glia 43, 175-184.

Hazell, A. S., Rao, K. V., Danbolt, N. C., Pow, D. V. and 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, 560-568.

Hazell, A. S., Todd, K. G. and Butterworth, R. F. (1998) Mechanisms of neuronal cell death in Wernicke's encephalopathy. Metab. Brain Dis. 13, 97-122.

Holowach, J., Kauffman, F., Ikossi, M.G., Thomas, C. and 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.

Jhala SS, Hazell AS. (2010) Modeling neurodegenerative disease pathophysiology in thiamine deficiency: consequences of impaired oxidative metabolism. Neurochem Int. 58(3):248-60.

Jhala SS, Wang D, Hazell AS. (2011) Loss of the glutamate transporter splice-variant GLT-1b in inferior colliculus and its prevention by ceftriaxone in thiamine deficiency. Neurochem Int. 2011 58(5):558-63.

Kimelberg, H.K., Bowman, C., Biddlecome, S. and Bourke, R.S., (1979) Cation transport and membrane potential properties of primary astroglial cultures from neonatal rat brains, Brain Res., 177. 533–550.

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

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

Lowry O.H., Rosebrough N.J, Farr A.L, Randall R.J., (1951) protein measurement with the folin phenol reagent. J Biol Chem. 193: 265-75.

McCandless, D.W. and Schenker, S. (1968) Encephalopathy of thiamine deficiency: Studies of

intracerebral mechanisms. J. Clin. Invest. 47, 2268-2280.

Muyderman H., Angehagen MSandberg M., Bjorklund U, Olsson T., Hansson E., Nilsson M., (2001) a1-adrenergic modulation of metabotropic glutamate receptor-induced calcium oscillations and glutamate release in astrocytes. J Biol Chem. 276: 46504–46514.

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

Pannunzio P, Hazell AS, Pannunzio M, Rao KV, Butterworth RF. (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: 286-92.

Pekny M., Nilsson M., (2005) Astrocyte activation and reactive gliosis. Glia. 50: 427-34.

Pow D.V., Sullivan R., Scott H., (2003) Antibody production and immunocytochemical localization of amino acid transporters. Methods Mol. Biol. 227, 213–244.

Rawunduzy A., Hansen A., Hansen TW., Nedergaard M., (1997) Effective reduction of infarct volume by gap junction blockade in a rodent model of stroke. J Neurosurg. 87:916–920.

S.M. Allan., N.J. Rothwell., (2001) Cytokines and acute neurodegeneration, Nat. Rev., Neurosci. 2

734– 744.

Schwartz JP, Lust WD, Shirazawa R, Passonneau JV. (1975) Glycolytic metabolism in cultured cells of the nervous system. III. The effects of thiamine deficiency and pyrithiamine on the C-6 glioma and C-1300 neuroblastoma cell lines. Mol Cell Biochem. 9:73-8.

Siushansian R., Bechberger JF., Cechetto DF., Hachinski VC., Naus CC., (2001) Connexin43 null mutation increases infarct size after stroke. J Comp Neurol. 440:387–394.

T. Liu., R.K. Clark., P.C. McDonnell., P.R. Young., R.F. White., F.C. Barone., G.Z. Feuerstein., (1994) Tumor necrosis factor-alpha expression in ischemic neurons, Stroke 25 1481–1488.

X. Wang, T.L. Yue., F.C. Barone R.F. White., R.C. Gagnon., G.Z. Feuerstein., (1994) Concomitant cortical expression of TNF-alpha and IL-1 beta mRNAs follows early response gene expression in transient focal ischemia, Mol. Chem. Neuropathol. 23 103– 114.

Zou JY., Crews FT., (2005) TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition. Brain Res. 1034: 11-24.

FIGURE LEGENDS

Figure 1. Representative cultures of astrocytes immunostained for GFAP. Cells exposed to dBcAMP for one week to induce the GLT-1 transporter protein were subsequently treated with either TD conditions for 10 days (B) or TD conditioned media for 24h (C). Compared to control astrocytes (A), cells exposed to TD conditions exhibited a reduction in processes, appearing to partially revert towards a less fibrous (non- dBcAMP treated) morphology, while treatment with conditioned media (CM) from TD treated astrocytes for 24h produced no obvious change in morphology. Bar, 50 μm.

Figure 2. Treatment of TD conditioned media in naïve astrocytes resulted in a decrease in glutamate uptake and increased glutamate release.

Figure 3. Effect of TD conditioned media on glutamate transporter levels in naïve astrocytes. Cells exposed to conditioned media showed loss of GLT-1 with immunoblotting (A) which was confirmed with flow cytometry analysis (B,C).

Figure 4. Effect of lactate on glutamate transporter integrity in astrocytes. A) Lactate levels in the media of astrocytes treated with TD over 10 days. Extracellular lacate concentration increased during TD. B) Treatment with TD conditioned media or lactic acid (25mM) both inhibited glutamate uptake to a similar extent. C) Immunoblot analysis of GLT-1 protein showed that lactic acid treatment for 24 h did not alter GLT-1 levels from controls.

Figure 5. I) Analysis of mitochondrial membrane potential (Δψm) in astrocytes with TD or after treatment of naïve astrocytes with TD conditioned media. Δψm was analyzed using the membrane potential-dependent aggregate-forming lipophilic cation JC-1. Red fluorescence indicates JC-1 aggregates (a) representing mitochondria with intact membrane potential whereas green fluorescence indicates JC-1 monomers (b) representing de-energized mitochondria. Images show treatment of A) naïve astrocytes treated with the mitochondrial membrane potential disrupter CCCP, B) control astrocytes, C) naïve astrocytes treated with TD, and D) naïve astrocytes treated with TD conditioned media. II) Flow cytometric determination of Δψm changes in control astrocytes (i), astrocytes exposed to TD (ii), and naïve astrocytes treated with TD conditioned media (iii). TD media treated astrocytes quantified by FACScan flow cytometry. Values represent mean intensity ± SEM of Alexa Fluor 488, green fluorescence, for n=6.

Figure 6. Influence of antioxidants on the effects of TD conditioned media on glutamate transport function in astrocytes. A) Co-treatment of naïve astrocytes with conditioned media and the antioxidants DTT or reduced glutathione (GSH) showed no effect on the inhibition of glutamate uptake produced by conditioned media treatment alone. B) Co-treatment with DTT or GSH ameliorated the increase in glutamate release produced by TD conditioned media on astrocytes.

Figure 7. Involvement of TNF-α dependent NFkB activation in glutamate transport dysfunction. TD increased TNF-α production in astrocytes (A) and resulted in increased activation of NFkB (B). Inhibition of TNF-α using an anti-TNF-α neutralizing antibody blocked the heightened activation of NFkB. Pharmacological inhibition of NFkB using the specific NFkB inhibitor PDTC (100μM) reduced the GLT-1 loss in TD astrocytes (C) and restored the glutamate uptake capacity of the TD

astrocytes (D).

Figure 8. Involvement of TNF-α dependent NFkB activation in glutamate transporter dysfunction in naïve astrocytes treated with TD conditioned media. Inhibition of TNF-α using anti-TNF-α neutralizing antibody or direct inhibition of NFkB using PDTC (100μM) showed no effects on glutamate uptake inhibition by TD conditioned media (TDcm) treated naïve astrocytes.

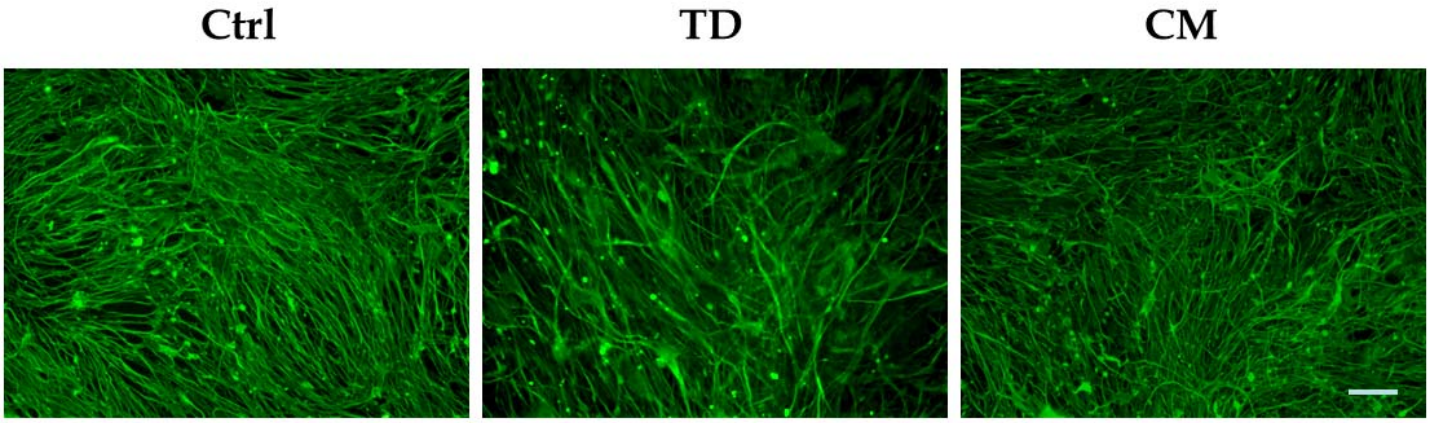
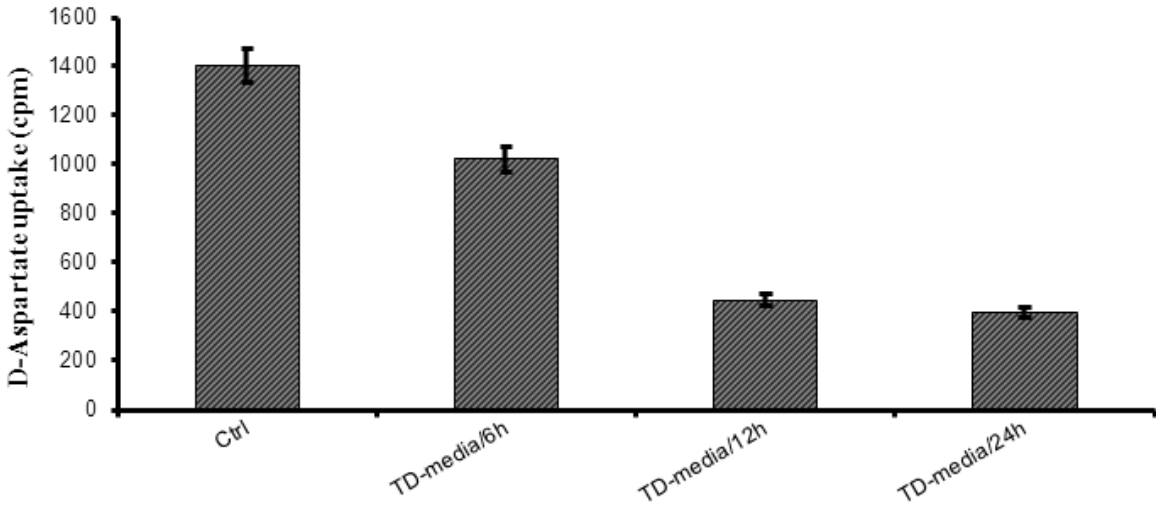


FIGURE 1

A)


D-Aspartate uptake (cpm)
1600
1400
1200
1000
800
600
400
200
0
Ctrl
TD-media/6h
TD-media/12h
TD-media/24h

B)

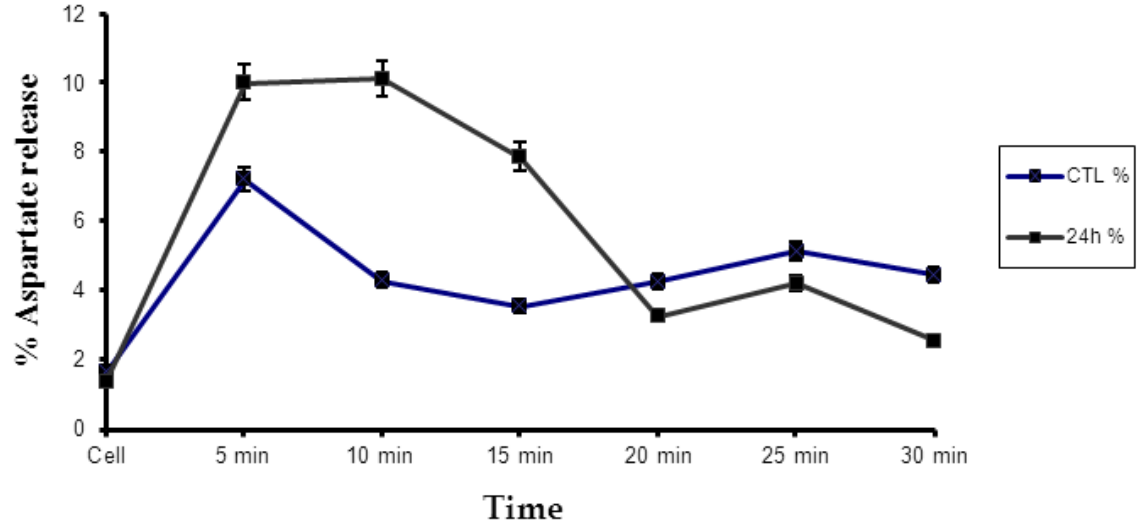

% Aspartate release
12
10
8
6
4
2
0
Cell
5 min
10 min
15 min
20 min
25 min
30 min
Time
CTL %
24h %

FIGURE 2

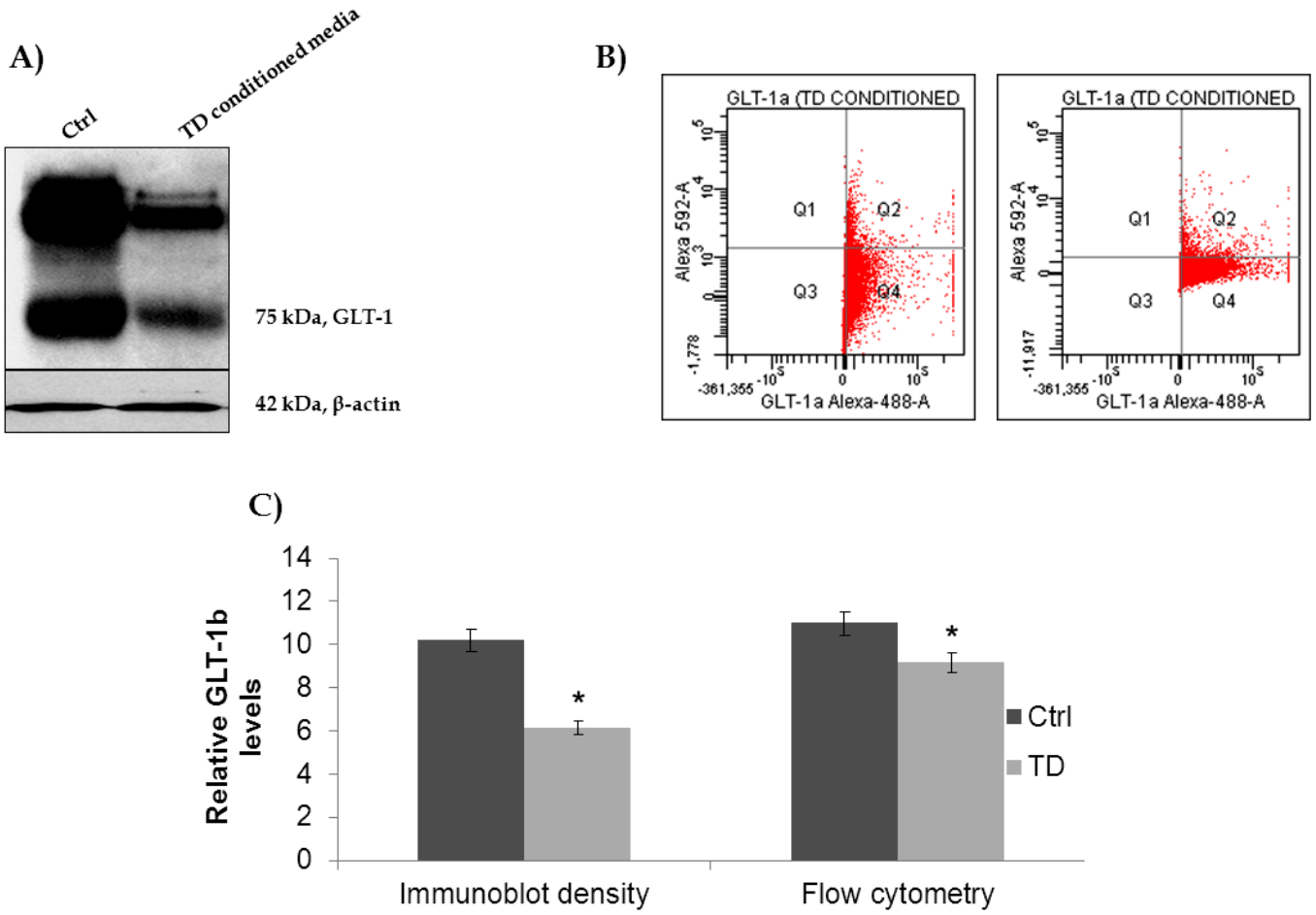

A)
Ctrl
TD conditioned media
75 kDa, GLT-1
42 kDa, β-actin
B)
GLT-1a (TD CONDITIONED
Alexa 592-A
GLT-1a Alexa-488-A
Q1
Q2
Q3
Q4
C)
Relative GLT-1b levels
Immunoblot density
Flow cytometry
Ctrl
TD

FIGURE 3

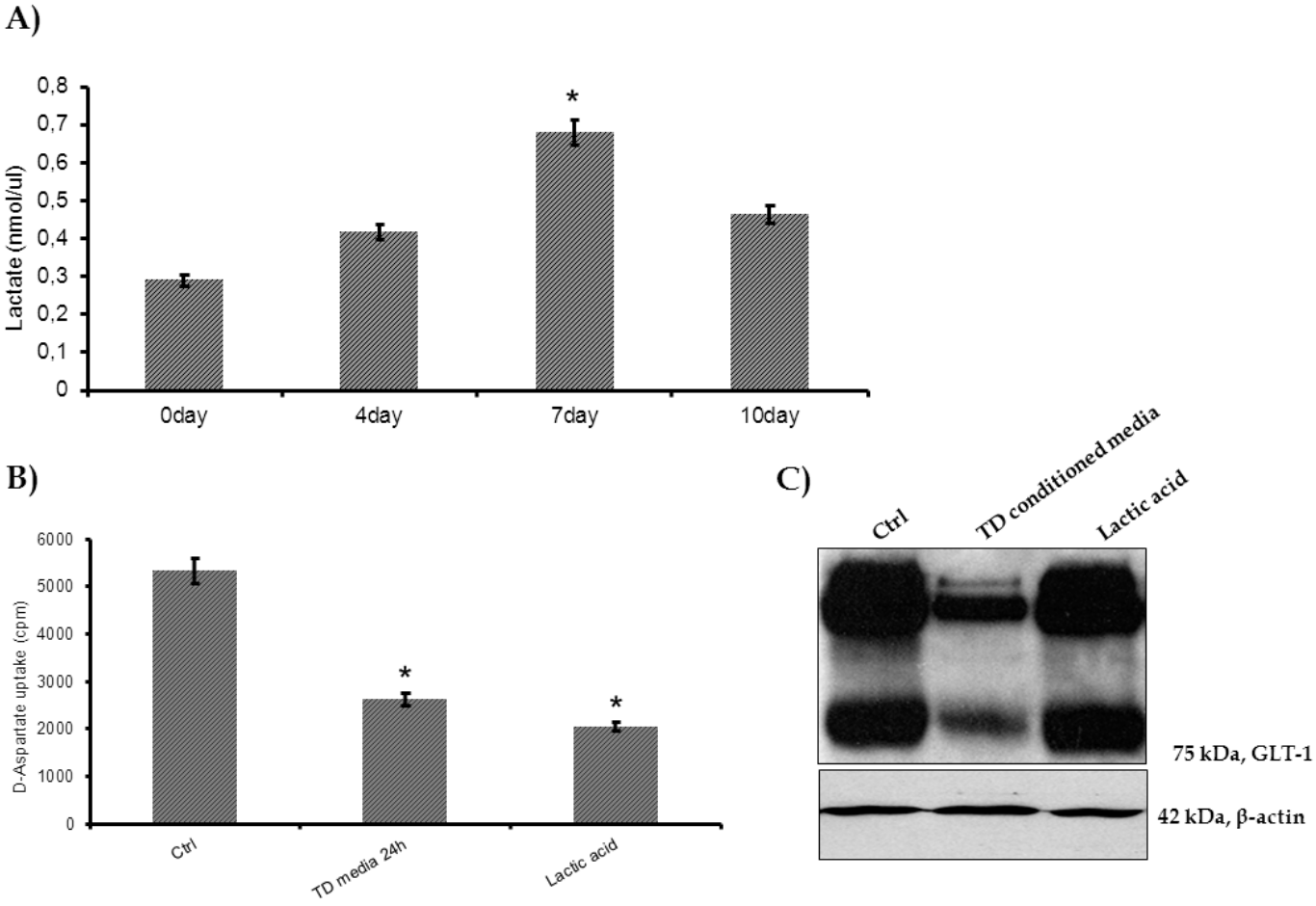

A)
Lactate (nmol/ul)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
*
0day
4day
7day
10day
B)
D-Aspartate uptake (cpm)
6000
5000
4000
3000
2000
1000
0
*
*
Ctrl
TD media 24h
Lactic acid
C)
Ctrl
TD conditioned media
Lactic acid
75 kDa, GLT-1
42 kDa, β-actin

FIGURE 4

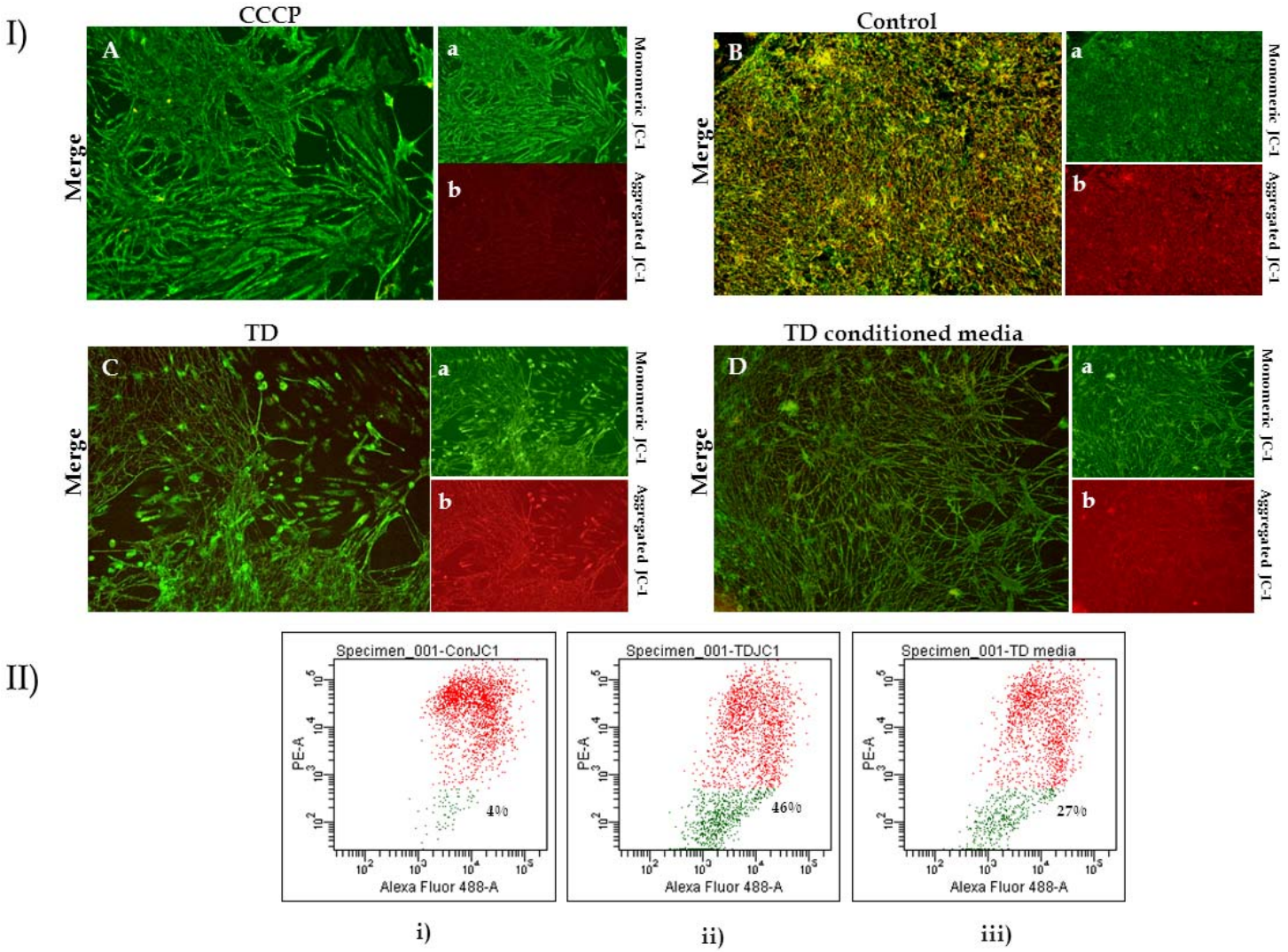

I)
CCCP
A
a
b
Merge
Monomeric JC-1
Aggregated JC-1
Control
B
TD
C
TD conditioned media
D
II)
Specimen_001-ConJC1
Specimen_001-TDJC1
Specimen_001-TD media
PE-A
Alexa Fluor 488-A
40%
46%
27%
i)
ii)
iii)

FIGURE 5

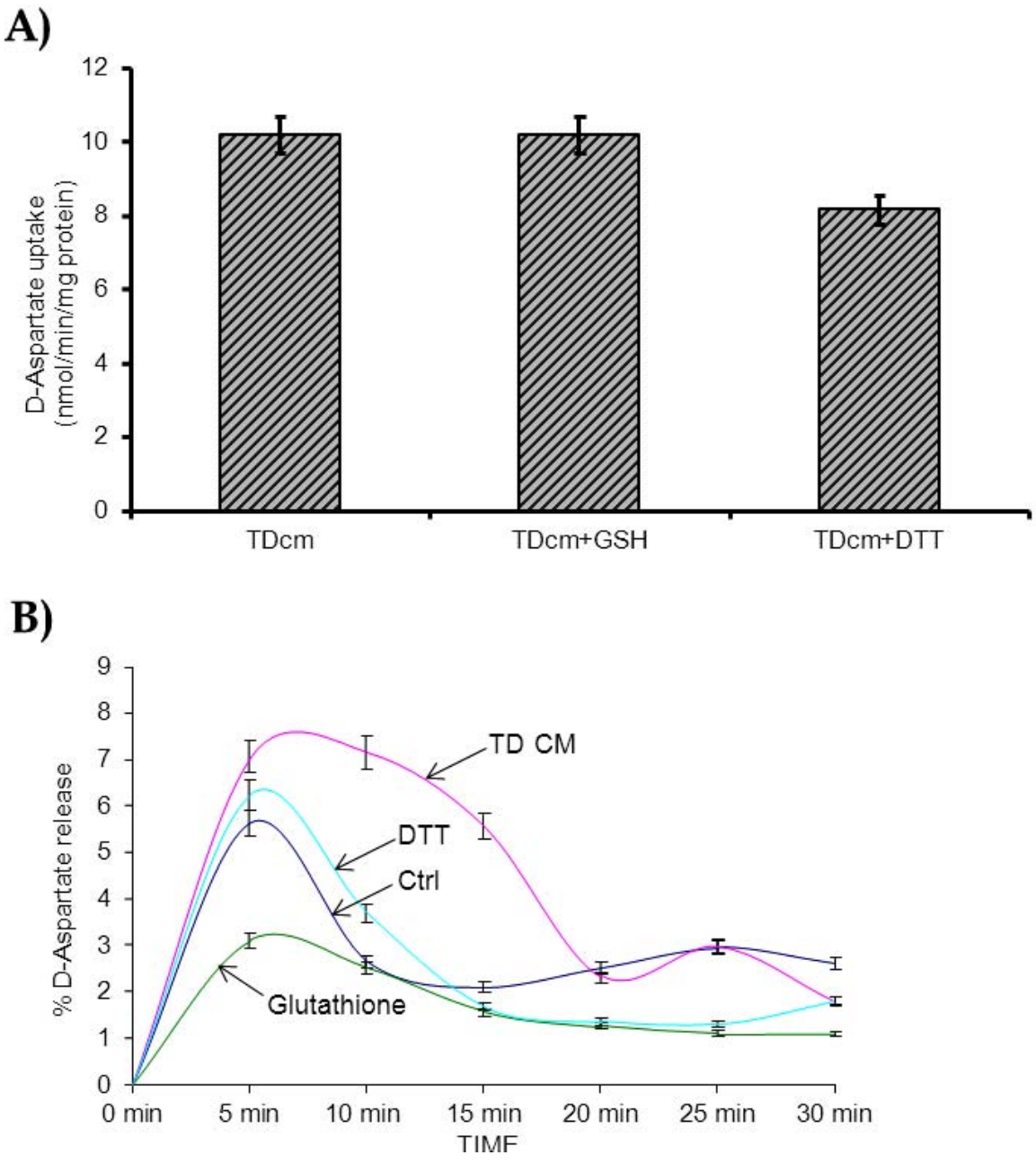

A)
D-Aspartate uptake (nmol/min/mg protein)
0
2
4
6
8
10
12
TDcm
TDcm+GSH
TDcm+DTT
B)
% D-Aspartate release
0
1
2
3
4
5
6
7
8
9
TD CM
DTT
Ctrl
Glutathione
0 min
5 min
10 min
15 min
20 min
25 min
30 min
TIME

FIGURE 6

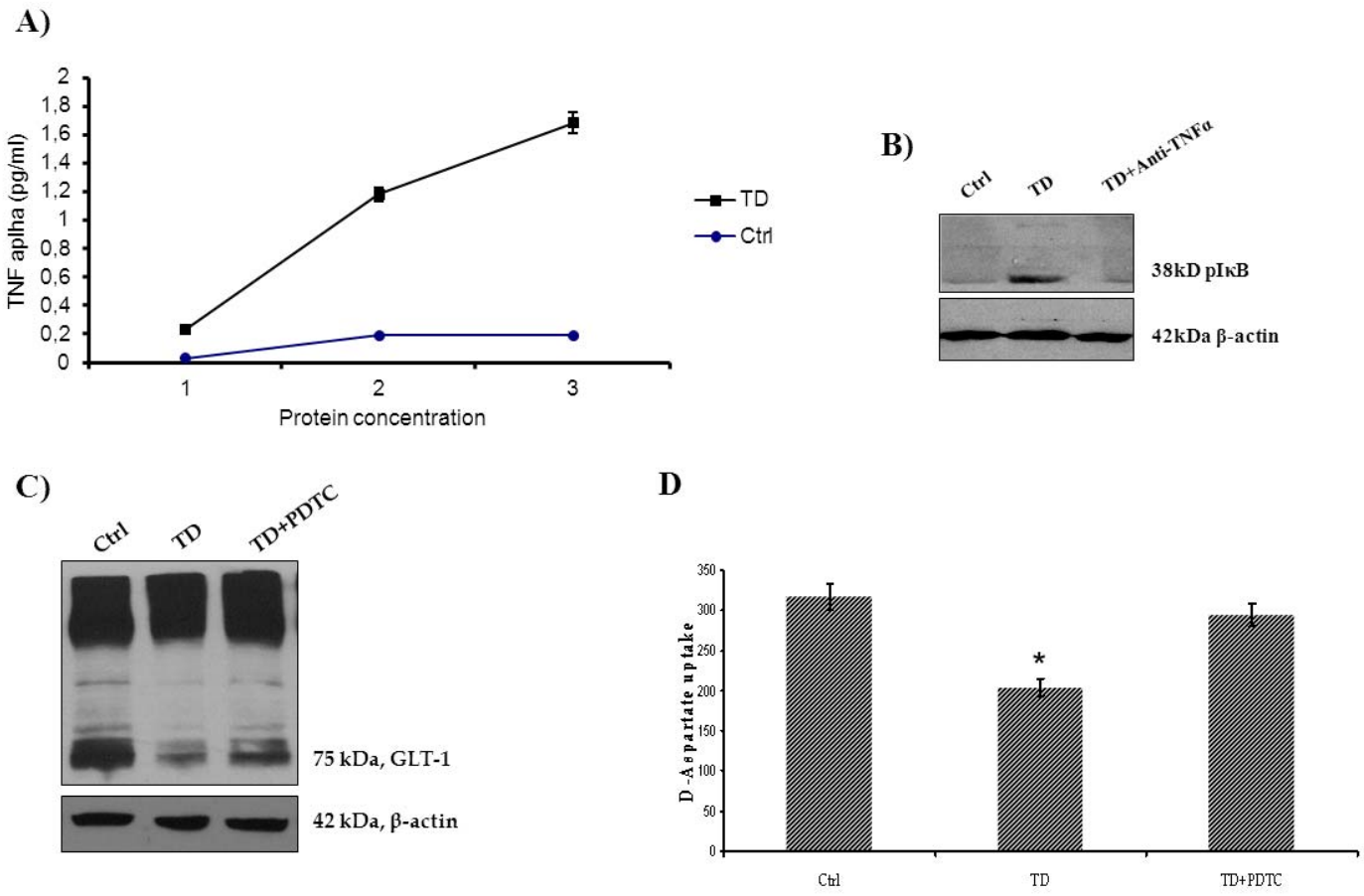

A)
TNF aplha (pg/ml)
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1
2
3
Protein concentration
TD
Ctrl
B)
Ctrl
TD
TD+Anti-TNFα
38kD pIκB
42kDa β-actin
C)
Ctrl
TD
TD+PDTC
75 kDa, GLT-1
42 kDa, β-actin
D
D-Aspartate uptake
350
300
250
200
150
100
50
0
*
Ctrl
TD
TD+PDTC

FIGURE 7

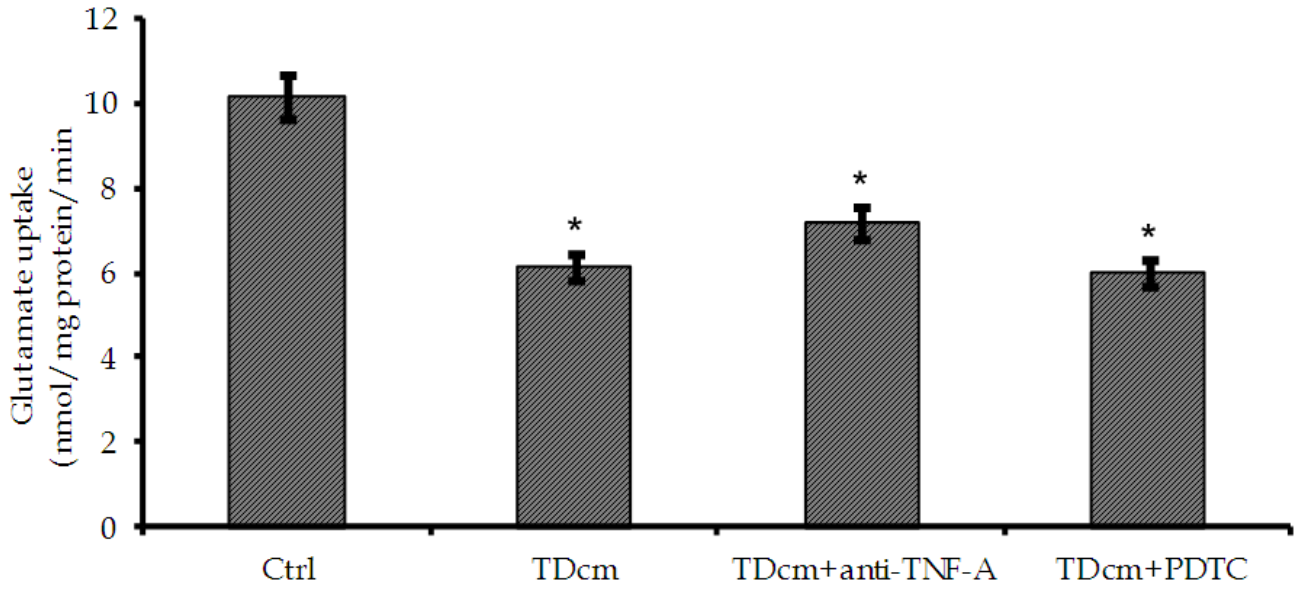

12
10
8
6
4
2
0
Glutamate uptake
(nmol/mg protein/min
*
*
*
Ctrl
TDcm
TDcm+anti-TNF-A
TDcm+PDTC

FIGURE 8

CHAPTER 5

GENERAL DISCUSSION

5.1 REGULATION OF ASTROCYTIC GLUTAMATE TRANSPORTERS DURING THIAMINE DEFICIENCY

This section constitutes a discussion and overview of the main findings of the three articles presented in the thesis.

5.1 Ceftriaxone protects against GLT1b splice variant loss in TD

Data presented in the first article examined the effects of ceftriaxone on the glutamate transporter splice variant GLT-1b in TD and in cultured astrocytes under conditions of TD. The results of this study suggest that TD results in the loss of GLT-1b in the vulnerable thalamus and inferior colliculus, while no change was observed in the non vulnerable frontal cortex. Similar findings were obtained with TD treated astrocyte cultures. Concomitantly, significant reductions in the uptake of D-aspartate (a non-metabolizable analog of glutamate) was observed. These findings extend earlier observations of suppressed high affinity glutamate/D-aspartate uptake and loss of glutamate transporters under conditions of TD described by Hazell et. al (2003). Recently, Rothstein and colleagues (2005) have investigated the effects of ceftriaxone, a β-lactam antibiotic on GLT-1 expression. The authors reported that ceftriaxone confers neuroprotection against ischemic and motor neuron degeneration by GLT-1 upregulation. We attempted to extend this hypothesis under conditions of TD. Results of our study indicate that ceftriaxone upregulates GLT1-b levels in the inferior colliculus, with no significant changes in the thalamus and frontal cortex in TD rats. In addition, ceftriaxone also stimulated glutamate uptake and upregulated GLT-1b levels in astrocytes during TD.

Decreased uptake of D-aspartate/glutamate may be a result of a loss in glutamate transporter uptake capacity or due to decreased expression of glutamate transporters.

Since protein levels of the GLT-1b splice variant were significantly altered under conditions of TD, this suggests that the TD suppressive effect on astrocytic glutamate uptake occurs at the level of transporter expression. Next, to extend this possibility we analysed the GLT-1b mRNA expression, the results of which showed no significant change in the expression pattern. Thus in contrast to GLT-1b protein levels, the lack of alteration in GLT-1b expression indicates that effects of TD on astrocytic glutamate uptake are likely determined by post-translational mechanisms. The decrease in glutamate transporter protein levels with no change in the mRNA expression is consistent with previous studies, e.g. glutamate-mediated increase in GLAST protein levels was not accompanied by an increase in the expression of mRNA (Swanson et .al., 1999). Such loss of glutamate transporters is regulated at different levels through different signalling pathways depending on the stimulating factors.

Ceftriaxone has proven to be a protective strategy against excitotoxicity and oxidative stress in stroke, cocaine relapse, hypobaric hypoxia and Huntington's disease. Consistent with the earlier findings of ceftriaxone mediated neuroprotection, results of our study suggest that ceftriaxone mediated neuroprotection against excitotoxicity also operates under conditions of TD.

These region specific effects of ceftriaxone are potentially due to selective vulnerability of certain brain areas to local differences of blood supply (Gibson et. al., 1997), differences in tissue requirements for TPP (Dreyfus, 1959), differences in GLT-1 content (Danbolt et. al., 1998) in the inferior colliculus and thalamus during TD. In

addition, activation of NF-κB by ceftriaxone, a transcription factor that plays a role in regulating immune responses and cell survival, has been proposed as an alternative mechanism in the regulation of glutamate transporter, GLT-1 by ceftriaxone. It has been reported that ceftriaxone acts as a potential stimulator of NF-κB activation and its translocation into the nucleus resulting in increased GLT-1 promoter activity (Lee et. al. 2008). However, TD results in no significant alterations in GLT-1 mRNA levels, thus it is likely that ceftriaxone mediated effects on the increased protein levels of GLT-1b is due to its effects on posttranslational mechanisms. Furthermore, it is interesting in this regard that palmitoylation, a process by which proteins are inserted into cellular membranes, is reduced for GLT-1 under certain pathological conditions (Huang and El-Husseini, 2005; Huang et. al., 2010), it would be interesting to see if ceftriaxone increases GLT-1 palmitoylation during TD.

5.2 Regulation of glutamate transporters by PARP-1 and NF-κB signaling pathways

The second article presented in my thesis deals with the mechanisms involved in the loss of glutamate transporters under conditions of TD. It is evident from past studies that NF-κB can act both as a positive or negative modulator of glutamate transporter regulation. The action of NF-κB as a positive regulator of glutamate transporters has been linked to ceftriaxone, brain-derived neurotrophic factor, β-amyloid, amitriptyline, EGF, TGF-α, and dbcAMP, as well as bromo-cAMP dependent upregulation of GLT-1 (Lee et al., 2008). On the contrary, inhibition of NF-κB signalling pathways has been shown to restore GLT-1 promoter activity during treatment with TNF-α. This was further confirmed with the identification of a consensus sequence for NF-κB binding within the

5′-untranslated region of the GLT-1 complementary DNA (cDNA) clones (Meyer et al. 1996). Thus, NF-κB activation is a diverse phenomenon in the regulation of glutamate transporters and it may act both as a repressor or activator depending on the nature of pathological stimuli. Based on the previous findings we hypothesized that NF-κB dependent signaling pathways contribute to the loss of glutamate transporters in TD, and NF-κB signaling in astrocytes in part is regulated by PARP-1 mechanisms. Support for this possibility was provided by our demonstration that TD results in activation of PARP-1 both *in vivo* and *in vitro* and inhibition of PARP-1 activity significantly blocked NF-κB activation and protects against the loss of GLT-1 transporters in the astrocytes.

Data presented in this study revealed a potential link between glutamate signaling and NF-kB activation in relation to poly (ADP) ribosylation under conditions of TD. Excessive oxidative DNA strand breaks lead to PARP-1-induced depletion of cellular NAD, glycolytic rate, ATP levels, and eventual cell death. Blocking PARP-1 activity using knock-out or siRNA for PARP-1 has shown protective effects on cell survival under various conditions, including streptozotocin-induced diabetes (Pieper et al., 1999) and cardiac and renal ischemia–reperfusion injuries (Pieper et al., 2000). While the mechanism involved in PARP-1 mediated cell death is not completely understood, a few studies have reported protective effects of PARP-1 inhibitors on the activation of NF-κB (Czapski et. al., 2004). Our flow cytometry and immunoblot data results suggest that PARP-1 inhibition using a pharmacological inhibitor, DPQ, significantly blocked the activation of NF-κB in the primary astrocyte culture under TD conditions. Blocking NF-κB activation showed no significant change in the PARP-1 activity, suggesting that

PARP-1 activation is an independent mechanism and is employed during metabolic inhibition such as that observed under TD conditions.

Overactivation of NF-κB is implicated in the pathogenesis of excitotoxicity and glutamate transporter dysregulation. Efficient glutamate transmission requires continued uptake of glutamate via energy dependent transporters located on the astrocytes, and our findings suggest that activation of astrocytic PARP-1 due to DNA damage leads to glutamate transporter downregulation by NF-κB dependent signaling pathways.

5.3 Effects of lactate accumulation on glutamate transporters during TD

Metabolic inhibition with substantial lactate production in brain is a classical feature of TD and WE. Lactic acid is known to cause astrocyte swelling, a feature of the disorder, and since swelling of these cells has previously been shown to inhibit glutamate uptake (Kimelberg et. al., 1990), we examined whether excessive lactate production during TD may contribute to excitotoxicity by inhibition of astrocytic glutamate transporter function. The findings presented in the third article demonstrate that conditioned media from TD astrocytes produced a downregulation of GLT-1 (GLT-1b splice variant) and loss of glutamate uptake capacity of the astrocytes. Measurement of lactate concentration in the media showed increased lactate levels under conditions of TD.

Previous studies have reported increased glutamate release and decrease in glutamate uptake after exogenous lactate administration in primary astrocyte cultures (Bender et. al., 1997). Given the bulk of extracellular lactate accumulation during TD, we wanted to determine whether the lactate accumulation during TD might be responsible

for glutamate transporter dysfunction. Thus we investigated the role of lactate accumulation in the regulation of glutamate transporters in astrocytes. Using a specific inhibitor for the monocarboxylic acid transporter which transports lactate, 4-CIN, we determined that lactate by itself is not a major contributor in glutamate transporter dysfunction. Thus lactate induced decrease in glutamate uptake may be due to the acidic factors associated with lactate accumulation.

It is evident that acidosis due to excessive accumulation of lactic acid may inhibit glutamate uptake directly by its effect on the transporters, possibly by affecting the charged groups on the glutamate carrier proteins thereby altering the tertiary structure of the transporter (Barbour et. al., 1988). Thus, decreased glutamate uptake could also result due to ionic imbalance. Hence, accumulation of lactic acid and other soluble factors may contribute to decreased glutamate uptake capacity and may be involved in GLT-1 loss in astrocytes during TD.

5.4 Effects of mitochondrial dysfunction on glutamate transporters in TD

Mitochondria are important integrators of cellular function that plays an important role in producing ATP through oxidative phosphorylation, control of cytosolic Ca^{2+} concentration and energy metabolism. Mitochondria are also one of the major cellular producers of reactive oxygen species (ROS). Many of these functions of mitochondria are regulated by the electrochemical gradient across the mitochondrial membrane or mitochondrial membrane potential ($\Delta\psi m$). Neuronal injury leading to excitotoxicity can produce a profound depolarization of mitochondrial membrane including alterations in intracellular calcium dynamics and the opening of mitochondrial permeability transition

pore (MPTP) (Ankarcrona et al., 1995). Mitochondrial permeability transition (MPT) is a key event in the pathology of TD due to altered Ca^{2+} homeostasis, enhanced ROS production and decreased ATP synthesis leading to neuronal loss in the specific brain regions (Leong et al., 1994; Bettendorff et. al., 1995).

Early mitochondrial damage is a critical downstream event in glutamate-mediated excitotoxicity (White and Reynolds 1996). We now propose that mitochondria may be an early target of injury in the cascade of events that leads to WE. This is confirmed with our findings that the exposure of naïve astrocytes to TD conditioned media for 12h to 24h leads to increased $\Delta\psi m$. While this finding in TD is perhaps not that surprising given our understanding that TD results in decreased ATP levels in vulnerable brain regions (Aikawa et al., 1984), indicating failure of energy metabolism, it is particularly interesting that 12-24h treatment of naïve astrocytes with TD conditioned media results in disruption/collapse of $\Delta\psi m$ in these cells. This suggests that the soluble factors responsible for downregulating GLT-1 are likely particularly efficacious in their effects on mitochondrial functions under conditions of TD. Thus, an early compromise in mitochondrial function (e.g., the induction of the permeability transition) may leave cells ill-prepared to deal with the later cellular energy drain associated with TD.

The reversibility of mitochondrial potential to normal levels after an insult depends on the extent of MPT. If MPT occurs to only a slight extent, the cell may recover, whereas if it occurs more it may undergo apoptosis. If it occurs to an even larger degree the cell is likely to undergo necrotic cell death (Honda and Ping, 2006). Given that severe energy depletion is a major event in the TD pathology, it appears that TD results in more severe impairment of mitochondrial function. This is supported by our present

findings that showed the effects of astrocytic soluble factors on Δψm, in addition enhanced activation of the PARP-1 enzyme that drain astroglial reserves of ATP and nicotinamide nucleotides. There are several studies in the past that indicate that the factors that increase the likelihood of MPT induction are altered during TD (Bettendorff et. al., 1995). Most importantly, TD induces a decrease in the activity of KGDH, leading to inhibition of mitochondrial energy metabolism and decreased ATP production, leading to MPT induction in this disease pathophysiology. Consequently, MPT increases ROS production, Ca^{2+} influx and causes Ca^{2+}overload that promote excitotoxic brain damage in TD pathophysiology. Earlier studies (Zhang et al., 1994) complement our present findings and invites speculation with respect to the mechanism of cell death under conditions of TD.

5.5 Effects of TNF-α production and NF-κB activity on glutamate transporters during TD

Increased expression and release of TNF-α has been reported in cerebrospinal fluid (CSF), plasma, or post mortem brain tissue in patients following stroke, brain injury, HIV dementia, alcoholism, and Alzheimer's disease, trauma, ischemia, and inflammatory diseases (Allan and Rothwell, 2001). Since TNF-α in the brain can also stimulate NF-κB activity which is implicated in the regulation of glutamate transporters, we investigated if TD progresses with increased release of TNF-α in the medium in order to see if a TNF-α mediated NF-κB dependent process is involved in the regulation of glutamate transporters in naïve astrocytes treated with conditioned media. Our data indicate that TNF-α is released into the media but that inhibition of TNF-α had no effect

on the reduced glutamate uptake capacity in naïve treated astrocytes. On the other hand, TNF-α inhibition in astrocytes with TD showed protection against the loss of GLT-1 protein and restored glutamate uptake capacity. However, TNF-α inhibition significantly reduced NF-κB activation in the astrocytes during TD, and inhibition of NF-κB ameliorated the decrease in GLT-1 due to TD. Thus, regulation of astrocytic glutamate transporters is determined by a TNF-α-mediated NF-κB dependent process.

5.6 Consequences of glutamate transporter downregulation in TD

Excitatory amino acid transporters are considered to be a potential target for neuroprotection against excitotoxicity. GLT-1 is considered as the major glutamate transporter responsible for clearance of glutamate from the extracellular space (Lehre and Danbolt, 1998). In addition, considerable evidence indicates that GLT-1 contributes to excitotoxicity and neuronal death in a number of neurological disorders, including ischemic stroke, traumatic brain injury, and amyotrophic lateral sclerosis (Allen et al., 2004; Maragakis and Rothstein, 2004). Previous studies indicate that extracellular glutamate concentration is increased in vulnerable brain regions in TD (Hazell et al., 1993, Langlais and Zhang, 1993). This effect of TD on glutamate transporter levels was also demonstrated in primary cultures of astrocytes (Hazell et. al., 2003). Furthermore, it has recently been demonstrated that loss of GLT-1 and GLAST also occurs in the cerebral cortex of human cases of WE (Hazell et. al., 2010). These studies indicate that downregulation of glutamate transporters likely leads to an excitotoxic event in TD.

Focal accumulation of lactic acid is a classical feature of TD and one of its major consequences is gliotoxicity (Navarro et. al., 2005). Astrocytes are particularly sensitive

to lactate and exposing them to extended periods of lactic acidosis leads to an inability of these cells to maintain ATP production (Bender et. al., 1997). The resulting decrease in ATP levels then causes a collapse of the ionic gradients across the astrocyte cell membrane due to suppression of Na^+ /K^+ ATPase activity, leading to movement of Na^+ into the cell along its concentration gradient. Elevated intracellular Na^+ concentration results in transport of glutamate from the astrocyte to the extracellular space (Danbolt 2001). Moreover, astrocytic swelling due to glutamate uptake or excessive K^+ spatial buffering can lead to depolarization of these cells, resulting in the release of glutamate via transporter reversal (Danbolt 2001). The major consequence of this transporter reversal is an increase in extracellular glutamate concentration which is a major causative factor implicated in excitotoxicity (Danbolt 2001).

Thus, severe impairment of reuptake of glutamate during TD could lead to elevated extracellular levels of glutamate. Alternatively, glutamate uptake in astrocytes is primarily mediated by GLT-1 and GLAST glutamate transporters (Danbolt 2001). Loss of these transporters has been reported in vulnerable areas of the brain of rats with TD and in astrocytes with TD (Hazell et. al., 2001; 2003; Jhala et. al., 2011). Additionally, increased activity of the cystine-glutamate exchanger can play a role in excitotoxicity by exporting glutamate out of the astrocyte into the extracellular space while enhancing cystine uptake for purposes of increased synthesis of the antioxidant glutathione in the cell in response to increasing oxidative stress (Lewerenz et al., 2009). Focal cerebral vulnerability is therefore a major consequence of TD which is associated with excitotoxic mediated brain damage, and elevated interstitial glutamate concentration as a result of the energy deficit is one of the key factors involved in the pathogenesis of this disorder.

5.7 Implications for TD-induced loss of glutamate transporters

Cerebral vulnerability to an excitotoxic insult is governed by the cellular processes responsible for the maintenance of glutamate (homeostatic) concentration in the extracellular space; these include glutamate transport/uptake and release mechanisms. Regulation of glutamate transporter activity in adverse conditions is a prerequisite for normal synaptic transmission. Any disturbance of these processes may lead to an irreversible process of cell death or excitotoxicity (Doble 1999; Danbolt 2001). Consequently, loss of glutamate uptake and the resultant elevation in extracellular glutamate levels plays a major role in the pathophysiology of TD (Fig. 6) (Hazell 2009). Elevations of extracellular glutamate may also be a contributing factor to seizure activity. Seizures can be observed at the late stage in experimental TD (Langlais et al., 2002) and are observed in rare cases of alcoholics with encephalopathy as SESA (Subacute encephalopathy with seizures in alcoholics) syndrome (Rothmeier et al., 2001; Fernández-Torre et al., 2006). Moreover, Tsuro et al. (2002) demonstrated that EAAT1-knockout mice were more susceptible to amygdala-kindled seizures and displayed a more severe and prolonged type of seizure. Thus, it would be of scientific importance to investigate the nature of seizure activity observed in TD and establish if seizures are a cause or consequence of loss of glutamate transporters.

Based on earlier studies, viable approaches to overcome excitotoxicity might be to upregulate the activity of the predominant GLT-1 subtype either at the level of small-molecule activators (e.g. ceftriaxone) or by independently promoting gene expression. In addition, better pharmacological tools are required to define the precise contributions of

GLT-1 to the regulation of extracellular glutamate concentration and to ultimately understand their potential role as drug targets.

Antioxidant *N*-acetylcysteine treatment has been shown to be a protective strategy against the loss of glutamate transporters in TD (Hazell et. al., 2010). Moreover, the antioxidant potential of ceftriaxone is confirmed in the disease model of ischemic brain injury (Lewerenz et al., 2009). In relation to these earlier findings along with implications of PARP-1 and TNF-α dependent mechanisms presented in the second and third articles, it is possible that oxidative stress is the major contributing factor to TD-induced suppression of glutamate transporter activity.

In the present studies it has been shown that impaired energy metabolism and its subsequent oxidative DNA damage can lead to overactivation of PARP-1 enzyme, enhanced lactate production and activation of TNF-α, leading to increased NF-κB signaling. NF-κB activation has been introduced as a major exacerbating factor in the pathogenesis of excitotoxicity in variety of neurodegenerative diseases, and is often considered as a major causal factor associated with excitotoxic insults. The experimental evidence provided in this thesis showing the interaction between energy impairment and excitotoxicity may therefore increase our understanding of how a vitamin deficiency can produce such devastating cerebral damage, and perhaps lead to new therapeutic approaches for the treatment of neurodegeneration as a whole.

5.8 Mechanism of selective brain damage in TD

Multiple mechanisms contribute to the selective brain lesions observed in WKS and experimental TD. Neuropathological evaluation of the brains of patients with WKS

reveals a highly selective damage primarily in diencephalic and brainstem structures. Moreover, bilateral symmetrical lesions are consistently observed in thalamic nuclei, inferior colliculi, inferior olivary nuclei, mammillary bodies, and lateral vestibular nuclei (Torvik et. al., 1985; 87). Other major structures, including cerebral cortex, caudate nuclei, and hippocampi manifest no significant damage in WKS (Troncoso et. al., 1981). This region selective brain damage is a consequence of a cascade of events in the pathology of TD. For example, brain structures that manifest selective vulnerability to TD are those with high thiamine turnover rates (Dreyfus, 1959) high rates of oxidative metabolism, selectively decreased activities of α- KGDH (Butterworth et. al., 1986), early microglial activation (Wang and Hazell, 2010) and increased free radical production (Hazell et. al., 1998). Additionally, increase in the extracellular glutamate concentration concomitant with the loss of astrocytic glutamate transporter, GLAST and GLT-1 localized to vulnerable brain regions have been reported earlier in TD (Hazell et al., 1993; 2001; Langlais and Zhang, 1993). Furthermore, evidence were also provided for the excitotoxic-like lesions in damaged areas of the brain (Armstrong-James et al., 1988), suggesting an involvement of glutamate-mediated excitotoxicity in pathophysiology. Major findings presented in this thesis project, suggesting the loss of the GLT-1 protein along with decreased glutamate transport kinetics both in TD astrocyte culture and animal model of the disorder, add further evidence for implication of a loss of glutamate transporter function in the selective vulnerability of brain damage in the pathophysiology of TD. Thus, the selective vulnerability to TD may be mediated by a glutamate-mediated excitotoxic process in affected structures, leading to alterations in mitochondrial

membrane potential due to inhibition of ATP synthesis and disturbances in calcium homeostasis.

5.9 Contribution to knowledge

A brief summary of the data presented in the three articles which constitute this thesis include the following:

Loss of glutamate transporter splice variant GLT-1b and its protection by ceftriaxone

Data provided in this thesis article demonstrate that TD results in the loss of glutamate transporter splice variant GLT-1b and thereby inhibits the glutamate uptake capacity of the astrocytes. Treatment with ceftriaxone significantly blocks this downregulation and restored glutamate uptake capacity. These findings suggest the enhanced interstitial brain glutamate levels observed in TD to some extent are a result of TD-induced downregulation of GLT-1b protein levels, and ceftriaxone may be a potential therapeutic strategy against the loss of these transporters.

PARP-1 dependent activation of NFκB signalling pathways regulate glutamate transporters during TD

The data presented in the second article suggest that impaired oxidative metabolism can lead to PARP-1 dependent activation of NF-κB signalling cascades leading to glutamate transporter dysfunction under conditions of TD. The major findings of this study suggest that excessive poly (ADP) ribosylation due to increased DNA damage promotes NF-κB activation and regulates glutamate transporters activity by protein modification.

Glutamate transporters are regulated by extracellular factors released from astrocytes

Findings presented in the first and second articles demonstrated the loss of glutamate transporters under conditions of TD and a role for NF-κB in the regulation of glutamate transporters. In the third article we report an alternative mechanism that may operate under conditions of TD but also involving NF-κB. Major results of this study suggest that TNF-α mediated NF-κB is involved in the regulation of astrocytes under TD conditions. In addition, soluble factors and metabolic related substances accumulate extracellularly, contributing to astrocytic glutamate transporter dysfunction, e.g. increased lactic acidosis, or by ionic imbalance that affects functional characteristics of the glutamate transporters.

Based on the data presented in this thesis it is evident that glutamate transporters in TD are regulated by posttranslational influences or through protein-protein interactions with specific proteins (Fig. 5). The GLT-1 is known to be regulated by phosphorylation, ubiquitination, and glycosylation and therefore it would be interesting to examine the effect of TD on these posttranslational mechanisms. In addition there are several interacting molecules that could be examined, e.g. Sept2 is a molecule that interacts with GLAST and downregulates its function (Kinoshita et. al., 2004). JM4 is a homologue to JWA that downregulates the function of EAAT1-4 by interacting with exon9 and preventing ER exit (Schweneker et. al., 2005; Ruggiero et. al., 2008). CNTF is a molecule that increases expression of GLAST at the plasma membrane (Escartin et. al., 2006). Analysis of these molecules would likely provide a more complete picture of the abnormalities in EAAT regulation in TD.

SUMMARY OF FINDINGS

The objective of this thesis was to study the mechanisms of glutamate transporter downregulation in TD encephalopathy. Results of the studies presented suggest that downregulation of glutamate transporters, both in animal and cell culture models, can be attenuated by the administration of ceftriaxone, a β-lactam antibiotic. Poly (ADP) ribose mediated NF-kB activation is a major signaling pathway now linked to the loss of glutamate transporters during TD. Furthermore, the release of soluble factors from astrocytes exposed to TD conditions can influence their functional integrity at the level of these glutamate transporters.

REFERENCES

Ahmed, A., Daida, Y., & Novotny, R. (2011). PS2-02: Micronutrient Deficiencies After Bariatric Surgery: Does Ethnicity Matter? Clin Med Res, 9(3-4), 165.

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.

Aisen, P. S., & Davis, K. L. (1994). Inflammatory mechanisms in Alzheimer's disease: implications for therapy. Am J Psychiatry, 151(8), 1105-1113.

Al-Attas, O. S., Al-Daghri, N. M., Alfadda, A., Abd Al-Rahman, S. H., & Sabico, S. (2011). Blood Thiamine and Derivatives as measured by High-Performance Liquid Chromatography: Levels and Associations in DM Patients with Varying Degrees of Microalbuminuria. J Endocrinol Invest.

Albrecht, J., Talbot, M., Kimelberg, H. K., & Aschner, M. (1993). The role of sulfhydryl groups and calcium in the mercuric chloride-induced inhibition of glutamate uptake in rat primary astrocyte cultures. Brain Res, 607(1-2), 249-254.

Alexander, L., Pijoan, M. and Myerson, A. (1938). Beriberi and Scurvy. Trans. Am. Neurol. Assoc. , 64, 135-139.

Allan, S. M., & Rothwell, N. J. (2001). Cytokines and acute neurodegeneration. Nat Rev Neurosci, 2(10), 734-744.

Allen, D. N., Goldstein, G., & Seaton, B. E. (1997). Cognitive rehabilitation of chronic alcohol abusers. Neuropsychol Rev, 7(1), 21-39.

Allen, N. J., Karadottir, R., & Attwell, D. (2004). Reversal or reduction of glutamate and GABA transport in CNS pathology and therapy. Pflugers Arch, 449(2), 132-142.

Anderson, S. H., Charles, T. J., & Nicol, A. D. (1985). Thiamine deficiency at a district general hospital: report of five cases. Q J Med, 55(216), 15-32.

Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., et al. (1995). Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron, 15(4), 961-973.

Armstrong-James, M., Ross, D. T., Chen, F., & Ebner, F. F. (1988). The effect of thiamine deficiency on the structure and physiology of the rat forebrain. Metab Brain Dis, 3(2), 91-124.

Arundine, M., & Tymianski, M. (2003). Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. Cell Calcium, 34(4-5), 325-337.

Avdulov, N. A., Chochina, S. V., Igbavboa, U., O'Hare, E. O., Schroeder, F., Cleary, J. P., et al. (1997). Amyloid beta-peptides increase annular and bulk fluidity and induce lipid peroxidation in brain synaptic plasma membranes. J Neurochem, 68(5), 2086-2091.

Baldwin, A. S., Jr. (1996). The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol, 14, 649-683.

Barbour, B., Brew, H., & Attwell, D. (1988). Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. Nature, 335(6189), 433-435.

Beauchesne, E., Desjardins, P., Hazell, A. S., & Butterworth, R. F. (2009). Altered expression of tight junction proteins and matrix metalloproteinases in thiamine-deficient mouse brain. Neurochem Int, 55(5), 275-281.

Beauchesne, E., Desjardins, P., Hazell, A. S., & Butterworth, R. F. (2009). eNOS gene deletion restores blood-brain barrier integrity and attenuates neurodegeneration in the thiamine-deficient mouse brain. J Neurochem, 111(2), 452-459.

Becher, A., White, J. H., & McIlhinney, R. A. (2001). The gamma-aminobutyric acid receptor B, but not the metabotropic glutamate receptor type-1, associates with lipid rafts in the rat cerebellum. J Neurochem, 79(4), 787-795.

Behrens, P. F., Franz, P., Woodman, B., Lindenberg, K. S., & Landwehrmeyer, G. B. (2002). Impaired glutamate transport and glutamate-glutamine cycling: downstream effects of the Huntington mutation. Brain, 125(Pt 8), 1908-1922.

Bender, A. S., Young, L. P., & Norenberg, M. D. (1997). Effect of lactic acid on L-glutamate uptake in cultured astrocytes: mechanistic considerations. Brain Res, 750(1-2), 59-66.

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

Bettendorff, L., Goessens, G., Sluse, F., Wins, P., Bureau, M., Laschet, J., et al. (1995). Thiamine deficiency in cultured neuroblastoma cells: effect on mitochondrial function and peripheral benzodiazepine receptors. J Neurochem, 64(5), 2013-2021.

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.

Bojinov, S. (1971). Encephalitis with acute Parkinsonian syndrome and bilateral inflammatory necrosis of the substantia nigra. J Neurol Sci, 12(4), 383-415.

Bowman, K., Goodhart, R. and Jolliffe, N. (1939). Observations of role of Vitamin B1 in the etiology and treatment of Korsakoff psychosis. J. Nerv. Ment. Dis. , 90, 569-575.

Butchbach, M. E., Tian, G., Guo, H., & Lin, C. L. (2004). Association of excitatory amino acid transporters, especially EAAT2, with cholesterol-rich lipid raft microdomains: importance for excitatory amino acid transporter localization and function. J Biol Chem, 279(33), 34388-34396.

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., & 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.

Calingasan, N. Y., Baker, H., Sheu, K. F., & Gibson, G. E. (1995). Blood-brain barrier abnormalities in vulnerable brain regions during thiamine deficiency. Exp Neurol, 134(1), 64-72.

Carrick, T., Dunlop, J., (1999). Protein kinase C modulation of the human excitatory amino acid transporter 2 subtype of glutamate transporter. Paper presented at the Society for Neuroscience

Casado, M., Bendahan, A., Zafra, F., Danbolt, N. C., Aragon, C., Gimenez, C., et al. (1993). Phosphorylation and modulation of brain glutamate transporters by protein kinase C. J Biol Chem, 268(36), 27313-27317.

Chadda, K., Raynard, B., Antoun, S., Thyrault, M., & Nitenberg, G. (2002). Acute lactic acidosis with Wernicke's encephalopathy due to acute thiamine deficiency. Intensive Care Med, 28(10), 1499.

Chan, H., Butterworth, R. F., & Hazell, A. S. (2004). Primary cultures of rat astrocytes respond to thiamine deficiency-induced swelling by downregulating aquaporin-4 levels. Neurosci Lett, 366(3), 231-234.

Chaudhry, F. A., Lehre, K. P., van Lookeren Campagne, M., Ottersen, O. P., Danbolt, N. C., & Storm-Mathisen, J. (1995). Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron, 15(3), 711-720.

Chen, J. C., Hsu-Chou, H., Lu, J. L., Chiang, Y. C., Huang, H. M., Wang, H. L., et al. (2005). Down-regulation of the glial glutamate transporter GLT-1 in rat hippocampus and striatum and its modulation by a group III metabotropic glutamate receptor antagonist following transient global forebrain ischemia. Neuropharmacology, 49(5), 703-714.

Choi, D. W. (1992). Excitotoxic cell death. J Neurobiol, 23(9), 1261-1276.

Collins, G. H. (1967). Glial cell changes in the brain stem of thiamine-deficient rats. Am J Pathol, 50(5), 791-814.

Colotta, F., Polentarutti, N., Sironi, M., & Mantovani, A. (1992). Expression and involvement of c-fos and c-jun protooncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines. J Biol Chem, 267(26), 18278-18283.

Combs, G. F. (1992). The vitamins: fundamental aspects in nutrition and health. . San Diego, California.: Academic Press.

Conradt, M., & Stoffel, W. (1997). Inhibition of the high-affinity brain glutamate transporter GLAST-1 via direct phosphorylation. J Neurochem, 68(3), 1244-1251.

Conradt, M., Storck, T., & Stoffel, W. (1995). Localization of N-glycosylation sites and functional role of the carbohydrate units of GLAST-1, a cloned rat brain L-glutamate/L-aspartate transporter. Eur J Biochem, 229(3), 682-687.

Conti, F., & Weinberg, R. J. (1999). Shaping excitation at glutamatergic synapses. Trends Neurosci, 22(10), 451-458.

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

Cooper, J. R., Roth, R. H., & Kini, M. M. (1963). BIOCHEMICAL AND PHYSIOLOGICAL FUNCTION OF THIAMINE IN NERVOUS TISSUE. Nature, 199, 609-610.

Czapski, G. A., Cakala, M., Kopczuk, D., & Strosznajder, J. B. (2004). Effect of poly(ADP-ribose) polymerase inhibitors on oxidative stress evoked hydroxyl radical level and macromolecules oxidation in cell free system of rat brain cortex. Neurosci Lett, 356(1), 45-48.

Danbolt, N. C. (2001). Glutamate uptake. Prog Neurobiol, 65(1), 1-105.

Danbolt, N. C., Chaudhry, F. A., Dehnes, Y., Lehre, K. P., Levy, L. M., Ullensvang, K., et al. (1998). Properties and localization of glutamate transporters. Prog Brain Res, 116, 23-43.

De Wardener, H. E., Lennox, B. (1947). Cerebral beriberi (Wernicke's Encephalopathy): review of 52 cases in a Singapore prisoner-of-war hospital. . Lancet, 1, 11-17.

Doble, A. (1999). The role of excitotoxicity in neurodegenerative disease: implications for therapy. Pharmacol Ther, 81(3), 163-221.

Dorandeu, F., Antier, D., Pernot-Marino, I., Lapeyre, P., & Lallement, G. (1998). Venom phospholipase A2-induced impairment of glutamate uptake: an indirect and nonselective effect related to phospholipid hydrolysis. J Neurosci Res, 51(3), 349-359.

Dragunow, M., Young, D., Hughes, P., MacGibbon, G., Lawlor, P., Singleton, K., et al. (1993). Is c-Jun involved in nerve cell death following status epilepticus and hypoxic-ischaemic brain injury? Brain Res Mol Brain Res, 18(4), 347-352.

Drejer, J., Meier, E., & Schousboe, A. (1983). Novel neuron-related regulatory mechanisms for astrocytic glutamate and GABA high affinity uptake. Neurosci Lett, 37(3), 301-306.

Dreyfus, P. M. (1959). The quantitative histochemical distribution of thiamine in normal rat brain. J Neurochem, 4, 183-190.

Dreyfus, P. M. (1959). The quantitative histochemical distribution of thiamine in normal rat brain. J. Neurochem., 4, 183-190.

Dumuis, A., Sebben, M., Haynes, L., Pin, J. P., & Bockaert, J. (1988). NMDA receptors activate the arachidonic acid cascade system in striatal neurons. Nature, 336(6194), 68-70.

Escartin, C., Brouillet, E., Gubellini, P., Trioulier, Y., Jacquard, C., Smadja, C., et al. (2006). Ciliary neurotrophic factor activates astrocytes, redistributes their glutamate transporters GLAST and GLT-1 to raft microdomains, and improves glutamate handling in vivo. J Neurosci, 26(22), 5978-5989.

Estus, S., Zaks, W. J., Freeman, R. S., Gruda, M., Bravo, R., & Johnson, E. M., Jr. (1994). Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J Cell Biol, 127(6 Pt 1), 1717-1727.

Feldman, E. B. (1988). Essentials of clinical nutrition. Philadelphia: F.A. Davis Company.

Fernandez-Torre, J. L., Agirre, Z., Martinez-Martinez, M., & Rodriguez, E. (2006). Subacute encephalopathy with seizures in alcoholics (SESA syndrome): report of an unusual case. Clin EEG Neurosci, 37(3), 215-218.

Figiel, M., & Engele, J. (2000). Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuron-derived peptide regulating glial glutamate transport and metabolism. J Neurosci, 20(10), 3596-3605.

Fox, J. M., & Duppel, W. (1975). The action of thiamine and its di- and triphosphates on the slow exponential decline of the ionic currents in the node of Ranvier. Brain Res, 89(2), 287-302.

Fukamachi, S., Furuta, A., Ikeda, T., Ikenoue, T., Kaneoka, T., Rothstein, J. D., et al. (2001). Altered expressions of glutamate transporter subtypes in rat model of neonatal cerebral hypoxia-ischemia. Brain Res Dev Brain Res, 132(2), 131-139.

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.

Garcia, J. H. (1975). The neuropathology of stroke. Hum Pathol, 6(5), 583-598.

Gegelashvili, G., Civenni, G., Racagni, G., Danbolt, N. C., Schousboe, I., & Schousboe, A. (1996). Glutamate receptor agonists up-regulate glutamate transporter GLAST in astrocytes. Neuroreport, 8(1), 261-265.

Gegelashvili, G., Danbolt, N. C., & Schousboe, A. (1997). Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. J Neurochem, 69(6), 2612-2615.

Genda, E. N., Jackson, J. G., Sheldon, A. L., Locke, S. F., Greco, T. M., O'Donnell, J. C., et al. (2011). Co-compartmentalization of the astroglial glutamate transporter, GLT-1, with glycolytic enzymes and mitochondria. J Neurosci, 31(50), 18275-18288.

Gibson, G. E., Calingasan, N. Y., Baker, H., Gandy, S., & Sheu, K. F. (1997). Importance of vascular changes in selective neurodegeneration with thiamine deficiency. Ann N Y Acad Sci, 826, 516-519.

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.

Ginsberg, S. D., Martin, L. J., & Rothstein, J. D. (1995). Regional deafferentation down-regulates subtypes of glutamate transporter proteins. J Neurochem, 65(6), 2800-2803.

Greenwood, J., Love, E. R., & Pratt, O. E. (1982). Kinetics of thiamine transport across the blood-brain barrier in the rat. J Physiol, 327, 95-103.

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., Adams, B. L., Hammond, B., Yuan, E. C., Guo, S. M., & Bennion, M. (1974). Effect of thiamine deprivation and thiamine antagonists on the level of gamma-aminobutyric acid and on 2-oxoglutarate metabolism in rat brain. J Neurochem, 22(5), 831-836.

Guenther, W., & Neu, I. S. (1984). Investigation of the blood-brain barrier for IgG in inflammatory syndromes of the central nervous system. Eur Neurol, 23(2), 132-136.

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

Hazell, A. S., Hakim, A.M. (1994). Increase in extracellular glutamate concentration is a Ca2+-independent process in the thalamus of the thiamine deficient rat. J. Neurochem. (Suppl), 62, S-104.

Hazell, A. S. (2007). Excitotoxic mechanisms in stroke: an update of concepts and treatment strategies. Neurochem Int, 50(7-8), 941-953.

Hazell, A. S. (2009). Astrocytes are a major target in thiamine deficiency and Wernicke's encephalopathy. Neurochem Int, 55(1-3), 129-135.

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., Hakim, A. M., Senterman, M. K., & Hogan, M. J. (1998). Regional activation of L-type voltage-sensitive calcium channels in experimental thiamine deficiency. J Neurosci Res, 52(6), 742-749.

Hazell, A. S., Itzhak, Y., Liu, H., & Norenberg, M. D. (1997). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) decreases glutamate uptake in cultured astrocytes. J Neurochem, 68(5), 2216-2219.

Hazell, A. S., McGahan, L., Tetzlaff, W., Bedard, A. M., Robertson, G. S., Nakabeppu, Y., et al. (1998). Immediate-early gene expression in the brain of the thiamine-deficient rat. J Mol Neurosci, 10(1), 1-15.

Hazell, A. S., Pannunzio, P., Rama Rao, K. V., Pow, D. V., & Rambaldi, A. (2003). Thiamine deficiency results in downregulation of the GLAST glutamate transporter in cultured astrocytes. Glia, 43(2), 175-184.

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.

Hazell, A. S., Sheedy, D., Oanea, R., Aghourian, M., Sun, S., Jung, J. Y., et al. (2009). Loss of astrocytic glutamate transporters in Wernicke encephalopathy. Glia, 58(2), 148-156.

Hazell, A. S., Sheedy, D., Oanea, R., Aghourian, M., Sun, S., Jung, J. Y., et al. (2010). Loss of astrocytic glutamate transporters in Wernicke encephalopathy. Glia, 58(2), 148-156.

Hazell, A. S., Todd, K. G., & Butterworth, R. F. (1998). Mechanisms of neuronal cell death in Wernicke's encephalopathy. Metab Brain Dis, 13(2), 97-122.

Herman, M. A., & Jahr, C. E. (2007). Extracellular glutamate concentration in hippocampal slice. J Neurosci, 27(36), 9736-9741.

Hoffman-LaRoche, F. (1994). Vitamins (Basics) (Vol. 1st edition). New York, USA: Seaboard Lithographers.

.

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.

Honda, H. M., & Ping, P. (2006). Mitochondrial permeability transition in cardiac cell injury and death. Cardiovasc Drugs Ther, 20(6), 425-432.

Huang, K., & El-Husseini, A. (2005). Modulation of neuronal protein trafficking and function by palmitoylation. Curr Opin Neurobiol, 15(5), 527-535.

Huang, K., Kang, M. H., Askew, C., Kang, R., Sanders, S. S., Wan, J., et al. (2010). Palmitoylation and function of glial glutamate transporter-1 is reduced in the YAC128 mouse model of Huntington disease. Neurobiol Dis, 40(1), 207-215.

Inage, Y. W., Itoh, M., Wada, K., & Takashima, S. (1998). Expression of two glutamate transporters, GLAST and EAAT4, in the human cerebellum: their correlation in development and neonatal hypoxic-ischemic damage. J Neuropathol Exp Neurol, 57(6), 554-562.

Indraccolo, U., Gentile, G., Pomili, G., Luzi, G., & Villani, C. (2005). Thiamine deficiency and beriberi features in a patient with hyperemesis gravidarum. Nutrition, 21(9), 967-968.

Itokawa, Y., & Cooper, J. R. (1970). Ion movements and thiamine. II. The release of the vitamin from membrane fragments. Biochim Biophys Acta, 196(2), 274-284.

Jackson, M., Song, W., Liu, M. Y., Jin, L., Dykes-Hoberg, M., Lin, C. I., et al. (2001). Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins. Nature, 410(6824), 89-93.

Jhala, S. S., & Hazell, A. S. (2010). Modeling neurodegenerative disease pathophysiology in thiamine deficiency: consequences of impaired oxidative metabolism. Neurochem Int, 58(3), 248-260.

Jhala, S. S., & Hazell, A. S. (2011). Modeling neurodegenerative disease pathophysiology in thiamine deficiency: consequences of impaired oxidative metabolism. Neurochem Int, 58(3), 248-260.

Jhala, S. S., Wang, D., & Hazell, A. S. (2011). Loss of the glutamate transporter splice-variant GLT-1b in inferior colliculus and its prevention by ceftriaxone in thiamine deficiency. Neurochem Int, 58(5), 558-563.

Kalandadze, A., Wu, Y., Fournier, K., & Robinson, M. B. (2004). Identification of motifs involved in endoplasmic reticulum retention-forward trafficking of the GLT-1 subtype of glutamate transporter. J Neurosci, 24(22), 5183-5192.

Kandel ER, Schwartz JH, Jessell TM. (2000). Principles of Neural Science, 4th ed. McGraw-Hill, New York. ISBN 0-8385-7701-6

Karuppagounder, S. S., Shi, Q., Xu, H., & Gibson, G. E. (2007). Changes in inflammatory processes associated with selective vulnerability following mild impairment of oxidative metabolism. Neurobiol Dis, 26(2), 353-362.

Ke, Z. J., Bowen, W. M., & Gibson, G. E. (2006). Peripheral inflammatory mechanisms modulate microglial activation in response to mild impairment of oxidative metabolism. Neurochem Int, 49(5), 548-556.

Kimelberg, H. K., Barron, K. D., Bourke, R. S., Nelson, L. R., & Cragoe, E. J. (1990). Brain anti-cytoxic edema agents. Prog Clin Biol Res, 361, 363-385.

Kinnersley, H. W., & Peters, R. A. (1930). Carbohydrate metabolism in birds: Brain localisation of lactic acidosis in avitaminosis B, and its relation to the origin of symptoms. Biochem J, 24(3), 711-722.

Kinoshita, N., Kimura, K., Matsumoto, N., Watanabe, M., Fukaya, M., & Ide, C. (2004). Mammalian septin Sept2 modulates the activity of GLAST, a glutamate transporter in astrocytes. Genes Cells, 9(1), 1-14.

Kis, B., Deli, M. A., Kobayashi, H., Abraham, C. S., Yanagita, T., Kaiya, H., et al. (2001). Adrenomedullin regulates blood-brain barrier functions in vitro. Neuroreport, 12(18), 4139-4142.

Knecht, K., Michalak, A., Rose, C., Rothstein, J. D., & Butterworth, R. F. (1997). Decreased glutamate transporter (GLT-1) expression in frontal cortex of rats with acute liver failure. Neurosci Lett, 229(3), 201-203.

Kril, J. J. (1996). Neuropathology of thiamine deficiency disorders. Metab Brain Dis, 11(1), 9-17.

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.

Langlais, P. J., Mandel, R. J., & Mair, R. G. (1992). Diencephalic lesions, learning impairments, and intact retrograde memory following acute thiamine deficiency in the rat. Behav Brain Res, 48(2), 177-185.

Langlais, P. J., McRee, R. C., Nalwalk, J. A., & Hough, L. B. (2002). Depletion of brain histamine produces regionally selective protection against thiamine deficiency-induced lesions in the rat. Metab Brain Dis, 17(3), 199-210.

Langlais, P. J., & Zhang, S. X. (1993). Extracellular glutamate is increased in thalamus during thiamine deficiency-induced lesions and is blocked by MK-801. J Neurochem, 61(6), 2175-2182.

Langlais, P. J., Zhang, S. X., & Savage, L. M. (1996). Neuropathology of thiamine deficiency: an update on the comparative analysis of human disorders and experimental models. Metab Brain Dis, 11(1), 19-37.

Le, O., Heroux, M., & Butterworth, R. F. (1991). Pyrithiamine-induced thiamine deficiency results in decreased Ca(2+)-dependent release of glutamate from rat hippocampal slices. Metab Brain Dis, 6(3), 125-132.

Lee, S. G., Su, Z. Z., Emdad, L., Gupta, P., Sarkar, D., Borjabad, A., et al. (2008). Mechanism of ceftriaxone induction of excitatory amino acid transporter-2 expression and glutamate uptake in primary human astrocytes. J Biol Chem, 283(19), 13116-13123.

Lehre, K. P., & Danbolt, N. C. (1998). The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. J Neurosci, 18(21), 8751-8757.

Lehre, K. P., Levy, L. M., Ottersen, O. P., Storm-Mathisen, J., & Danbolt, N. C. (1995). Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci, 15(3 Pt 1), 1835-1853.

Leong, D. K., Le, O., Oliva, L., & Butterworth, R. F. (1994). Increased densities of binding sites for the "peripheral-type" benzodiazepine receptor ligand [3H]PK11195 in vulnerable regions of the rat brain in thiamine deficiency encephalopathy. J Cereb Blood Flow Metab, 14(1), 100-105.

Levitan, I. B. (2006). Signaling protein complexes associated with neuronal ion channels. Nat Neurosci, 9(3), 305-310.

Lewerenz, J., Albrecht, P., Tien, M. L., Henke, N., Karumbayaram, S., Kornblum, H. I., et al. (2009). Induction of Nrf2 and xCT are involved in the action of the neuroprotective antibiotic ceftriaxone in vitro. J Neurochem, 111(2), 332-343.

Liang, Z., Valla, J., Sefidvash-Hockley, S., Rogers, J., & Li, R. (2002). Effects of estrogen treatment on glutamate uptake in cultured human astrocytes derived from cortex of Alzheimer's disease patients. J Neurochem, 80(5), 807-814.

Lin, C. L., Bristol, L. A., Jin, L., Dykes-Hoberg, M., Crawford, T., Clawson, L., et al. (1998). Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. Neuron, 20(3), 589-602.

Lisovoski, F., Blot, S., Lacombe, C., Bellier, J. P., Dreyfus, P. A., & Junier, M. P. (1997). Transforming growth factor alpha expression as a response of murine motor neurons to axonal injury and mutation-induced degeneration. J Neuropathol Exp Neurol, 56(5), 459-471.

Lonsdale, D. (1990). Thiamine deficiency and sudden deaths. Lancet, 336(8711), 376.

Lundy, D. F., & McBean, G. J. (1995). Pre-incubation of synaptosomes with arachidonic acid potentiates inhibition of [3H]D-aspartate transport. Eur J Pharmacol, 291(3), 273-279.

Manz, H. J., & Robertson, D. M. (1972). Vascular permeability to horseradish peroxidase in brainstem lesions of thiamine-deficient rats. Am J Pathol, 66(3), 565-576.

Maragakis, N. J., & Rothstein, J. D. (2004). Glutamate transporters: animal models to neurologic disease. Neurobiol Dis, 15(3), 461-473.

Marie, H., & Attwell, D. (1999). C-terminal interactions modulate the affinity of GLAST glutamate transporters in salamander retinal glial cells. J Physiol, 520 Pt 2, 393-397.

Marie, H., Billups, D., Bedford, F. K., Dumoulin, A., Goyal, R. K., Longmore, G. D., et al. (2002). The amino terminus of the glial glutamate transporter GLT-1 interacts with the LIM protein Ajuba. Mol Cell Neurosci, 19(2), 152-164.

Marks, J. (1975). A guide to the vitamins: their role in New York Academy of Sciences, 1982, health and disease. (Vol. 378, pp. 137-144). Lancaster, UK.

: Medical & Technical Publishing Co. Ltd.

Martin, L. J., Brambrink, A. M., Lehmann, C., Portera-Cailliau, C., Koehler, R., Rothstein, J., et al. (1997). Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum. Ann Neurol, 42(3), 335-348.

Masliah, E., Alford, M., DeTeresa, R., Mallory, M., & Hansen, L. (1996). Deficient glutamate transport is associated with neurodegeneration in Alzheimer's disease. Ann Neurol, 40(5), 759-766.

Masliah, E., Alford, M., Mallory, M., Rockenstein, E., Moechars, D., & Van Leuven, F. (2000). Abnormal glutamate transport function in mutant amyloid precursor protein transgenic mice. Exp Neurol, 163(2), 381-387.

Masumoto, K., Esumi, G., Teshiba, R., Nagata, K., Nakatsuji, T., Nishimoto, Y., et al. (2009). Need for thiamine in peripheral parenteral nutrition after abdominal surgery in children. JPEN J Parenter Enteral Nutr, 33(4), 417-422.

Mathern, G. W., Mendoza, D., Lozada, A., Pretorius, J. K., Dehnes, Y., Danbolt, N. C., et al. (1999). Hippocampal GABA and glutamate transporter immunoreactivity in patients with temporal lobe epilepsy. Neurology, 52(3), 453-472.

Mathew, P., Graham, D. I., Bullock, R., Maxwell, W., McCulloch, J., & Teasdale, G. (1994). Focal brain injury: histological evidence of delayed inflammatory response in a

new rodent model of focal cortical injury. Acta Neurochir Suppl (Wien), 60, 428-430.

Matrana, M. R., & Davis, W. E. (2009). Vitamin deficiency after gastric bypass surgery: a review. South Med J, 102(10), 1025-1031.

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.

Meyer, T., Speer, A., Meyer, B., Sitte, W., Kuther, G., & Ludolph, A. C. (1996). The glial glutamate transporter complementary DNA in patients with amyotrophic lateral sclerosis. Ann Neurol, 40(3), 456-459.

Morgan, J. I., & Curran, T. (1986). Role of ion flux in the control of c-fos expression. Nature, 322(6079), 552-555.

Moriyama, Y., & Omote, H. (2008). Vesicular glutamate transporter acts as a metabolic regulator. Biol Pharm Bull, 31(10), 1844-1846.

Mulholland, P. J. (2006). Susceptibility of the cerebellum to thiamine deficiency. Cerebellum, 5(1), 55-63.

Muller, C. S., Haupt, A., Bildl, W., Schindler, J., Knaus, H. G., Meissner, M., et al. (2010). Quantitative proteomics of the Cav2 channel nano-environments in the mammalian brain. Proc Natl Acad Sci U S A, 107(34), 14950-14957.

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.

Murphy, T. H., Worley, P. F., & Baraban, J. M. (1991). L-type voltage-sensitive calcium channels mediate synaptic activation of immediate early genes. Neuron, 7(4), 625-635.

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.

Ohtsubo, K., & Marth, J. D. (2006). Glycosylation in cellular mechanisms of health and disease. Cell, 126(5), 855-867.

Olney, J. W. (1971). Glutamate-induced neuronal necrosis in the infant mouse hypothalamus. An electron microscopic study. J Neuropathol Exp Neurol, 30(1), 75-90.

Pawlak, J., Brito, V., Kuppers, E., & Beyer, C. (2005). Regulation of glutamate transporter GLAST and GLT-1 expression in astrocytes by estrogen. Brain Res Mol Brain Res, 138(1), 1-7.

Pellerin, L. (2005). How astrocytes feed hungry neurons. Mol Neurobiol, 32(1), 59-72.

Pentschew, A., & Garro, F. (1966). Lead encephalo-myelopathy of the suckling rat and its implications on the porphyrinopathic nervous diseases. With special reference to the permeability disorders of the nervous system's capillaries. Acta Neuropathol, 6(3), 266-278.

Phillips, S. C., Harper, C. G., & Kril, J. (1987). A quantitative histological study of the cerebellar vermis in alcoholic patients. Brain, 110 (Pt 2), 301-314.

Phillips, S. C., Harper, C. G., & Kril, J. J. (1990). The contribution of Wernicke's encephalopathy to alcohol-related cerebellar damage. Drug Alcohol Rev, 9(1), 53-60.

Pieper, A. A., Verma, A., Zhang, J., & Snyder, S. H. (1999). Poly (ADP-ribose) polymerase, nitric oxide and cell death. Trends Pharmacol Sci, 20(4), 171-181.

Pieper, A. A., Walles, T., Wei, G., Clements, E. E., Verma, A., Snyder, S. H., et al. (2000). Myocardial postischemic injury is reduced by polyADPripose polymerase-1 gene disruption. Mol Med, 6(4), 271-282.

Qin, L., He, J., Hanes, R. N., Pluzarev, O., Hong, J. S., & Crews, F. T. (2008). Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. J Neuroinflammation, 5, 10.

Ritter, S. L., Asay, M. J., Paquet, M., Paavola, K. J., Reiff, R. E., Yun, C. C., et al. (2011). GLAST stability and activity are enhanced by interaction with the PDZ scaffold NHERF-2. Neurosci Lett, 487(1), 3-7.

Robertson, D. M., Wasan, S. M., & Skinner, D. B. (1968). Ultrastructural features of early brain stem lesions of thiamine-deficient rats. Am J Pathol, 52(5), 1081-1097.

Rothmeier, J., Friese, M., Willemsen, F., & Froescher, W. (2001). Subacute encephalopathy with seizures in chronic alcoholism (SESA syndrome). Clin Electroencephalogr, 32(4), 186-190.

Rothstein, J. D., Dykes-Hoberg, M., Pardo, C. A., Bristol, L. A., Jin, L., Kuncl, R. W., et al. (1996). Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron, 16(3), 675-686.

Rothstein, J. D., Martin, L., Levey, A. I., Dykes-Hoberg, M., Jin, L., Wu, D., et al. (1994). Localization of neuronal and glial glutamate transporters. Neuron, 13(3), 713-725.

Rothstein, J. D., Patel, S., Regan, M. R., Haenggeli, C., Huang, Y. H., Bergles, D. E., et al. (2005). Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature, 433(7021), 73-77.

Ruggiero, A. M., Liu, Y., Vidensky, S., Maier, S., Jung, E., Farhan, H., et al. (2008). The endoplasmic reticulum exit of glutamate transporter is regulated by the inducible mammalian Yip6b/GTRAP3-18 protein. J Biol Chem, 283(10), 6175-6183.

Sauberlich, H. E., Dowdy, R. P., & Skala, J. H. (1973). Laboratory tests for the assessment of nutritional status. CRC Crit Rev Clin Lab Sci, 4(3), 215-340.

Schanne, F. A., Kane, A. B., Young, E. E., & Farber, J. L. (1979). Calcium dependence of toxic cell death: a final common pathway. Science, 206(4419), 700-702.

Schlag, B. D., Vondrasek, J. R., Munir, M., Kalandadze, A., Zelenaia, O. A., Rothstein, J. D., et al. (1998). Regulation of the glial Na+-dependent glutamate transporters by cyclic AMP analogs and neurons. Mol Pharmacol, 53(3), 355-369.

Scholz, W. (1949). Histologische und topische Veränderungen und Vulnerabilitäts verhältnisse im menschlichen Gehirn bei Sauerstoffmangel, Ödem und plasmatischen Infiltration. I. Problemstellung und feingewebliche Situation. . Arch Psychiatr Nervenkr, 181, 621-665.

Schousboe, A. (1981). Transport and metabolism of glutamate and GABA in neurons are glial cells. Int Rev Neurobiol, 22, 1-45.

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.

Schweneker, M., Bachmann, A. S., & Moelling, K. (2005). JM4 is a four-transmembrane protein binding to the CCR5 receptor. FEBS Lett, 579(7), 1751-1758.

Sebrell, J. W. (1962). In A clinical evaluation of thiamine deficiency. (Vol. 98, pp. 563-567): Annals of the New York Academy of Sciences.

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

Siegel GJ, Agranoff BW, Albers RW, et al., editors. (1999). Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th edition. Philadelphia: Lippincott-Raven. ISBN-10: 0-397-51820.

Sitcheran, R., Gupta, P., Fisher, P. B., & Baldwin, A. S. (2005). Positive and negative regulation of EAAT2 by NF-kappaB: a role for N-myc in TNFalpha-controlled repression. EMBO J, 24(3), 510-520.

Slotboom, D. J., Konings, W. N., & Lolkema, J. S. (1999). Structural features of the glutamate transporter family. Microbiol Mol Biol Rev, 63(2), 293-307.

Snell R.S. (2009). Clinical Neuroanatomy, 7th ed. Lippincott Williams & Wilkins. ISBN-10: 0781794277

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

Stella, N., Tence, M., Glowinski, J., & Premont, J. (1994). Glutamate-evoked release of arachidonic acid from mouse brain astrocytes. J Neurosci, 14(2), 568-575.

Swank, R. L. (1940). AVIAN THIAMIN DEFICIENCY : A CORRELATION OF THE PATHOLOGY AND CLINICAL BEHAVIOR. J Exp Med, 71(5), 683-702.

Swanson, R. A., Liu, J., Miller, J. W., Rothstein, J. D., Farrell, K., Stein, B. A., et al. (1997). Neuronal regulation of glutamate transporter subtype expression in astrocytes. J Neurosci, 17(3), 932-940.

Tamahara, S., Inaba, M., Sato, K., Matsuki, N., Hikasa, Y., & Ono, K. (2002). Non-essential roles of cysteine residues in functional expression and redox regulatory pathways for canine glutamate/aspartate transporter based on mutagenic analysis. Biochem J, 367(Pt 1), 107-111.

Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., et al. (1997). Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science, 276(5319), 1699-1702.

Terasawa, M., Nakahara, T., Tsukada, N., Sugawara, A., & Itokawa, Y. (1999). The relationship between thiamine deficiency and performance of a learning task in rats. Metab Brain Dis, 14(3), 137-148.

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. (1998). Evaluation of the role of NMDA-mediated excitotoxicity in the selective neuronal loss in experimental Wernicke encephalopathy. Exp Neurol, 149(1), 130-138.

Torvik, A. (1985). Two types of brain lesions in Wernicke's encephalopathy. Neuropathol Appl Neurobiol, 11(3), 179-190.

Torvik, A. (1987). Brain lesions in alcoholics: neuropathological observations. Acta Med Scand Suppl, 717, 47-54.

Torvik, A. (1987). Topographic distribution and severity of brain lesions in Wernicke's encephalopathy. Clin Neuropathol, 6(1), 25-29.

Tran, N. D., Correale, J., Schreiber, S. S., & Fisher, M. (1999). Transforming growth factor-beta mediates astrocyte-specific regulation of brain endothelial anticoagulant factors. Stroke, 30(8), 1671-1678.

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.

Trotti, D., Danbolt, N. C., & Volterra, A. (1998). Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? Trends Pharmacol Sci, 19(8), 328-334.

Tsuru, N., Ueda, Y., & Doi, T. (2002). Amygdaloid kindling in glutamate transporter (GLAST) knockout mice. Epilepsia, 43(8), 805-811.

Vallejo-Illarramendi, A., Domercq, M., & Matute, C. (2005). A novel alternative splicing form of excitatory amino acid transporter 1 is a negative regulator of glutamate uptake. J Neurochem, 95(2), 341-348.

Vemuganti, R., Kalluri, H., Yi, J. H., Bowen, K. K., & Hazell, A. S. (2006). Gene expression changes in thalamus and inferior colliculus associated with inflammation, cellular stress, metabolism and structural damage in thiamine deficiency. Eur J Neurosci, 23(5), 1172-1188.

Victor M., A. R. a. C. G. (1989). The Wernicke-Korsakoff syndrome and related neurologic disorders due to alcoholism and malnutrition. (2nd ed. ed.). Philadelphia, PA.: FA Davis.

Volterra, A., Trotti, D., Floridi, S., & Racagni, G. (1994). Reactive oxygen species inhibit high-affinity glutamate uptake: molecular mechanism and neuropathological implications. Ann N Y Acad Sci, 738, 153-162.

Vortmeyer, A. O., & Colmant, H. J. (1988). Differentiation between brain lesions in experimental thiamine deficiency. Virchows Arch A Pathol Anat Histopathol, 414(1), 61-67.

Wang, D., & Hazell, A. S. (2010). Microglial activation is a major contributor to neurologic dysfunction in thiamine deficiency. Biochem Biophys Res Commun, 402(1), 123-128.

Warnock, L. G., & Burkhalter, V. J. (1968). Evidence of malfunctioning blood-brain barrier in experimental thiamine deficiency in rats. J Nutr, 94(2), 256-260.

Watanabe, I., Tomita, T., Hung, K. S., & Iwasaki, Y. (1981). Edematous necrosis in thiamine-deficient encephalopathy of the mouse. J Neuropathol Exp Neurol, 40(4), 454-471.

Watanabe M, R. M., Kalandadze A, Rothstein JD. (2003). GPS1, interacting protein with GLT-1. Paper presented at the Society for Neuroscience.

Watanabe M, U. T., Jackson M, Ruggiero AM, Sawa A, Kalandadze A, Robinson MB., Rothstein JD. (2004). CSN1 Downregulates GLT-1 activity. . Paper presented at the Society for Neuroscience.

Wendel, U., Lombeck, I., & Bremer, H. J. (1983). Maple-syrup-urine disease. N Engl J Med, 308(18), 1100-1101.

White, R. J., & Reynolds, I. J. (1996). Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. J Neurosci, 16(18), 5688-5697.

William, R. R. (1961). Toward the conquest of beriberi. . Cambridge, Massachusets.: Harvard University Press.

Witt, E. D. (1985). Neuroanatomical consequences of thiamine deficiency: a comparative analysis. Alcohol Alcohol, 20(2), 201-221.

Yamamoto, Y., & Gaynor, R. B. (2004). IkappaB kinases: key regulators of the NF-kappaB pathway. Trends Biochem Sci, 29(2), 72-79.

Yamashita, M., & Yamamoto, T. (1995). Wernicke encephalopathy with symmetric pericentral involvement: MR findings. J Comput Assist Tomogr, 19(2), 306-308.

Yernool, D., Boudker, O., Jin, Y., & Gouaux, E. (2004). Structure of a glutamate transporter homologue from Pyrococcus horikoshii. Nature, 431(7010), 811-818.

Yi, J. H., & Hazell, A. S. (2006). Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. Neurochem Int, 48(5), 394-403.

Zelenaia, O., Schlag, B. D., Gochenauer, G. E., Ganel, R., Song, W., Beesley, J. S., et al. (2000). Epidermal growth factor receptor agonists increase expression of glutamate transporter GLT-1 in astrocytes through pathways dependent on phosphatidylinositol 3-kinase and transcription factor NF-kappaB. Mol Pharmacol, 57(4), 667-678.

Zelenaia, O. A., & Robinson, M. B. (2000). Degradation of glial glutamate transporter mRNAs is selectively blocked by inhibition of cellular transcription. J Neurochem, 75(6), 2252-2258.

Zigmond M.J., Bloom F.E., Landis S.C., Roberts J.L., Squire L.R. (1999). Fundamental neuroscience, Ist ed. Academic Press, ISBN-10: 0127808701

Zhang, J., Dawson, V. L., Dawson, T. M., & Snyder, S. H. (1994). Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. Science, 263(5147), 687-689.

Zhang, S. X., Weilersbacher, G. S., Henderson, S. W., Corso, T., Olney, J. W., & Langlais, P. J. (1995). Excitotoxic cytopathology, progression, and reversibility of thiamine deficiency-induced diencephalic lesions. J Neuropathol Exp Neurol, 54(2), 255-267.

Zhou, J., & Sutherland, M. L. (2004). Glutamate transporter cluster formation in astrocytic processes regulates glutamate uptake activity. J Neurosci, 24(28), 6301-6306.

Zink, M., Schmitt, A., Henn, F. A., & Gass, P. (2004). Differential expression of glutamate transporters EAAT1 and EAAT2 in mice deficient for PACAP-type I receptor. J Neural Transm, 111(12), 1537-1542.