THE PATHOGENESIS OF NEURONAL LOSS IN THIAMINE DEFICIENCY ENCEPHALOPATHY

by

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The pathogenesis of neuronal loss in thiamine deficiency encephalopathy

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ABBREVIATIONS

ACPD	*Trans*-1-amino-cyclopentane-1,3-dicarboxylate
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ANC	adenine nucleotide carrier
AOCC	agonist-operated calcium channel
AP4	1-2-amino-4-phosphonobutyrate
AP5	D-amino-5-phosphonopentanoate
ATP	adenosine triphosphate
BBB	blood-brain barrier
Cal	calmodulin
Ca^{2+}-ATPase	calcium-adenosine triphosphatase
CBP	calcium binding protein
CPP	3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate
EAA	excitatory amino acid
ED1	macrophage lysosomal antigen
GABA	γ-amino butyric acid
α-KGDH	α-ketoglutarate dehydrogenase
GFAP	glial fibrillary acidic protein
Na^{+}-K^{+}-ATPase	sodium-potassium-adenosine triphosphatase
NMDA	N-methyl-D-aspartate
PCP	phencyclidine
PDHC	pyruvate dehydrogenase complex
PPIX	protoporphyrin IX
PTBR	"peripheral-type" benzodiazepine receptor
TD	thiamine deficiency

TK	transketolase
TDP	thiamine diphosphate
TDPase	thiamine diphosphatase
TMP	thiamine monophosphate
TMPase	thiamine monophosphatase
TPKase	thiamine pyrophosphokinase
TTP	thiamine triphosphate
TTPase	thiamine triphosphatase
VDAC	voltage-dependent anion channel
VSCC	voltage-sensitive calcium channel
WE	Wernicke's encephalopathy
WKS	Wernicke-Korsakoff Syndrome

Abstract

A deficiency of thiamine (Vitamin B1) is associated with the Wernicke-Korsakoff Syndrome (WKS), which is characterized neuropathologically by lesions of selective regions in the brainstem and diencephalon. Administration of pyrithiamine to rats results in neuropathological changes similar in nature and distribution to lesions seen in WKS in humans. The following work is concerned with neuronal loss associated with thiamine-deficiency (TD) and the potential use of "peripheral-type" benzodiazepine receptor (PTBR) ligands in the detection of brain lesions in WKS. PTBRs have been localized on the outer mitochondrial membranes of glial cells, and it has been suggested that densities of PTBR binding sites can be used as a marker of reactive gliosis following neuronal loss.

In a study of the topographic distribution of lesions in TD, autoradiography studies with the PTBR ligand ^{3}H-PK11195 were performed in parallel with histological studies. At an early stage (day 7) of thiamine deficiency, densities of ^{3}H-PK11195 binding sites in presymptomatic TD animals (animals that showed no neurological symptoms of thiamine deficiency) were not significantly different from controls. Histological studies of these animals revealed only mild gliosis in selective brain regions of these animals. However, at a later stage of thiamine deficiency (day 12-14), when animals were neurologically symptomatic, significantly increased densities of ^{3}H-PK11195 binding sites were evident in vulnerable brain regions including, the inferior olive, inferior colliculus and thalamus. Increased densities of PTBRs were spatially coincident with histological evidence of neuronal loss and marked gliosis. Immunohistochemical studies with antibodies to astrocytes (anti-glial fibrillary acidic protein; GFAP) and macrophages (anti-ED1) revealed increased numbers of ED1-positive cells in inferior olive, inferior colliculus and medial thalamus of presymptomatic TD animals (prior to major histopathological lesions), which was suggestive of an early macrophage response in these brain regions. Studies of symptomatic TD animals revealed marked increases in GFAP-immunostaining and

sustained increases in ED1-immunostaining in vulnerable brain regions. Increased densities of ^{3}H-PK11195 were found to be spatially and temporally coincident with increased GFAP-immunostaining, suggesting that increased densities of PTBRs in TD were due mainly to reactive gliosis. Immunohistochemical studies with an antibody to serum albumin revealed increased permeability of the blood-brain barrier in presymptomatic TD animals, which suggested that increased ED1-immunostaining was due in major part to infiltrating blood-borne macrophages. This finding could have important implications for elucidating of pathophysiologic mechanisms involved in selective neuronal death in this TD. It has been suggested that neuronal cell loss in TD is due to glutamate-induced excitotoxicity mediated by the N-methyl-D-aspartate (NMDA) receptor. In a study of the effect of the NMDA-receptor antagonist MK801 in TD animals, histological studies revealed similar degrees of neuronal loss in MK801-treated and untreated TD animals. Decreased densities of ^{3}H-PK11195 binding sites were seen in the medial thalamus of MK801-treated TD animals, however, histological and immunohistochemical studies of ththis brain region revealed extensive neuronal loss and an absence of GFAP-positive astrocytes within the areas of the lesions. Thus, the absence of a glial reaction in the thalamus was reflected by decreased densities of PTBRs. Non-invasive brain imaging techniques may offer a potential diagnostic aid for WKS, a condition that is difficult to diagnose during life. In autoradiographic studies using radioligands for central (neuronal) and "peripheral-type" (glial) benzodiazepine receptors, it was found that increased densities of binding sites for the PTBR ligand (^{3}H-PK11195) closely paralleled the topographic distribution of neuronal cell loss. In contrast, binding sites for the central benzodiazepine receptor (^{3}H-Ro 15 1788) showed poor spatial correlation with the neuronal loss in TD animals. These results suggest that ^{11}C-PK11195 may afford a useful PET ligand for the assessment of brain damage in WKS patients.

Resumé

Le syndrome de Wernicke-Korsakoff (SWK) est associé à une déficience en thiamine (vitamine B1) sévère et s'accompagne de lésions cellulaires sélectives observables dans des régions spécifiques du tronc cérébral et du diencéphale. L'administration chronique de pyrithiamine chez le rat reproduit des changements neuropathologiques similaires aux lésions observées chez l'humain atteint du SWK. Le ligand ^{3}H-PK11195 est un marqueur des récepteurs aux benzodiazépines de type péripherique (RBTP) qu'on retrouve principalement sur la membrane mitochondriale externe des cellules gliales. Comme une perte de cellules neuronales s'accompagne généralement d'une astrocytose réactive dans les régions affectées, une augmentation du nombre de RBTP pourrait indiquée un dommage cellulaire dans le cerveau de sujets atteints du SWK. Le présent travail étudie la relation qui existe entre la perte neuronale causée par une déficience en thiamine et l'utilisation potentielle du ligand ^{3}H-PK11195 comme moyen de détection de ces lésions dans le SWK.

Chez le rat déficient en thiamine démontrant des symptômes de détérioration neurologique, une augmentation significative de la densité des RBTP dans les régions vulnérables comme l'olive inférieure, le colliculus inférieur et le thalamus a été observée par autoradiographie quantitative utilisant le ligand ^{3}H-PK11195. Cette augmentation région-spécifique du nombre de RBTP est parallèle à la distribution des sites de lésions neuronales et d'astrocytose. Aucun changement ne fut observé chez les animaux déficients en thiamine qui étaient présymptomatiques (i.e. à un stade avant l'apparition de lésions histopathologiques majeures). Par ailleurs des études immunohistochimiques utilisant des anticorps reconnaissant spécifiquement les astrocytes (anti-glial fibrillary acidic protein; GFAP) ou les macrophages (anti-ED1) ont révélé une augmentation du nombre de macrophages dans l'olive inférieure, le colliculus inférieur et le thalamus médian chez les animaux présymptomatiques déficients en thiamine. Ces observations suggèrent une réponse précoce des macrophages dans ces régions vulnérables qui ne démontrent encore

aucune lésions à ce stade. Chez les animaux symptômatiques déficient en thiamine, une augmentation très significative de l'immunoréactivité contre le GFAP et le ED1 a été observée dans ces mêmes régions du cerveau. L'augmentation de la densité des RBTP rapportée plus haut coïncide étroitement avec changements temporels et région-spécifiques de l'immunoréactivité de la protéine GFAP. Ceci suggère que l'augmentation des RBTP dans le cerveau d'animaux symptômatiques déficients en thiamine pourrait être causée principalement par une augmentation du nombre d'astrocytes (astrocytose) dans les régions où il y perte neuronale. D'autre part, à l'aide d'un anticorps reconnaissant l'albumine sérique, des études immunohistochimiques chez les animaux présymptomatiques déficients en thiamine ont démontré une augmentation de la perméabilité de la barrière hématoencéphalique. Ceci suggère que l'augmentation de l'immunoréactivité à l'anticorps ED1 pourrait être causée principalement par l'infiltration de macrophages provenant de la périphérie. Ces observations pourraient contribuer à élucider les mécanismes impliqués dans la perte neuronale sélective associée à la déficience en thiamine.

Les cellules microgliales, lorsque activée, libèrent une grande quantité de glutamate *in vitro*. Ces études suggèrent que la perte neuronale associée à une déficience en thiamine pourrait être causée par l'excitotoxicité glutamatergique médiée par les récepteurs à NMDA (N-méthyl-D-aspartate). Cependant, dans le présent rapport, aucune neuroprotection n'a pu être démontrée dans le cerveau de rats déficients en thiamine au stade préconvulsif, traités avec la drogue MK801 (antagoniste des récepteurs à NMDA). En effet, aucune différence ne fut observée quant au degré de perte neuronale et d'astrocytose, et quant à la densité des RBTP dans l'olive inférieure et le colliculus inférieur d'animaux déficients en thiamine traités avec le MK801 ou non. Curieusement, une diminution de la densité des sites liant le ^{3}H-PK11195 associée à des lesions neuronales sévères et à l'absence d'immunoréactivité pour la protéine astrocytaire GFAP ont été observés dans le thalamus médian des animaux traités avec le MK801. Cette absence de réponse gliale dans le thalamus médian pourrait

être responsable de la diminution de la densité des RBTP observée dans cette région de mort cellulaire.

Le développement de techniques diagnostiques non invasives pourrait offrir un outil très important pour l'évaluation clinique de patients atteints du SWK. Des études autoradiographiques utilisant les ligands radioactifs spécifiques aux RBTP (glial; ^{3}H-PK11195) et aux récepteurs à benzodiazepines de type central (RBTC, neuronal; ^{3}H-Ro15 1788) ont démontré que l'augmentation région-spécifique des RBTP correspond étroitement aux régions de perte neuronale, alors que les changemants de densité des RBTC a une corrélation très faible avec la distribution des lésions neuronales associées à la déficience en thiamine. Les résultats de nos études autoradiographiques avec le ligand ^{3}H-PK11195 dans la déficience en thiamine expérimentale suggèrent donc que ^{11}C-PK11195 utilisé comme ligand pour l'analyse par tomographie à émission de positron (PET) pourrait devenir un outil précieux dans l'évaluation cliniques des lésions cérébrales chez les patients atteints du SWK.

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PREFACE

This work represents a collection of studies concerned with the pathogenesis and detection of neuronal loss associated with thiamine-deficiency encephalopathy.

This thesis is comprised of 6 chapters. Chapter 1 is a review of the literature concerning thiamine, thiamine-deficiency disorders and mechanisms implicated in the pathogenesis of neuronal loss seen in such disordes. Chapter 2 examines the use of a "peripheral-type" benzodiazepine receptor ligand as a marker of neuronal loss. Chapter 3 describes results of studies of the blood-brain barrier and glial reaction in thiamine-deficiency. In Chapter 4 the potential neuroprotective effects of a glutamate-receptor antagonist were investigated. Chapter 5 contains a comparison of central- and peripheral-benzodiazepine receptor ligands as markers of neuronal loss. Chapter 6 is a general discussion and interpretation of the results contained in the preceding chapters.

Chapter 2 was co-authored by Miss Oanh Le who assisted with the pyrithiamine-treatment and feeding of animals, Dr Luis Oliva (Neuropathologist) who performed the neuronal cell counts, and Dr Roger Butterworth. This work was published in the Journal of Cerebral Blood Flow and Metabolism in 1994 (J. Cereb. Blood Flow and Metab. 14; 100-105). Chapter 3 was co-authored by Dr Luis Oliva, who assisted with the assessment of histological and immunohistochemical results, Dr Jillian Kril contributed to the blood-brain studies by assisting with pyrithiamine-treatment and perfusion fixation of animals, and Dr Roger Butterworth. This work has been submitted to Brain Research for publication. Chapters 4 and 5 were co-authored by Dr Luis Oliva, who once again performed the neuronal cell counts, and Dr Roger Butterworth. Chapter 4 has been submited to Neuroscience Letters for publication. Chapter 5 has been accepted by Alcoholism: Clinical and Experimental Research, and is currently "In press".

The work contained within this thesis represents an original contribution of knowledge to the elucidation of ultrastructural and glial events in early thiamine deficency, as well as the potential use of a novel ligand in the detection of neuronal loss associated with thiamine deficiency disorders. Studies of the integrity of the blood-brain barrier, and the glial reaction, in thiamine deficiency were performed; results of these studies may contribute to the elucidation of events leading to neuronal loss in thiamine deficiency disorders. The distribution of brain lesions was assessed by using a selective ligand for the "peripheral-type" benzodiazepine receptor. The results of these studies may have implications for non-invasive techniques as diagnostic aids in Wernicke's encephalopathy in humans.

Dorothy Leong

CHAPTER 1

Introduction

1.1 Biochemical aspects of thiamine

1.1.1 Sources, human requirements and absorption

Thiamine (Vitamin B1) is available from both vegetable and meat products. Rich sources of the vitamin include Brewer's yeast, wheat germ, spinach, meat, dried peas and beans and whole grain cereals (Victor et al., 1989). The recommended daily allowance of thiamine is 1-1.5 mg for the average adult. Since body tissues have a limited capacity to store the vitamin, requirements of thiamine are greater during ingestion of disproportionate large amounts of carbohydrate, and in conditions of increased metabolic rate, diarrhea or infection (Victor et al., 1989). In the normal rat the highest levels of thiamine are found in the liver, followed by the kidney, heart, brain and muscle (9, 7.6, 7.4, 3.0, 2.3 μg/g fresh tissue weight, respectively) (Dreyfus, 1959).

Studies have revealed a dual system for thiamine absorption in the small intestine; at low or physiologic thiamine concentrations, transport occurs by an active saturable process, but at higher thiamine concentrations transport occurs by passive diffusion (Hoyumpa et al., 1982). Thiamine entry into brain from blood occurs mainly by a saturable carrier-mediated transport mechanism (Spector, 1976; Greenwood et al., 1982). In addition there is also a non-saturable (passive diffusion) component that provides less than 10% of total thiamine uptake (Greenwood et al., 1982). The normal rate of influx of thiamine by the carrier-mediated mechanism into brain has been found to be of a similar order of magnitude to the turnover rate of thiamine in brain (Rindi et al., 1980; Greenwood et al., 1982), suggesting a very small margin of spare capacity. Thus low plasma concentrations of thiamine can result in a rate of influx into the brain insufficient to meet the needs of cerebral tissues, making the brain particularly vulnerable to thiamine deficiency (Thomson et al., 1987).

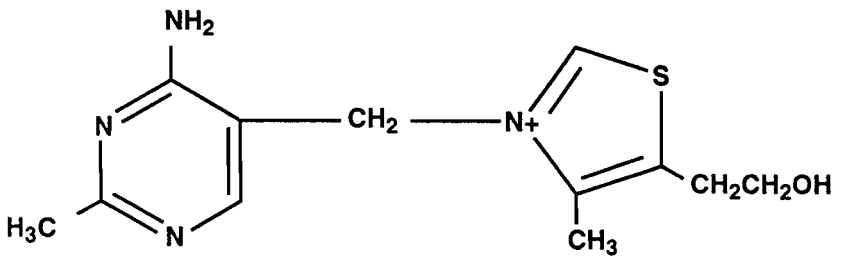


Figure 1- Chemical structure of thiamine

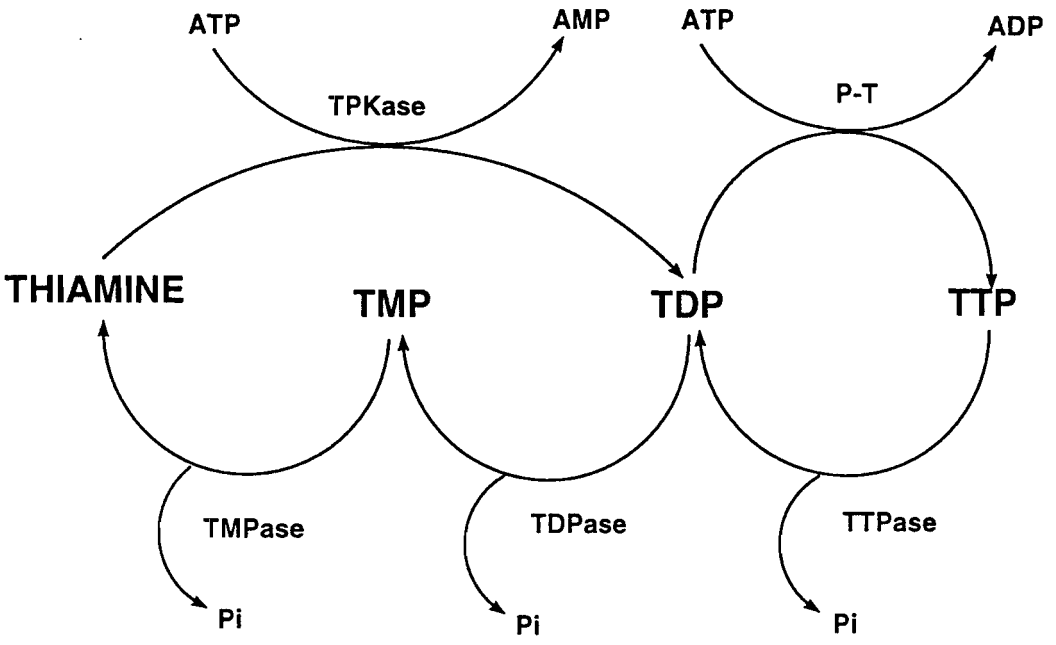


Figure 2- Thiamine phosphorylation and dephosphorylation. TMP, TDP, TTP; thiamine mono-, di-, triphosphate, TMPase, TDPase, TTPase; thiamine mono-, di-, triphosphatase, P-transferase; phosphotransferase, TPKase; thiamine pyrophosphokinase, AMP, ADP, ATP; adenosine mono-, di-, triphosphate.

1.1.2 Thiamine and thiamine phosphate esters

Thiamine is made up of pyrimidine and thiazole moieties which are joined by a methylene bridge (Figure 1). In order for thiamine to be biologically active it must be phosphorylated to thiamine diphosphate (TDP) (Ochoa and Peters, 1938). In this conversion, which is carried out by thiamine pyrophosphokinase, adenosine triphosphate (ATP) acts as the pyrophosphate donor (see Figure 2).

In the brain, approximately 80-85% of thiamine is in the TDP form (Rindi and deGiusseppe., 1961; Ishii et al., 1979; Heroux and Butterworth, 1995); the remainder is in the thiamine monophosphate (TMP) or thiamine triphosphate (TTP) ester forms. While TDP is an enzyme cofactor involved in carbohydrate metabolism (Kinnersley and Peters, 1930; Horecker and Smyrniotis, 1953), TTP appears to be involved in membrane excitability and nerve conduction (Cooper and Pincus, 1979; Bettendorff, 1994). No function for TMP in the brain has been proposed.

1.2 The Wernicke-Korsakoff Syndrome

1.2.1 Clinical signs, incidence and treatment

A deficiency of thiamine may result in a condition known as the Wernicke-Korsakoff Syndrome (WKS). Wernicke's encephalopathy (WE) is clinically characterized by ophthalmoplegia, ataxia and a global confusional state; while Korsakoff's psychosis is characterized by memory loss (Victor et al., 1989). Wernicke's encephalopathy and Korsakoff's psychosis probably represent successive stages of a single disease process. Korsakoff's psychosis usually follows WE in the WKS; it has been reported that 85% of patients who survive the acute illness of WE later demonstrate symptoms of Korsakoff's psychosis (Victor et al., 1989).

Although most commonly encountered in chronic alcoholics (Harper, 1979, 1983), Wernicke's encephalopathy has also been described in other conditions such as gastrointestinal carcinoma, hyperemesis gravidarum, malnutrition (including anorexia nervosa and fasting) and AIDS (Ebels, 1978; Devathasan and Koh, 1982; Haid et al., 1982; Handler and Perkins, 1982; Nightingale et al., 1982; Butterworth et al., 1991). Thiamine deficiency in alcoholics results from inadequate dietary intake (Leevy et al., 1965; Neville et al., 1968), reduced gastrointestinal absorption (Tomasulo et al., 1968; Balaghi and Neal, 1977; Hoyumpa et al., 1975, 1978; Thomson and Majumdar, 1981), and depletion of liver and brain stores of the vitamin (Baker et al., 1964; Abe and Itokawa, 1977).

From autopsy studies the incidence of WE has been reported to be 2.8% (of 4677 cases, Royal Perth Hospital; Harper, 1983), 1.9% (of 3548 cases, Cleveland Metropolitan Hospital; Victor and Laureno, 1978), 1.7% (of 1600 cases, Bellevue Hospital, New York; Cravioto et al., 1961) and 0.8% (of 8735 cases, Ulleval Hospital, Norway; Torvik et al., 1982).

Wernicke's encephalopathy is a treatable condition, and following thiamine administration (50 to 100 mg daily), ophthalmoplegia is generally rapidly reversed (within days); while nystagmus, ataxia and mental confusion respond more gradually (within weeks to months); recovery of memory loss is minimal in the majority of patients (Victor et al., 1989)..

It has been suggested that the prompt reversal of ocular palsies by thiamine indicates that neurological defects are due to a biochemical abnormality that has stopped short of significant structural change, and that since recovery from amnesia is slow and incomplete, this implies that memory loss, once fully developed, depends on a structural rather than a "biochemical lesion" (Victor et al., 1989).

1.2.2 Neuropathology of Wernicke's encephalopathy

Postmortem examination of patients with WE show characteristic bilaterally symmetrical lesions within selective regions of the diencephalon and brainstem, though the mechanisms responsible for the selective vulnerability of certain regions remains unknown. Lesions are found in periventricular areas of the diencephalon, periaqueductal region of the midbrain, structures in the floor of the fourth ventricle, and in the cerebellum (Cravioto et al., 1961; Harper, 1979; Victor et al., 1989).

The mammillary bodies appear to be particularly susceptible to thiamine deficiency; histopathological studies often describe shrunken mammillary bodies with a brownish discolouration (Cravioto et al., 1961; Harper, 1979; Torvik et al., 1982; Victor et al., 1989). In a comprehensive study of the WKS by Victor and colleagues (1989), neuropathologic studies revealed mammillary body lesions in 46 of 62 cases, and in 15 of the 46 affected cases, the mammillary bodies were reduced to half of their normal size. The medial thalamus was also frequently involved; the most vulnerable area was the medial dorsal nuclei. In the midbrain the periaqueductal region was the most affected. Lesions were also seen in the medial-vestibular nucleus. Examination of the cerebellum revealed atrophy of the folia of the superior vermis, and nearly complete loss of Purkinje cells over the entire folia (Torvik et al., 1982).

Microscopically the lesions varied depending on the age of the lesion, the severity of the disease, and the brain region involved (Victor et al., 1989). In general, acute lesions were characterized by slight loss of neurones, axis cylinders, myelin sheaths, and proliferation of pleomorphic microglia, prominence of blood vessels, including proliferation of capillaries and hypertrophy of endothelial cells; and alterations of astrocytes. In chronic lesions there was loss of parenchymal elements and the main reactive cells present were

fibrous astrocytes. Endstage tissue had a loose vacuolated appearance although neurones may still have been present (Harper, 1979, 1983; Victor et al., 1989).

Other neuropathological studies have also described differences in the topographic distribution and severity of lesions in acute and chronic WE (Torvik et al., 1982; Torvik, 1987). In general, acute cases show more extensive and severe lesions than the chronic cases. In acute cases lesions are seen in the mammillary bodies, thalamus, and subependymal structures along the ventricles and aqueduct, whereas in the chronic cases lesions were found mainly in the mammillary bodies and thalamus (Torvik, 1987). Examination of acute cases revealed swelling of the capillary endothelium, destruction of the neuropil, macrophage response, astrocytic reaction and perivascular haemorrhages within the mammillary bodies. In chronic cases there was evidence of previous destruction of the neuropil with sponginess of the tissue and gliosis in the centre of the mammillary bodies. There was no swelling of the vascular endothelium in these cases (Torvik et al., 1982).

The occurrence of two types of brain lesions in WE has been described (Torvik, 1985). Lesions in the mammillary bodies and subependymal structures along the ventricles and aqueduct were characterized by severe endothelial swelling, destruction of the neuropil and neuronal sparing, whereas in the thalamus and inferior olives there was evidence of neuronal disintegration, sparing of the neuropil, and mild swelling of capillary endothelium. Furthermore, the neuronal disintegration in the thalamus and inferior olives was found to resemble that seen in anoxic necrosis.

1.3 Experimental thiamine deficiency

1.3.1 Pyrithiamine: a central thiamine antagonist

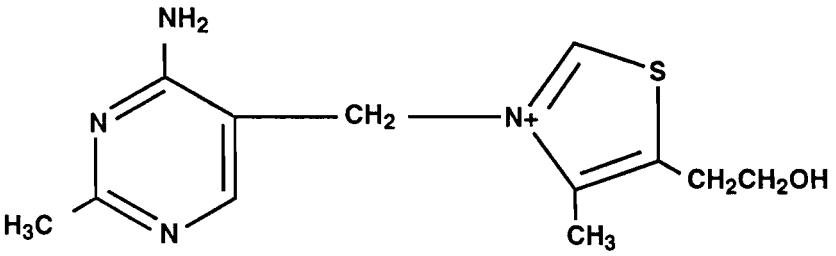
Pyrithiamine is a centrally acting thiamine antagonist (Rindi and Perri, 1961; Figure 3), that has several biochemical actions including the inhibition of conversion of thiamine to its active form (TDP), by acting as a substrate for thiamine pyrophosphokinase (TPK) (Gubler, 1968; Rindi and Perri, 1961; Rogers, 1970), as well as inhibiting the transport of thiamine into the brain (Spector, 1976) It has also been reported that pyrithiamine can displace thiamine from the vagus nerve of the rabbit (Cooper, 1968).

1.3.2 Thiamine deficiency in animals

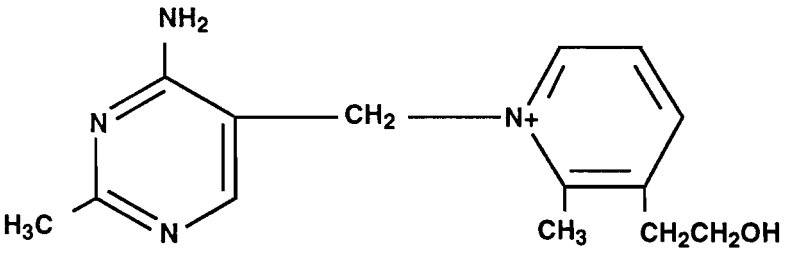
Administration of pyrithiamine to rats, along with a thiamine-deficient diet, results in neurological signs of thiamine deficiency including weight loss, hypothermia, loss of righting reflex, ataxia and opisthotonus. In the advanced stages of thiamine deficiency convulsions ultimately lead to death of the animal (Dreyfus and Victor, 1961; Troncoso et al., 1981; Vortmeyer and Colmant, 1988).

Neuropathological examination of pyrithiamine-induced thiamine-deficient rats shows lesions that are similar in nature and distribution to those seen in WE in humans (Papp et al., 1981; Troncoso et al., 1981; Armstrong-James et al., 1988). Lesions are found in the thalamus, mammillary bodies, collicular plate, vestibular nuclei and inferior olive. Studies of the severest lesions reveal oedema, necrosis of the neurones and neuropil, and haemorrhages surrounding the capillaries (Troncoso et al., 1981).

Similar to WE in humans, histological studies have also revealed two types of lesions in pyrithiamine-induced thiamine deficient rats (Vortmeyer and Colmant, 1988). Lesions of the inferior colliculus and vestibular nuclei were characterized by bullous spongiform appearance of the neuropil and severely damaged oedematous neurones. Examination of



Thiamine



Pyrithiamine

Figure 3- Chemical structures of thiamine and pyrithiamine

the thalamus and inferior olives revealed shrunken neurones with eosinophilic cytoplasm which resembled ischaemic neuronal necrosis.

There are various opinions concerning the primary lesion in thiamine deficiency. In studies of dietary-deprived thiamine deficient rats, Tellez and Terry (1968) found the earliest changes to be in the presynaptic terminals and axons. However, previous reports suggest that the initial cellular changes in thiamine deficient rats and mice consist of glial modifications including oedematous swelling of astrocytes, oligodendrocytes and myelin sheaths (Collins, 1967; Robertson et al., 1968,; Tellez and Terry, 1968; Watanabe and Kanabe, 1978). In a study of the brainstem of dietary-deprived thiamine deficient rats, Collins (1967) described the earliest changes as cytoplasmic and nuclear swelling of the glial cells, which in perivascular regions was associated with an alteration of the capillary basement membrane. Robertson et al (1968) also studied the brainstem of thiamine deprived rats and reported that early lesions consisted of intracellular swelling of perivascular glial foot processes. More advanced lesions involved swelling of the myelin sheaths and the extracellular compartment. In studies of pyrithiamine-induced thiamine deficient mice the early lesion was found to consist of oedema involving astrocytes, oligodendrocytes and myelin sheaths (Watanabe and Kanabe, 1978).

1.4 TDP-dependent enzymes and the "biochemical lesion" in thiamine deficiency

TDP is the cofactor for three enzymes involved in cerebral glucose utilization and energy metabolism; two citric acid cycle enzymes, the pyruvate dehydrogenase complex (PDHC) and α-ketoglutarate dehydrogenase (α-KGDH); and transketolase (TK), a constituent enzyme of the pentose-phosphate pathway, that produces pentoses for nucleotide synthesis and NADPH for biosynthesis of fatty acids (Figure 4).

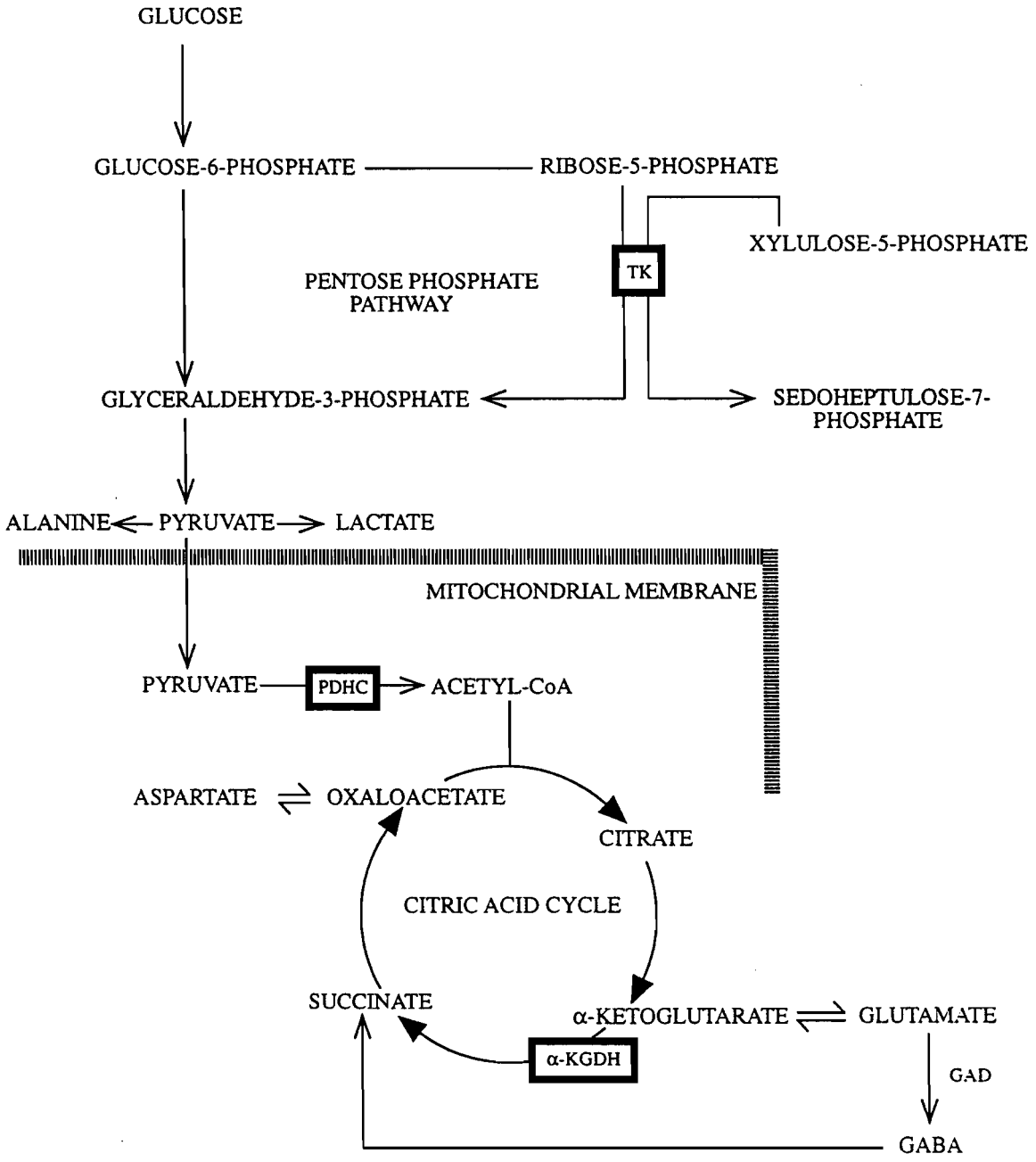


Figure 4- Thiamine-dependent enzymes involved in cerebral glucose metabolism. TK; transketolase, PDHC; pyruvate dehydrogenase complex, α-KGDH, α-ketoglutarate dehydrogenase.

Studies by Peters et al. (1936) demonstrated that thiamine-deficiency in pigeons resulted in opistothonus and the accumulation of lactate in the brainstem of affected birds. The addition of small amounts of thiamine to the thiamine-deficient brain tissue *in vitro* corrected the metabolic defect, which led Peters to suggest that the reversible or "biochemical lesion" in thiamine deficiency was due to a defect in the oxidation of pyruvate. There are reports of decreased PDHC actvities in the brainstem of dietary thiamine-deprived rats (Dreyfus and Hauser, 1965; Pincus and Wells, 1972; Butterworth et al., 1985). However, reductions of PDHC activity are only noted after the onset of neurological symptoms of thiamine deprivation. Studies to date in pyrithiamine-induced thiamine-deficient rats have failed to reveal significant reductions in PDHC activities in any brain region (Gibson et al., 1984; Butterworth et al., 1986; Elnageh and Gaitonde, 1988).

Activities of α-ketoglutarate dehydrogenase (α-KGDH) are decreased in severe thiamine deficiency, and such decreases precede the onset of neurological symptoms (Gibson et al., 1984; Butterworth et al., 1986). In addition, α-KGDH activities are restored to normal following thiamine-treatment and reversal of the neurological symptoms of thiamine deficiency (Butterworth et al., 1986; Butterworth and Héroux, 1989). Decreased activity of α-KGDH would be expected to result in a decrease of citric acid cycle intermediates beyond the α-ketoglutarate step, and subsequent decreased entry of pyruvate into the citric acid cycle, resulting in accumulation of lactate and alanine. A study of pyrithiamine-induced thiamine deficient rats revealed decreased activities of α-KGDH and concomitant increases of alanine in brain (Butterworth and Héroux, 1989). These findings suggest that the reversible symptoms of thiamine deficiency (i.e. the "biochemical lesion") results from reversible decreases of α-KGDH.

Activities of transketolase (TK) are also decreased in thiamine deficiency (McCandless et al., 1976; Gibson et al., 1984; Giguere and Butterworth, 1987). However, such decreases

are not restored to normal values following thiamine-treatment sufficient for normalization of neurological function (Gibson et al., 1984; Giguere and Butterworth, 1987); suggesting that changes in TK activity may not be related to the reversible symptoms of thiamine deficiency, and therefore are not involved in the "biochemical lesion" in this disorder.

1.5 Mechanisms of neuronal cell death in thiamine deficiency

Several mechanisms have been proposed to explain the selective neuronal loss seen in thiamine deficiency. Such mechanisms include:

(i) Impaired cerebral energy metabolism (Aikawa et al., 1984)

(ii) Focal accumulation of lactic acid and subsequent pH changes (Hakim and Pappius, 1983)

(iii) Region-selective breakdown of the blood-brain barrier (Harata and Iwasaki, 1995; Calingasan et al., 1995)

(iv) Glutamate-excitotoxicity mediated by the N-methyl-D-aspartate (NMDA) receptor (Langlais and Mair, 1990)

1.5.1 Impaired cerebral energy metabolism

The brain depends on an adequate supply of glucose as an energy source and glucose utilization is thiamine-dependent. Thiamine deficiency and hence the lack of cofactor results in reduced activities of thiamine-dependent enzymes of the citric acid cycle, which would be expected to result in decreased synthesis of high energy phosphates (such as ATP and phosphocreatine). In particular, activities of α-KGDH are reduced in the brain during thiamine deficiency (Gibson et al., 1984; Butterworth et al., 1986), and this rate-limiting enzyme is important for the maintenance of cellular energy metabolism (Butterworth, 1989). It has been suggested that selective neuronal cell loss in thiamine deficiency may result from reduced activities of thiamine-dependent enzymes which cause mitochondrial abnormalities and impairment of cerebral energy metabolism (Parker et al., 1984; Aikawa et

al., 1984). Studies of thiamine-deficient rats revealed that levels of total high energy phosphate were reduced to 89% of controls in the diencephalon and 91% in the lower brainstem before the onset of neurological signs of thiamine deficiency, and to 76% and 79%, respectively, after the onset of symptoms (Aikawa et al., 1984).

Autoradiographic studies using ^{14}C-deoxyglucose have revealed changes in local cerebral glucose utilization (LCGU) in brains of thiamine-deficient animals (Hakim and Pappius, 1981, 1983; Sharp et al., 1982). As thiamine deficiency progressed over a 11 day period, a gradual decline in LCGU was seen in a number of brain regions, this decline was followed by a significant rise in LCGU, which preceded another decline in LCGU, clinical symptoms of thiamine deficiency and histological lesions (Hakim and Pappius, 1983). It was suggested that the rise in LCGU was due to increased glycolysis resulting from an attempt to maintain ATP levels (the "Crabtree Effect") which were compromised by the reduced activity of citric acid cycle enzymes. Such changes were seen in selective brain regions, suggesting that some structures, including those known to be histologically affected, were metabolically more vulnerable to thiamine-deficiency (Hakim and Pappius, 1983).

1.5.2 Focal accumulation of lactic acid and H^+

Early studies of thiamine deficiency described decreased activities of PDHC and increased levels of brain lactate (Kinnersley and Peters, 1930; McCandless and Schenker, 1968; McCandless, 1982), and it has been suggested that focal accumulation of lactate, and consequent acidosis, may contribute to neuronal loss in thiamine deficiency (Hakim and Pappius, 1983; Hakim, 1984). In addition, when the rate of ATP breakdown exceeds that of synthesis and levels of ATP decrease, large quantities of H^+ accumulate and intracellular acidification occurs (Erecinska and Silver, 1989).

In an autoradiographic study using ^{14}C-dimethyloxazolidinedione (DMO) to measure regional cerebral pH, acidosis was seen in many of the brain regions that had shown the previously mentioned rise in LCGU (Hakim, 1984). Acidosis has been implicated in neuronal damage (Myers, 1979; Siesjo, 1988a; Siesjo et al., 1993; Golman et al., 1989), and may cause injury in a number of ways including promoting formation of free radicals, which can mediate lipid peroxidation and disruption of membranes (Rhencrona et al., 1989); causing denaturation of proteins and nucleic acids (Kalimo et al., 1981); causing cell swelling leading to osmolysis (Siesjo, 1985); inhibiting mitochondrial energy metabolism (Ljunggren et al., 1974; Hillered et al., 1985); and may also facilitate Ca^{2+} release from intracellular stores (Busa and Nuccitelli, 1984; Abercrombie and Hart, 1986). In support of the role of acidosis in thiamine deficient-induced brain damage, studies using nimodipine, a Ca^{2+} channel blocker, reveal a reduction in acidosis in vulnerable brain regions of thiamine deficient animals. By blocking the voltage-dependent calcium channel, nimodipine enabled optimal use of the Na^+/H^+ exchanger to reduce the proton load in the thiamine-deficient brain (Vogel and Hakim, 1988).

1.5.3 The blood-brain barrier

The "blood-brain barrier" (BBB) functions to provide a protected environment for the brain by maintaining the ionic homeostasis around synapses (Goldstein and Betz, 1986; Risau, 1991; Abbott, 1992). The BBB refers to the anatomical and physiological complex that controls the movement of substances from the extracellular fluid of the body to that of the brain (Nolte, 1988). Endothelial cells of brain capillaries, that form the BBB, are joined by *tight junctions*, and are devoid of fenestrations and transcapillary channels, and have very few pinocytotic vesicles (Bradbury, 1984; Goldstein and Betz, 1986; Risau, 1991). Closely associated with the cerebral capillary endothelium are the basement membrane, astrocytic foot processes which ensheath the capillaries, pericytes and other perivascular cells located between the endothelium and glia (Bradbury, 1984; Risau, 1991; Abbott et

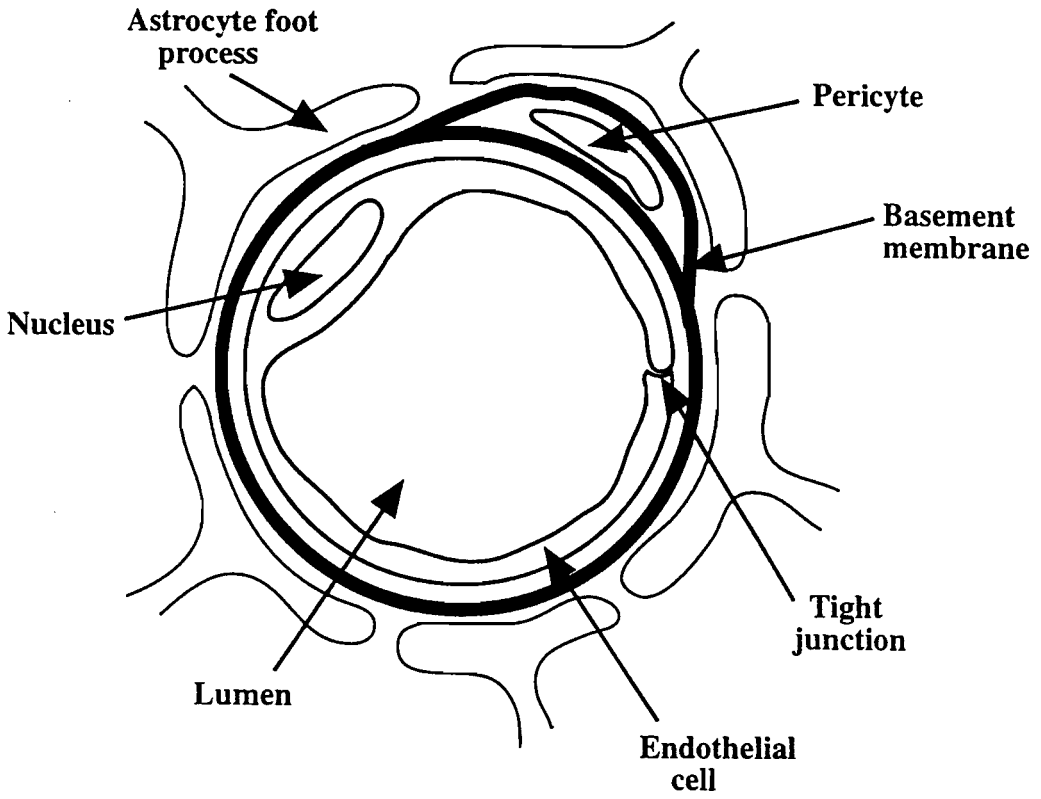


Figure 5- Schematic representation of the blood-brain barrier showing the tight junction of the cerebral capillary endothelial cell, and the associated basement membrane and astrocytic foot processes.

al., 1992; Figure 5). These features of the cerebral capillary endothelium permit selective entry of many types of molecules into the brain; the tight junctions of cerebral capillary endothelium are virtually impermeable to large proteins such as albumin (MW 68 900) and horseradish peroxidase (HRP; MW 40 000) (Reese and Karnovsky, 1967; Bradbury, 1984; Abbott et al., 1992).

1.5.3.1 The blood-brain barrier in thiamine deficiency

Vascular changes have been described in both human WE (Harper, 1979; Torvik, 1985; Okeda et al., 1995) and thiamine-deficient rats (Troncoso et al., 1981). Okeda et al. (1995) described the vascular changes as luminal dilatation and endothelial swelling of almost all vessels, and petechial haemorrhages and fibrinoid degeneration involving selectively the arterioles and capillaries on the arterial side. Such changes in vasculature have suggested that increased permeability of the blood-brain barrier may play a role in the pathogenesis of selective neuronal loss seen in thiamine-deficiency. Previous studies have revealed breakdown of the blood-brain barrier in selective brain regions of thiamine-deficient mice and rats (Warnock and Burkhalter, 1968; Robertson and Manz, 1971; Manz and Robertson, 1972; Watanabe et al., 1981; Phillips and Cragg, 1984). In two recent studies increased permeability of the blood-brain barrier was seen at early stages of thiamine-deficiency, preceding the appearance of major histological lesions in both pyrithiamine-induced thiamine-deficient mice (Harata and Iwasaki, 1995) and rats (Calingasan et al., 1995). Disruption of the blood-brain barrier has also been described in WE patients; Magnetic Resonance Imaging studies reveal damage of the blood-brain barrier in periventricular areas in acute WE (Schroth et al., 1991).

1.5.4 Excitatory amino acid receptors

The excitatory amino acid (EAA) neurotransmitters glutamate and aspartate are a major class of neurotransmitters in the mammalian central nervous system (Fagg and Foster, 1983; Cotman et al., 1987). There are at least five types of EAA receptors in the CNS, defined by preferred agonists, and accordingly designated as (i) kainate, (ii) quisqualate (also called α-amino-3-hydroxy-5-methyl-ioxazole-4-propionic acid; AMPA), (iii) NMDA (N-methyl-D-aspartate), (iv) AP4 (1-2-amino-4-phosphonobutyrate) which appears to be an inhibitory autoreceptor, and (v) ACPD receptors (trans-1-aminocyclopentane-1-3-dicarboxylic acid) (Cotman and Iversen, 1987; Monaghan et al., 1989; Cooper et al., 1991). Kainate and AMPA receptors are permeable to Na^+ and K^+ and are thought to be involved in fast synaptic transmission (Rothman and Olney, 1986; Siesjo, 1988b). Two of the EAA receptors can regulate intracellular Ca^{2+}; NMDA-receptor ion channels allow the passage of Na^+, K^+ and Ca^{2+}, and as a metabotropic receptor, the ACPD receptor can activate inositol phosphate metabolism leading to mobilization of internal Ca^{2+} stores (Cotman and Monoghan, 1989).

The NMDA receptor-ion channel complex is comprised of multiple components including (1) a transmitter recognition site for glutamate or NMDA, (2) a regulatory site which binds glycine, (3) a channel site which binds phencyclidine (PCP), (4) a voltage-dependent binding site for Mg^{2+} and (5) an inhibitory site which binds Zn^{2+} (Watkins and Evans, 1981; Fadda et al., 1988; Cotman and Monaghan et al., 1989; Mayer et al., 1989) (Figure 6). Autoradiographic studies have revealed two classes of NMDA receptors which are differentially labeled by ^{3}H-glutamate and ^{3}H-CPP [3-((+)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate] and differ in their regulation by glycine and anatomical distribution in the CNS (Monaghan et al., 1988).

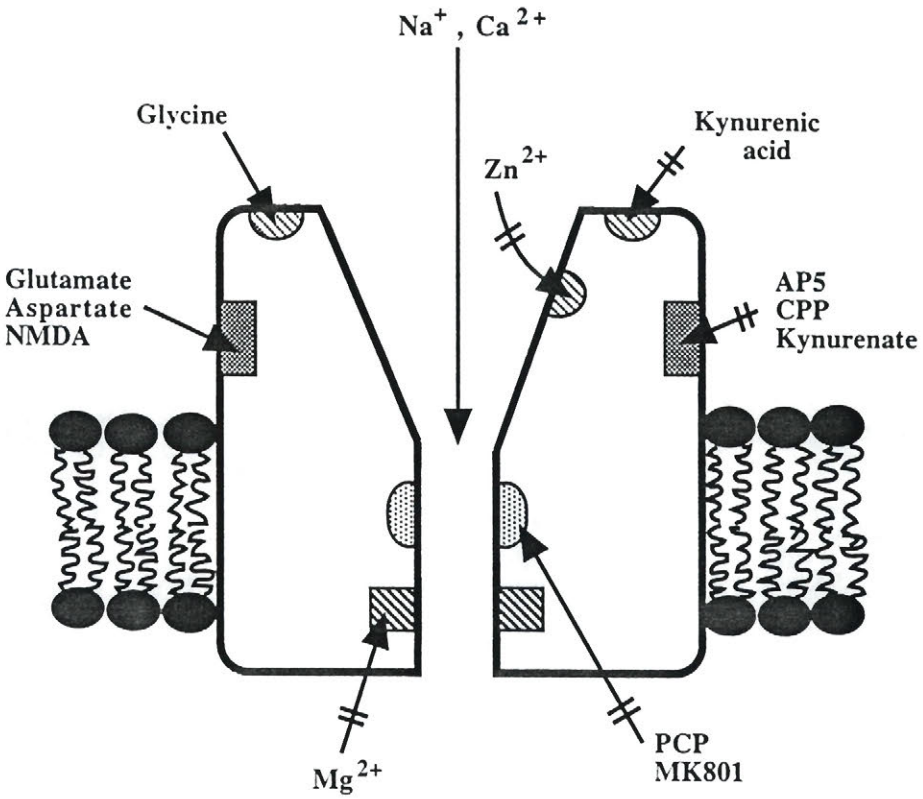


Figure 6- The N-methyl-D-aspartate (NMDA)-receptor ion channel complex showing (1) a recognition site where glutamate, aspartate and NMDA bind and act as agonists, and at which AP5 (D-amino-5-phosphonopentanoate), CPP [((±)-2-carboxypiperazine-4-yl)propyl-1-phosphonic acid] and kynurenate act as competitive antagonists, (2) a binding site where glycine binds to act as a allosteric modulator, and where kynurenic acid displaces glycine binding competitively, (3) a binding site at which zinc acts as a noncompetitive antagonist. There are binding sites in the pore of the channel including (1) a voltage-dependent binding site for magnesium and (2) a binding site for PCP (phencyclidine) and MK801. (Adapted from Mayer et al., 1989).

1.5.4.1 NMDA-receptor mediated glutamate-excitotoxicity

Elevated extracellular concentrations of glutamate are thought to play a role in neuronal damage seen in a number of neurological disorders including anoxia-ischaemia, hypoglycemia, Huntington's disease and epilepsy (McGeer and McGeer, 1976; Collins and Olney, 1982; Benveniste et al., 1984; Ben-Ari, 1985; Rothman and Olney, 1986). It has been suggested that "glutamate-excitotoxicity" results from the prolonged depolarizing action of glutamate, and related excitotoxins, at postsynaptic EAA receptors on neurones; in particular, the NMDA receptor is thought to be involved (Olney et al., 1971; Choi et al., 1988). At normal resting membrane potential the ion channel of the NMDA-receptor complex is blocked by Mg^{2+} in a voltage-dependent manner (Mayer et al., 1984; Nowak et al., 1984); however upon partial depolarization of the cell, resulting from glutamate acting at non-NMDA receptors (Siesjo et al., 1989), this voltage-dependent block is removed, allowing the entry of Na^+ and Ca^{2+} (MacDermott et al., 1986; Mayer and Westbrook, 1987). This can in turn lead to a large increase in intracellular Ca^{2+} which is cytotoxic (Nicotera et al., 1990). Excitotoxicity is thought to lead to neuronal death through two mechanisms (Rothman and Olney, 1987; Choi, 1994). Firstly, an acute component characterized by prolonged depolarization and Na^+ entry into the cell which pulls Cl^- in along an electrochemical gradient, which leads to further cation (mostly Na^+) influx to maintain electroneutrality. The entry of Na^+ and Cl^- draws water into the cell leading to cell swelling, osmotic lysis and rapid neurodegeneration (Rothman, 1985; Olney et al., 1986; Choi, 1987). However, it has been suggested that a second component, characterized by delayed cell degeneration and involving Ca^{2+}, may be the more significant cause of neuronal cell death by glutamate excitotoxicity (Griffiths et al., 1983; Simon et al., 1984; Choi, 1985; Cheung et al., 1986; Garthwaite and Garthwaite, 1986; Garthwaite et al., 1986; Rothman et al., 1987; Michaels and Rothman, 1990).

1.5.4.2 Cellular calcium homeostasis

The cytosolic concentration of calcium is maintained at approximately 10^{-7}M, while the concentration of calcium in the extracellular fluid is close to 10^{-3}M (Cheung et al., 1986; Siesjo, 1988b); this concentration difference creates a steep electrochemical gradient and negative membrane potential. Calcium can be extruded from cells by a calmodulin-activated Ca^{2+}-ATPase (Shatzmann, 1975; Carafoli, 1987) and the electrogenic Na^{+}/Ca^{2+} exchanger in the plasma membrane (Blaustein, 1977; Carafoli, 1987). In addition, intracellular calcium can be sequestered by organelles including endoplasmic reticulum and mitochondria (Weber et al., 1966; Lehninger, 1970; Carafoli, 1987; Blaustein, 1988), or bound to calmodulin or other calcium binding proteins (Cheung, 1980; Rhoads et al., 1985; Carafoli, 1987) (see Figure 7). In neurones, synaptic vesicles also appear to act as additional sequestration sites (Fifkova et al., 1983; Griffiths et al., 1983). In addition to entering through agonist-operated calcium channels (AOCC) which open in response to glutamate, such as the NMDA-receptor, calcium may also enter cells through voltage-sensitive calcium channels (VSCCs) (see Figure 7). There are four major types of VSCCs which open in response to depolarization: N (neuronal) and P (Purkinje cell) types which are found mainly on presynaptic endings, where they modulate the release of transmitters in response to presynaptic infflux of Na^{+} and depolarization; and the L (long-lasting) and T (transient) types which are mainly localized on postsynaptic membranes (Hess, 1990; Hoffman et al., 1994; Siesjo, 1994).

1.5.4.3 Intracellular calcium and neuronal damage

Elevated intracellular concentrations of calcium are cytotoxic for many reasons, which are mediated through actions on enzymes including lipases, phosphatases (or protein kinases), proteases and endonucleases, causing vascular and membrane damage, irreversible damage to mitochondria, degradation of neurofilaments, and ultimately cell death (Farber et al., 1981; Cheung et al., 1986; Choi et al., 1989; Nicotera et al., 1990; Siesjo, 1994).

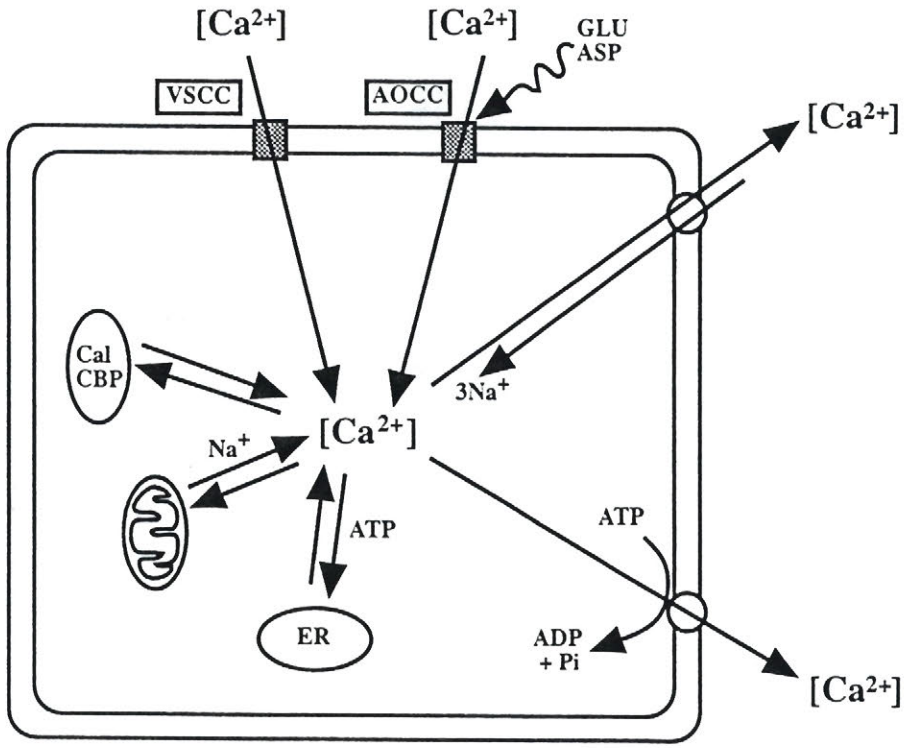


Figure 7- A schematic illustration of Ca^{2+} transport, binding and sequestration. Ca^{2+} entry occurs via voltage sensitive- and agonist operated- calcium channels (VSCC and AOCC), and exits by the ATP driven Na^{+}/Ca^{2+} exchanger. Also shown are Ca^{2+} binding to calmodulin (Cal) and calcium binding protein (CBP), and sequestration by mitochondria and endoplasmic reticulum. (Adapted from Siesjo, 1988).

Activation of phospholipase A_2 (PLA_2) results in the production of lysophospholipids and free fatty acids (including arachidonic acid) and platelet activating factor (PAF), which result in membrane and vascular dysfunction, as well as inhibiting mitochondrial uptake of Ca^{2+} (Lenzen et al., 1989; Rustenbeck and Lenzen, 1989). Activation of a receptor-linked phospholiase C (PLC) leads to the formation of diacylglycerol (DAG), and elevated levels of calcium may act with DAG to activate protein kinase C (PKC) leading to membrane alterations or phosphorylation of receptors and ion channels, which could enhance cell excitability and augment calcium influx (Kaczmarek, 1987; Connor et al., 1988). Activation of calcium-dependent neutral proteases (calpains) lead to proteolytic damage to the cytoskeleton and disassembly of microtubules which will disrupt axonal transport (Siman and Noszek, 1988).

Increased concentrations of Ca^{2+} have also been linked to the production of oxygen free radicals which can cause lipid peroxidation resulting in membrane and vascular damage (Chan and Fishman, 1980; Siesjo and Bengtsson, 1989), as well as further glutamate release (Pellegrini-Giampietro et al., 1988). Elevated levels of Ca^{2+} are also asscociated with formation of free radicals; activation of PLA_2 and PLC leads to the production of oxygen free radicals when arachidonic acid is further metabolized by cyclo-oxygenases and lipoxygenases (Malis et al., 1990). Oxygen free radicals induce mitochondrial damage associated with increased membrane permeability and decreased ATP synthetase activity (Malis and Bonventre, 1986). A major source of superoxide in postischemic tissue is xanthine oxidase which is converted from xanthine dehydrogenase by a Ca^{2+}-activated protease (McCord, 1985). Stimulation of NMDA receptors causes the release of nitric oxide which can react with superoxide to form peroxynitrite leading to the formation of hydroxyl radicals (Garthwaite et al., 1988; Beckman et al., 1990; Bredt and Snyder, 1992). Elevated intracellular Ca^{2+} can also activate endonucleases which degrade genomic DNA (Kure et al., 1991). In addition, calcium may also stimulate release of glutamate,

thereby propagating glutamate neurotoxicity in a positive-feedback manner (Rothman et al., 1987; Choi et al., 1989).

1.5.4.4 NMDA-receptor antagonists and neuroprotection

The availability of glutamate receptor antagonists and their potential neuroprotective and therapeutic effects have undergone much investigation (Simon et al., 1984; Meldrum, 1985; Albers et al., 1989; Bullock and Fujisawa, 1992). MK801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine hydrogen maleate] or dizocilpine, is a noncompetitive NMDA-receptor antagonist which has anticonvulsant, central sympathomimetic, and anxiolytic effects (Clineschmidt et al., 1982; Kemp et al., 1986; Wong et al., 1986). MK801 acts at a site within the NMDA receptor-ion channel resulting in a use-dependent block of the receptor, ie it is most effective when the receptor is activated (Wong et al., 1986, 1988; Foster and Wong, 1987). Within the channel, MK801 acts at a site distinct from the site where Mg^{2+} acts to block the receptor (Nowak et al., 1984; Mayer et al., 1984) and prevent the influx of Ca^{2+}. MK801 is a lipophilic molecule that is able to cross the blood-brain barrier, where its uptake is determined by the rate of cerebral blood flow (McCulloch and Iversen, 1991; Wallace et al., 1992). MK801 has been found to be effective in preventing neuronal loss in ischaemia (McDonald et al., 1987; Park et al, 1988; Olney et al., 1989; Swan and Meldrum, 1990; Gill et al., 1991; Lin et al, 1993), excitotoxin-induced brain damage (Foster et al., 1987; Olney et al., 1987; McDonald et al., 1990), hypoglycemia (Wieloch, 1985; Papagapiou and Auer, 1990; Nellgard and Wieloch, 1992), and animal seizure models (Leander et al., 1988; McNamara et al., 1988).

1.5.4.5 Excitotoxicity and thiamine deficiency

It has also been proposed that glutamate excitotoxicity may play a role in neuronal loss in thiamine deficiency (Langlais, 1995). The nature of the lesions due to thiamine deficiency

are of the ischaemic type (Torvik, 1985; Vortmeyer and Colmant, 1988), and studies have revealed increased extracellular levels of glutamate in vulnerable brain regions of thiamine deficient rats (Hazell et al., 1993; Langlais and Zhang, 1993). Elevated levels of glutamate may result from the energy impairment associated with thiamine deficiency (Aikawa et al., 1984; Langlais, 1995); depletion of energy metabolism can result in increased release of glutamate (Kaupinnen et al., 1988), and may also result in a loss of the voltage-dependent block of the NMDA receptor allowing the entry of Na^+ and Ca^{2+}, resulting in cytotoxic levels of calcium. Consistent with the possible role of excitotoxicity in neuronal loss in thiamine deficiency, administration of MK801 has been found to result in significant attenuation of necrotic damage in the thalamus of thiamine deficient rats (Langlais and Mair, 1990).

1.6 The "peripheral-type" benzodiazepine receptor- a novel marker of neuronal loss

1.6.1 Location of "peripheral-type" benzodiazepine receptors

Benzodiazepines are widely prescribed drugs because of their anxiolytic, anticonvulsant and sedative properties. Two classes of benzodiazepine receptors, which differ in tissue distribution, structure, function and pharmacology, have been identified in mammalian tissues (Mohler and Okada, 1977; Braestrup et al., 1977; Braestrup and Squires, 1977; Henn and Henke, 1978; Syapin and Skolnick, 1979). The two types of receptors, classified as "central" and "peripheral" types, can be distinguished by their different binding specificities (Gallagher et al., 1981); the benzodiazepines clonazepam and flumazenil (Ro15-1788) show high affinity for the central receptor, but have only a very low affinity for the peripheral receptor (Braestrup and Squires, 1977, 1978), while the "peripheral-type" benzodiazepine receptor (PTBR) can be identified by the benzodiazepine Ro5-4864 (Marangos et al., 1982), as well as the isoquinoline carboxamide PK11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide] which shows

high affinity for this receptor (Le Fur et al., 1983). While PTBRs have been found in peripheral tissues such as adrenals, lung, kidney, heart and the pituitary (Marangos et al., 1982; Taniguchi et al., 1982; Benavides et al., 1983a; Anholt et al., 1985), autoradiographic studies also reveal high densities of PTBRs in the brain, particularly in the olfactory bulb, neurohypophysis, choroid plexus and ependymal linings of the ventricles (Benavides et al., 1983; Richards and Mohler, 1984; Gehlert et al., 1985). Central benzodiazepine receptors are primarily located on neurones (Gallagher et al., 1981), but in the brain PTBRs are predominantly found on non-neuronal cells (McCarthy and Harden, 1981; Marangos et al., 1982; Sher and Machen, 1984; Schoemaker et al., 1982), including astrocytes (Bender and Hertz, 1985a; Tardy et al., 1981), microglia (Park et al., 1994) and cells of monocytic lineage, including macrophages (Zavala et al., 1984, 1987; Benavides et al., 1989; Ferrarese et al., 1990). It has been suggested that the PTBR is an integral protein of the outer mitochondrial membrane (Anholt et al., 1986a, b; Antkiewicz-Michaluk, 1988a), although PTBRs have also been localized on membranes of red blood cells (Olson et al., 1988a).

1.6.2 Structure of "peripheral-type" benzodiazepine receptors

Central benzodiazepine receptors constitute part of the γ-aminobutyric $acid_A$ ($GABA_A$) receptor-chloride complex where they modulate the affinity of the GABA receptor, and are responsible for the anxiolytic and anticonvulsant properties of benzodiazepines (Costa and Guidotti, 1979; Tallman et al., 1978, 1980). In contrast, the PTBR shows no association with the GABA receptor (Marangos et al., 1982; Schoemaker et al., 1983). Early studies proposed that the PTBR was a membrane protein complex composed of at least two subunits including, a 30-35 kDa subunit that contained the binding site for benzodiazepines, and a 17-18 kDa subunit that bound isoquinoline carboxamide derivatives (Doble et al., 1987; Antkiewicz-Michaluk, 1988b; Snyder et al., 1990). More recently it has been suggested the PTBR is comprised of at least three subunits (Figure 8); 18, 30 and

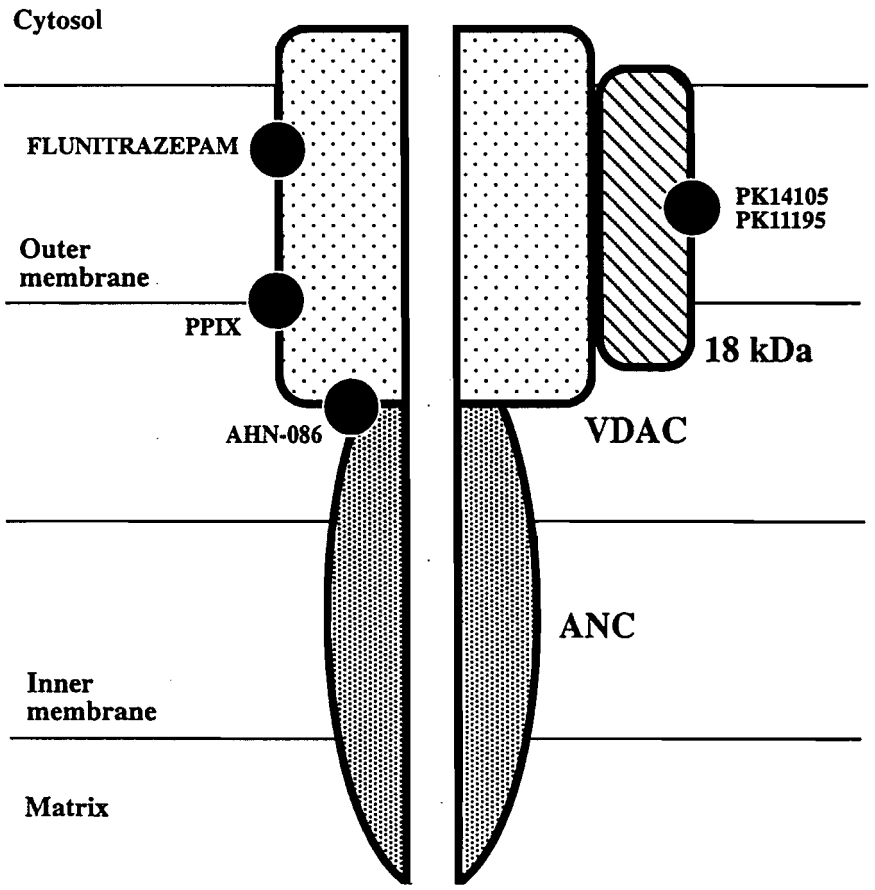


Figure 8- The "peripheral-type" benzodiazepine receptor (PTBR) showing the proposed three proteins that comprise the active receptor; the 18 kDa protein containing the recognition site for isoquinoline carboxamides (PK11195 and PK14105), which is associated with a voltage-dependent anion channel (VDAC) that binds benzodiazepines flunitrazepam and AHN-086; AHN-086 also binds to the closely associated adenosine nucleotide carrier (ANC). Protoporphyrin IX (PPIX) has been shown to inhibit ligand binding to the PTBR. (Adapted from McEnery, 1992).

32 kDa proteins, where the 18 kDa protein or isoquinoline binding protein (IBP) is associated with a 32 kDa protein, identified as a voltage-dependent anion channel (VDAC), also known as porin, in the outer mitochondrial membrane; and a 30 kDa protein or adenine nucleotide carrier (ANC) in the inner mitochondrial membrane (McEnery et al., 1992; Garnier et al., 1994). In this model the 30 and 32 kDa proteins can be labeled by benzodiazepines; flunitrazepam has been used to label a 32 kDa protein (Snyder et al., 1987), and AHN-086, a derivative of Ro5-4864, labels both 30 and 32 kDa proteins (Lueddens et al., 1986; McCabe et al., 1989, McEnery et al., 1992). The 18 kDa IBP has been purified (Antkiewicz-Michaluk et al., 1988b; Riond et al., 1989) and the corresponding complementary DNA (cDNA) cloned from rat, bovine and human tissue (Sprengel et al., 1989; Parola et al., 1991; Riond et al., 1991). The IBP appears to be comprised of five transmembrane α-helicle domains localized to the outer mitochondrial membrane (Sprengel et al., 1989; Bernassau et al., 1993). A recent study has also revealed the presence of a 10 kDa protein (Blahos et al., 1995), thus the PTBR appears to be a hetero-oligomeric complex for which the effector mechanism remains to be elucidated.

1.6.3 Function of "peripheral-type" benzodiazepine receptors

The function of the PTBR is unknown, but there is evidence to suggest a role in steroidogenic tissues (Muhkin et al., 1989; Papadopoulos, 1993; Gavish, 1995; Papadopoulos and Brown, 1995), where the PTBR is thought to mediate the intramembrane transport of cholesterol from the outer to the inner mitochondrial membrane; the rate limiting step in steroidogenesis (Krueger and Papadopoulos, 1990). In a recent model of the PTBR it was suggested that the five transmembrane α helicle domains of the 18 kDa IBP protein form a pore through which cholesterol is translocated to the inner mitochondrial membrane where cholesterol is converted to pregnenolone, the parent compound of steroid hormones (Bernassau et al., 1993). More recently Papadopoulos et al. (1994) have suggested that clusters of approximately five 18 kDa IBP proteins in

assoociation with a VDAC form a pore through which cholesterol can pass to reach the inner mitochondrial membrane.

The actual role of PTBRs in glial cells is unknown, however, studies have shown that mitochondria of glial cells, mainly oligodendrocytes, are capable of producing pregnenolone (Hu et al., 1987; Jung-Testas et al., 1989). Although the glial cell cultures used in the latter study also contained astrocytes (Jung-Testas et al., 1989). Doble et al. (1985) have described an association between PTBRs and voltage-sensitive calcium channels (VSCC). Furthermore, isoquinoline carboxamides are capable of modulating the activity of VSCC (Mestre et al., 1986), and dihydropyridines (VSCC ligands) have been shown to interact with the PTBR in glial cell cultures and binding studies (Cantor et al., 1984; Bender and Hertz, 1985b).

Other studies have shown that peripheral-type benzodiazepines have a variety of effects on cell growth and differentiation (Matthew et al., 1981; Wang et al., 1984a, b; Morgan et al., 1985), and increased densities of PTBR binding sites have been seen in brain tumours (Starosta-Rubenstein et al., 1987; Benavides et al., 1988; Olson et al., 1988; Ferrarese et al., 1989; Black et al., 1990; Ikezaki et al., 1990; Cornu et al., 1992; Miettinen et al., 1995). These findings along with the mitochondrial localization of the PTBR could suggest a role for this receptor as a potential modulator of intermediary metabolism (Anholt, 1986).

Increased densities of PTBRs have also been found in conditions of acute stress (Basile et al., 1987; Okun et al., 1988; Karp et al., 1989), and neuronal loss (for review see Myers, 1993). In studies assessing neuronal damage caused by intrastriatal injection of excitotoxins, increased densities of PTBR binding sites were found in membrane preparations of rat striatal tissue (Schoemaker et al., 1982; Owen et al., 1983; Gehlert et

al., 1985; Benavides et al., 1987); such increases of PTBRs were found to mirror decreased activities of marker enzymes for GABAergic and cholinergic neurones (Owen et al., 1983; Benavides et al., 1987). In addition, autoradiographic studies using the PTBR-specific ligand ^{3}H-PK11195 have revealed increased densities of PTBRs, which show good spatial correlation with histological assessment of neuronal loss, in experimental cerebral focal and global ischaemia (Dubois et al., 1988; Benavides et al., 1990; Demerle-Pallardy et al., 1991; Myers et al., 1991a, b; Stephenson et al., 1995) and excitotoxin-induced lesions in rat brain (Dubois et al., 1988; Benavides et al., 1990; Price et al., 1990). Increased densities of PTBRs have also been reported in a number of neuropathological conditions such as Alzheimer's disease (Owen et al., 1983; McGeer et al., 1988; Diorio et al., 1991), Huntington's disease (Schoemaker et al., 1982), multiple sclerosis (Benavides et al., 1988) and cerebral infarction (Benavides et al., 1988; Ramsay et al., 1992). Since PTBRs have been localized to glial cells and reactive gliosis is a consequence of neuronal loss, it has been suggested that PTBRs can be used as a marker of neuronal damage, ie an increase in density of PTBRs is an index of reactive gliosis that follows neuronal damage (Benavides et al., 1987, 1990).

1.7 Reactive gliosis

Various forms of injury to the brain including infection, ischaemia, trauma, or other insult, will provoke reactive gliosis, which is characterized by astrocytic proliferation, microglial activation and macrophage invasion; glial cells that appear to play important roles in the wound healing process in the brain (Norton et al., 1992; Landis, 1994).

1.7.1 Astrocytes

Astrocytes constitute up to 25% of cells and 35% of total mass of the CNS (Eng et al., 1992). The astrocyte is a multifunctional cell which plays many important roles in the CNS, where it provides structural support for the neurones, maintains the ionic composition of the extracellular space, metabolizes neurotransmitters, guides migrating neurones during development, and contributes to the structure of the blood-brain barrier (Kimelberg and Aschner, 1993). In addition, in response to injury in the CNS, reactive astrocytes form scar tissue by proliferating and extending numerous processes which show increased expression of Glial Fibrillary Acidic Protein (GFAP), the major subunit of astrocyte intermediate filaments (Eng, 1985, 1988; Lindsay, 1986; Miller et al., 1986; O'Callaghan, 1991; Landis, 1994). GFAP immunohistochemistry affords a useful technique for the study of the astroglial response to neuronal injury (Bignami and Dahl, 1976; Amaducci et al., 1981; Eng, 1985, 1988).

1.7.2 Macrophages and microglia

Mononuclear phagocytes (macrophages and microglia) play an important role in the early glial response to brain injury (Perry et al., 1993) where they function to phagocytose dead cells and cellular debris and may also play a role in wound-healing and scar formation (Thomas, 1992). There are two populations of macrophages in most tissues; one population associated with circulating precursor cells, and the other being resident within the tissue (Jordan and Thomas, 1988). Macrophages are generated in the bone marrow and circulate as monocytes in the blood before being delivered to target tissues where they differentiate into macrophages (Jordan and Thomas, 1988; Perry and Gordon, 1988; 1991). As a result of injury to the brain, the number of macrophages may be dramatically increased by rapid recruitment of blood monocytes to the site of injury (Jordan and Thomas, 1988; Perry and Gordon, 1988). Injury to the brain may also result in activation of microglial cells, which are the resident macrophages of the brain (Jordan and Thomas,

1988; Perry and Gordon, 1988, 1991; Dickson et al., 1993). Microglia express antigens found on macrophages, and share other properties in common with cells of the mononuclear phagocytic system (Perry and Gordon, 1991; Dickson et al., 1993). Four different forms of parenchymal microglia, corresponding to different morphological and functional states of a single cell type, have been identified and termed amoeboid, ramified, activated and reactive microglia (Jordan and Thomas, 1988; Thomas, 1992; Flaris et al., 1993). It is believed that blood monocytes are the precursors of amoeboid microglia, which are active macrophages during development, and the precursors of inactive ramified (resting) microglia. Following injury to the brain, ramified microglia draw in their processes and are converted firstly to activated microglia which are partially phagocytic, and then to the highly phagocytic reactive microglia (macrophages), which move to the site of injury or surround affected neurones (Thomas, 1992; Altman, 1994).

In addition to the change in morphology of the microglial cell, there is also an upregulation of antigenic molecules characteristic of macrophages, including the major histocompatibility (MHC) class I and II antigens, leucocyte common antigen and CD4 (Perry et al., 1993; Altman, 1994). At present there are no antigens uniquely expressed by microglia that make them distinct from other macrophage populations (Perry and Gordon, 1991). The ED1 antibody selectively labels a cytoplasmic antigen in mononuclear phagocytes, (i.e. monocytes and macrophages) (Dijkstra et al., 1985). It has also been reported that activated and reactive (phagocytic) microglia express the ED1 antigen (Graeber et al., 1990; Flaris et al., 1993; Altman, 1994). The ED1 antibody reacts with large, round oval cells (Polman et al., 1986; Sminia, 1986).

In addition to their function in removing dead cells and cellular debris, microglia-derived macrophages appear to also play a role in wound-healing within the CNS (Thomas, 1992). Previous studies suggest that cytokines released from macrophages may contribute to the

formation of the astroglial scar (Giulian and Baker, 1985; Giulian, 1990). Macrophages and microglia can release cytokines such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (Dinarello, 1984; Giulian et al., 1986, 1988; Selmaj et al., 1990; Benveniste, 1992), which induce astrocyte proliferation, and increased concentrations of these cytokines are encountered at sites of neuronal injury (Giulian and Lachman, 1985; Taupin et al., 1993). Furthermore, it has been shown that the inhibition of the secretory and phagocytic activity of activated microglia prevents astrogliosis following a penetrating wound to the brain (Giulian et al., 1989), and improves functional recovery from ischaemic injury to the spinal cord (Giulian and Robertson, 1990). It has been suggested that activated and reactive microglia participate in wound healing through not only regulation of gliosis in scar formation, but also by playing a role in neuronal regeneration and neovascularization (Thomas, 1992). It has been reported that IL6 released from microglia and astrocytes stimulate nerve growth factor for regeneration (Frei et al., 1989; Fontana et al., 1993), and that macrophages condition the non-neuronal cell surface for neurite growth (David et al., 1990). Macrophages release IL1 and transformimg growth factor β1 (TGF β1), two cytokines involved in angiogenesis (formation of new blood vessels) (Perry et al., 1993). Astrocytes also release TGF β1 and proliferate in response to IL1 (Benveniste, 1992; Fontana et al., 1993; Oppenheim and Saklatvala, 1993).

1.8 Non-invasive brain imaging techniques

1.8.1 Computed tomography

Computed tomography (CT) involves directing x-ray beams through the skull at a number of different angles and comparing and processing the resulting collection of density profiles with a computer (Nolte, 1988). In CT scanning multiple narrow x-ray beams are rotated 180° around one side of a patient's head while x-ray detectors are rotated around the opposite side in an identical path. At each degree of rotation, x-ray tube and detectors make

a series of transmission readings. The radiodensity of a region of tissue is calculated by summing the transmission readings of all beams passing through that region. The image from each "slice" of brain is a matrix computed from thousands of intersecting radiation intensity measurements, visually displayed as areas of relatively dark or light (Martin and Brust, 1985).

1.8.2 Positron emission tomography

Positron emission tomography (PET) utilizes certain isotopes that decay with the emission of positrons (eg ^{11}C, ^{13}N, ^{15}O and ^{18}F). The emitted positron is an unstable particle which quickly collides with an electron and the two particles are annihilated, producing two gamma rays that travel in opposite directions from one another. By surrounding a patient's head with a ring of gamma-ray detectors, it is possible to localize the site of gamma emission within the brain, which is how PET scanning is used to provide images of the brain (Martin and Brust, 1985; Nolte, 1988). With PET it is possible to study structure as well as function in the living brain. Glucose utilization can be studied in regions of the brain with ^{18}F-deoxyglucose which is taken up by neurones and metabolized by hexokinase like glucose. However, unlike glucose, phosphorylated deoxyglucose is not metabolized further and so accumulates within active brain cells. Thus, ^{18}F-deoxyglucose uptake into neurones provides an index of brain activity. PET techniques can also be used in similar studies of cerebral blood flow (with ^{15}O) and neurotransmitter receptor localization with ^{11}C-ligands (Martin and Brust, 1985).

1.8.3 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is another non-invasive technique that allows great resolution of anatomical detail in the living brain. MRI is based upon the resonance of atomic nuclei placed in a strong magnetic field. Atomic nuclei with an odd number of either protons or neutrons behave like small magnets, and tend to align themselves with an

externally applied magnetic field. A radiofrequency pulse can disrupt the alignment of the atomic nuclei. Termination of the radiofrequency pulse causes the atomic particles to realign themselves according to the direction of the magnetic field, and in doing so release energy in the form of radiofrequency signals. The energy emitted at a particular frequency from a particular area of tissue can provide information about the number of atoms of a particular type in that area of tissue. The rate at which the nuclei realign themselves occurs with characteristic relaxation time constants, T1 and T2; T1 and T2 refer to longitudinal and transverse relaxation times, respectively (Jacobson, 1987). Hydrogen atoms give strong magnetic resonance signals, and the brain is mostly made of water; grey matter has a higher water concentration than does white matter, so MRI can differentiate grey and white matter in detail in computer-generated "slices" of a living brain (Martin and Brust, 1985; Nolte, 1988).

1.8.4 Imaging of brain damage and the diagnosis of WE

WE in humans is difficult to diagnose and cases are frequently missed by routine clinical neurologic evaluation in both alcoholic and non-alcoholic patients (Torvik et al., 1982; Harper, 1983; Butterworth et al., 1991). The classical clinical triad of symptoms (ophthalmoplegia, ataxia, global confusional state) may occur in only a minority of WE patients (Harper et al., 1986). In a study of 131 cases of WE identified at autopsy, only 26 cases had been identified during life (Harper, 1983); and retrospective analysis of the clinical signs in 97 of these patients revealed that 16% showed the classical clinical triad, while 19% had no documented clinical signs (Harper et al., 1986). The discrepancy between clinical and pathological indentification of WE may suggest that chronic brain changes result from repeated subclinical episodes of WE (Harper et al., 1986; Bowden, 1990).

Diagnosis of WE has recently improved by non-invasive diagnostic procedures such as CT and MRI which enable the macroscopic visualization of structural brain changes. CT studies of acute WE reveal areas of decreased density in diencephalic regions (McDowell and Leblanc, 1984; Mensing et al., 1984; Kitaguchi et al., 1987). CT studies have also revealed severely reduced local cerebral blood flow in the thalamus, basal ganglia and limbic system of alcoholics with WKS (Hata et al., 1987). MRI studies reveal T2 signal hyperintensity, indicative of increased water content, in areas around the 3rd ventricle, aqueduct and 4th ventricle, which correlate with subsequently defined pathologic lesions in WE (Gallucci et al., 1990; Yokote et al., 1991). In chronic WE, neuropathologic studies frequently reveal shrinkage of mammillary bodies (Victor et al., 1989) and MRI has been used to detect mammillary body atrophy in WE (Charness and DeLaPaz, 1987; Yokote et al., 1991; D'Aprile et al., 1994). MRI has also successfully detected breakdown of the blood-brain barrier in acute WE (Schroth et al., 1991).

PET may offer an alternative (or additional) non-invasive technique for the diagnosis and assessment of neuronal cell loss in WE. Autoradiographic studies using ^{14}C-deoxyglucose have revealed reduced local cerebral glucose utilization in the pyrithiamine-induced thiamine deficient rat (Hakim and Pappius, 1983). Similarly, PET studies using ^{18}F-fluoro-2-deoxy-D-glucose reveal a 22% decrease of brain glucose utilization in alcoholic Korsakoff patients (Kessler et al., 1984). PET studies using ^{11}C-Ro15-1788, a ligand for the central benzodiazepine receptor, have been used in the evaluation of brain lesions in patients with Huntington's Disease (Holthoff et al., 1993) and epilepsy (Savic et al., 1993, 1994; Prevett et al., 1995); and to study the effects of alcohol on benzodiazepine receptor binding in non-WE alcoholics and healthy volunteers (Pauli et al., 1992; Litton et al., 1993; Farde et al., 1994).

Significant increases in densities of ^{3}H-PK11195 binding sites have been reported to occur in a number of conditions including cerebral ischaemia (Myers et al., 1991), Huntington's Disease (Schoemaker et al., 1982) and Alzheimer's Disease (Diorio et al., 1991), and consequently it has been suggested that alterations in the densities of these sites offer a useful indirect approach to the assessment of neuronal loss in these conditions (Benavides et al., 1987). ^{11}C-PK11195 is a PET ligand which has been used for the imaging of human gliomas (Starosta-Rubenstein et al., 1987; Black et al., 1989, 1990; Junck et al., 1989, 1991), glioblastomas (Pappata et al., 1991) and in studies of brain damage in cerebral infarcts (Junck et al., 1990; Myers, 1993). PET studies using ^{11}C-PK11195 may also be of value in the assessment brain damage in WE patients.

CHAPTER 2

Increased densities of binding sites for the "peripheral-type"benzodiazepine receptor (PTBR) ligand ^{3}H-PK11195 in vulnerable regions of the rat brain in thiamine deficiency encephalopathy

ABSTRACT

Quantitative receptor autoradiography was used to evaluate the density of high affinity binding sites for the "peripheral-type" benzodiazepine receptor (PTBR) ligand ^{3}H-PK11195 in brain regions of the rat at different stages of pyrithiamine-induced thiamine deficiency encephalopathy, an experimental model of the Wernicke-Korsakoff Syndrome (WKS). Assessment of the density of ^{3}H-PK11195 binding sites in thiamine-deficient animals showing no neurological signs of thiamine deficiency encephalopathy revealed no significant alterations compared to pair-fed control animals in any brain region studied. Densities of ^{3}H-PK11195 binding sites were, however, significantly increased in brain regions of the rat at the symptomatic stage, where increased densities were seen in the inferior colliculus (233% increase, $p<0.001$), inferior olivary nucleus (154% increase, $p<0.001$) and thalamus (up to 107% increase, $p<0.001$). Histological studies of these same brain regions revealed evidence of neuronal cell loss and concomitant gliosis. Densities of ^{3}H-PK11195 binding sites in non-vulnerable brain regions that showed no histological evidence of neuronal loss, such as the cerebral cortex, hippocampus and caudate-putamen, were not significantly different from those in control animals. Increased densities of binding sites for the PTBR ligand probably reflect glial proliferation and are consistent with an excitotoxic mechanism in the pathogenesis of neuronal cell loss in thiamine deficiency encephalopathy. Positron Emission Tomography (PET) using ^{11}C-PK11195 could offer a potentially useful diagnostic tool in WKS in humans.

INTRODUCTION

Thiamine, in the form of thiamine pyrophosphate, is a cofactor for several enzymes involved in cerebral glucose metabolism, including the pyruvate dehydrogenase complex (PHDC), the α-ketoglutarate dehydrogenase complex (α-KGDH) and transketolase (TK), which are important for energy production and synthesis of neurotransmitters such as glutamate, γ-aminobutyric acid (GABA) and aspartate. Pyrithiamine-induced thiamine deficiency in the rat results in region-selective metabolic abnormalities including reduced activities of α-KGDH (Butterworth et al, 1986) and increased concentrations of brain lactate (Hakim 1984) and alanine (Butterworth and Héroux, 1989). Metabolic perturbations are particularly evident in brain regions such as the thalamus, a region of the brain that ultimately manifests neuronal cell loss in this model of thiamine deficiency encephalopathy (Troncoso et al, 1981; Aikawa et al, 1984). The nature and distribution of the brain lesions in pyrithiamine-treated rats closely resembles those reported in the Wernicke-Korsakoff Syndrome (WKS) in humans.

"Peripheral-type" benzodiazepine receptors (PTBRs) are highly localized on non-neuronal cells, particularly on the outer membrane of mitochondria in astrocytes (Syapin and Skolnick, 1979; McCarthy and Harden, 1981; Tardy et al, 1981; Bender and Hertz, 1985, 1987; Anholt et al, 1986; Basile and Skolnick, 1986). Densities of PTBRs have been shown to be increased in membrane preparations from rat striata following intrastriatal injection of excitotoxic compounds (Schoemaker et al, 1982; Owen et al, 1983; Benavides et al, 1987); as well as in postmortem brain tissue from patients with Huntington's disease (Schoemaker et al, 1982) and Alzheimer's disease (Owen et al, 1983; Diorio et al, 1991). The increases in densities of PTBRs following excitotoxic lesions were found to be mirrored by decreases in activities of neuronal marker enzymes choline acetyltransferase and glutamic acid decarboxylase (Benavides et al, 1987), and by histological evidence of neuronal loss and increased gliosis (Schoemaker et al, 1982). Since glial proliferation,

following neuronal degeneration, could be responsible for the increase in PTBRs, it has been suggested that levels of PTBRs can be used as an indirect index of neuronal damage (Benavides et al, 1987).

Experimental thiamine deficiency is characterized by pathologic lesions, symmetrical in distribution, and found selectively in brain regions including the thalamus, mammillary bodies, inferior olive and inferior colliculi (Troncoso et al, 1981). Recent evidence suggests that glutamate-induced excitotoxicity may play a key role in the pathogenesis of neuronal cell death in thiamine deficiency (Langlais and Mair, 1990). As part of a series of investigations of the pathophysiologic mechanisms implicated in thiamine deficiency encephalopathy, quantitative receptor autoradiography was used to measure the densities of ^{3}H-PK11195 binding sites in vulnerable and non-vulnerable brain regions of the rat at different stages of thiamine deficiency encephalopathy.

MATERIALS AND METHODS

Treatment Groups-

Male Sprague-Dawley rats weighing 175-200 g were used for the experiments described. All animals were housed individually in the André-Viallet Clinical Research Centre's animal quarters under constant conditions of temperature, humidity, and light cycles. The following treatment groups were used for the studies described.

Group 1- Pair-fed controls.

Rats were fed a thiamine-deficient diet (ICN, Nutritional Biochemical, Cleveland, OH, U.S.A.) pair-fed to equal food consumption to that of rats in Groups 2 and 3. In addition, all rats received thiamine subcutaneously in a dose of 10 µg/ 100 g body weight/ day.

Group 2- PT presymptomatic.

Rats were fed a thiamine-deficient diet and administered pyrithiamine subcutaneously (50 µg in 0.2 ml of saline/ 100 g body weight/ day) for 7 days. At this time rats showed no evidence of neurological abnormalities (normal gait, normal righting reflexes).

Group 3- PT symptomatic.

Rats were fed a thiamine-deficient diet and administered pyrithiamine subcutaneously (50 µg in 0.2 ml of saline/ 100 g body weight/day). Neurological status was evaluated daily (Butterworth and Héroux, 1989) until loss of righting reflex was evident; this stage was generally between days 12 and 14.

Quantitative receptor autoradiography-

Rats were decapitated and the brains were removed rapidly and frozen in isopentane chilled on dry ice. Frozen brains were mounted onto microtome chucks. Brain sections (20 µm thick) were cut using a Microtome cryostat at -18°C and were thaw-mounted onto gelatin-coated glass microscope slides. Brain sections were kept at -70°C prior to use in binding experiments.

For binding experiments brain sections were incubated for 60 min at 25°C in Tris-HCl buffer (170 mM, pH=7.4) containing 1 nM ^{3}H-PK11195 (specific activity 86 Ci/ mmol, NEN). Non-specific binding was defined by incubating adjacent sections in the presence of 1 µM PK11195. The incubation was terminated by rinsing sections three times for 5 min in ice-cold incubation buffer. Sections were then dipped briefly in cold distilled water and dried rapidly under a stream of cold air.

Autoradiograms were prepared by apposing sections together with tissue-calibrated standards of known ^{3}H-concentrations (Amersham microscales) to ^{3}H-sensitive Hyperfilm

(Amersham) for 4 weeks. Films were developed and tissue concentrations of ^{3}H-PK11195 were measured by quantitative densitometry analysis using a MCID computer-based densitometer and image analysis system (Imaging Research Inc., Ontario, Canada). The amount of ^{3}H-PK11195 bound to various brain regions was calculated from the specific activity of the ligand. Specific binding in each area was calculated by subtracting from the total binding the amount bound in corresponding regions in adjacent sections incubated for non-specific labeling.

Histological Analysis-

Sections adjacent to those used for autoradiography were stained with cresyl violet using standard histological techniques and then viewed by light microscopy. Neuronal cell counts were made by a neuropathologist (L.O.) who was blinded to the treatment group. Measurements were made within fields measuring 500 x 500 μm, in sections observed at 250 x magnification.

Statistical Analysis-

Data are presented as mean ± SD. Statistical comparisons of binding site densities and neuronal cell counts between pair-fed control and pyrithiamine-treated groups were performed with the two-tailed Student's t test.

RESULTS

In agreement with previous reports (Gibson et al, 1984; Butterworth and Héroux, 1989), daily administration of pyrithiamine to rats on a thiamine-deficient diet resulted, within 14 days, in neurological signs of thiamine deficiency such as loss of righting reflex. Pair fed control animals showed no such neurological symptoms. Measurement of thiamine status using the erythrocyte transketolase assay revealed significant thiamine deficiency in pyrithiamine-treated animals in all cases (data not shown).

Autoradiographic Studies-

Assessment of the density of ^{3}H-PK11195 binding sites in presymptomatic pyrithiamine-treated animals revealed no significant differences between treated animals and pair-fed control animals (Table 1).

At the symptomatic stage of thiamine deficiency encephalopathy, significant region-selective increases in densities of ^{3}H-PK11195 binding sites were seen in the brains of pyrithiamine-treated animals (Table 2, Figure 1). Significant increases were seen in the inferior colliculus (increased by 233%, $p<0.001$), inferior olivary nucleus (increased by 154%, $p<0.001$), medial geniculate nucleus of the thalamus (increased by 107%, $p<0.001$) and the mammillary nucleus of the hypothalamus (increased by 83%, $p<0.001$). More modest but statistically significant increases were also observed in the posterior nuclei and ventroposterior nucleus of the thalamus. There were no significant alterations of ^{3}H-PK11195 binding sites in cerebral cortex, hippocampus, nor caudate-putamen.

Histopathological studies-

Neuronal cell counts were made within corresponding brain areas of control and treated animals. Examination of histological sections from presymptomatic animals revealed evidence of neuronal cell loss and mild to moderate gliosis (Table 3). Histological changes in the brains of symptomatic pyrithiamine-treated animals were characterized by pallor of the neuropil (Figure 2) and by neuronal cell loss in well demarcated areas including the medial geniculate nucleus of the thalamus, inferior olive and inferior colliculus (Table 3, Figure 3). In these same areas there was evidence of marked gliosis. Neither neuronal loss nor gliosis was seen in brain regions such as the caudate-putamen and frontal cortex in any of the treatment groups (Table 3).

Table 1- ^{3}H-PK11195 binding site densities in brain regions of presymptomatic pyrithiamine-treated and pair-fed control rats.

Brain region	^{3}H-PK11195 binding (pmol/g tissue) Pair-fed control (n)	PT-treated (n)
Cerebral cortex		
Motor cortex	49.0 ± 9.54 (6)	62.1 ± 24.7 (5)
Somatosensory cortex	40.7 ± 7.89 (6)	53.0 ± 14.3 (5)
Auditory cortex	42.9 ± 10.2 (5)	53.7 ± 8.16 (5)
Frontal cortex	59.3 ± 14.5 (6)	58.5 ± 23.8 (5)
Caudate-putamen	23.1 ± 3.46 (6)	31.9 ± 5.81 (5)
Limbic		
Hippocampus-CA1	35.8 ± 7.44 (6)	52.7 ± 23.7 (6)
CA3	29.5 ± 5.81 (6)	43.9 ± 20.5 (6)
Thalamus		
Medial geniculate nucleus	39.2 ± 8.01 (5)	51.1 ± 16.8 (6)
Posterior nuclei	33.1 ± 10.9 (5)	48.5 ± 24.1 (6)
Ventral posterior nucleus	25.4 ± 10.3 (6)	43.4 ± 23.7 (5)
Hypothalamus		
Dorsomedial nucleus	56.0 ± 8.45 (6)	73.9 ± 11.3 (6)
Mammillary nucleus	104.8 ± 19.9 (5)	110.2 ± 10.5 (6)
Midbrain		
Inferior colliculus	60.5 ± 8.03 (6)	75.2 ± 18.6 (6)
Substantia nigra	46.8 ± 15.2 (5)	63.1 ± 16.7 (6)
Pons and Medulla		
Inferior olivary nucleus	96.0 ± 6.83 (5)	105.3 ± 26.4 (6)
Superior olivary nucleus	73.4 ± 26.5 (6)	101.9 ± 23.5 (6)
Lateral vestibular nucleus	65.7 ± 10.0 (5)	70.9 ± 21.6 (6)
Medial vestibular nucleus	85.5 ± 18.8 (5)	89.8 ± 21.3 (6)
Facial nucleus	99.3 ± 15.1 (6)	110.0 ± 24.2 (5)
Raphe nucleus	57.7 ± 20.8 (5)	56.7 ± 15.6 (5)
Cerebellar Cortex	71.8 ± 19.7 (5)	74.9 ± 28.3 (6)

Values represent mean ± SD (pmol/g of tissue) specific binding of ^{3}H-PK11195 in presymptomatic pyrithiamine-treated and pair-fed control rats. n, number of animals per group. There were no significant differences between the two groups (Student's t test, two-tailed).

Table 2- ^{3}H-PK11195 binding site densities in brain regions of symptomatic pyrithiamine-treated and pair-fed control rats.

Brain region	^{3}H-PK11195 binding: Pair-fed control (n) (pmol/g tissue)	PT-treated (n) (pmol/g tissue)	% increase	
Cerebral cortex				
Motor cortex	61.8 ± 8.56 (6)	75.1 ± 20.9 (6)	21	
Somatosensory cortex	64.8 ± 10.6 (6)	64.1 ± 18.4 (6)	0	
Auditory cortex	60.9 ± 11.9 (6)	66.8 ± 17.0 (6)	10	
Frontal cortex	56.3 ± 10.8 (6)	62.8 ± 13.8 (6)	11	
Caudate-putamen	34.3 ± 3.18 (6)	36.5 ± 3.56 (6)	6	
Limbic				
Hippocampus-CA1	64.1 ± 12.9 (6)	66.7 ± 17.3 (6)	4	
CA3	55.4 ± 7.76 (6)	65.7 ± 18.4 (6)	18	
Thalamus				
Medial geniculate nucleus	55.0 ± 11.7 (6)	114.0 ± 22.8 (6)	107	**
Posterior nuclei	47.3 ± 12.1 (6)	92.8 ± 36.7 (6)	96	*
Ventral posterior nucleus	56.0 ± 6.22 (6)	88.8 ± 18.2 (5)	58	*
Hypothalamus				
Dorsomedial nucleus	73.5 ± 22.0 (6)	87.2 ± 23.4 (6)	19	
Mammillary nucleus	85.2 ± 25.2 (5)	156.3 ± 25.0 (6)	83	**
Midbrain				
Inferior colliculus	69.2 ± 7.52 (6)	230.4 ± 13.2 (6)	233	**
Substantia nigra	63.8 ± 18.8 (6)	74.4 ± 31.3 (6)	17	
Pons and Medulla				
Inferior olivary nucleus	84.4 ± 14.0 (6)	214.7 ± 19.6 (6)	154	**
Superior olivary nucleus	99.9 ± 25.2 (6)	136.8 ± 36.7 (6)	37	
Lateral vestibular nucleus	63.2 ± 19.0 (6)	95.9 ± 11.1 (6)	52	*
Medial vestibular nucleus	93.0 ± 26.0 (6)	108.7 ± 25.6 (6)	17	
Facial nucleus	101.1 ± 11.7 (6)	121.0 ± 31.9 (6)	20	
Raphe nucleus	65.3 ± 6.84 (6)	71.0 ± 23.3 (6)	9	
Cerebellar Cortex	67.9 ± 15.7 (6)	77.2 ± 9.81 (6)	14	

Values represent mean ± SD (pmol/g of tissue) specific binding of ^{3}H-PK11195 in symptomatic pyrithiamine-treated and pair-fed control rats. n, number of animals per group. *$p<0.05$, **$p<0.001$, significant difference between pyrithiamine-treated and pair-fed control groups by Student's t test (two-tailed).

Table 3- Neuronal cell counts and degree of gliosis in pyrithiamine-treated rats

Brain region	Pair-fed controls (n)	PT-treated (n)	% loss	Degree of gliosis
Presymptomatic stage				
Inferior olive	52.5 ± 6.45 (5)	42.0 ± 1.41 (6)	20*	+
Lateral vestibular	29.2 ± 8.92 (5)	15.7 ± 4.27 (6)	46*	+/++
Inferior colliculus	62.0 ± 4.08 (6)	45.5 ± 6.56 (6)	27*	+
Med. geniculate n.	65.7 ± 10.4 (5)	33.2 ± 7.59 (6)	49**	++
Thalamus	77.4 ± 3.13 (6)	45.8 ± 1.64 (6)	41**	++
Caudate-Putamen	85.0 ± 6.27 (6)	83.0 ± 4.97 (5)	-	0
Frontal cortex	97.5 ± 4.65 (6)	101 ± 4.24 (5)	-	0
Symptomatic stage				
Inferior olive	62.8 ± 6.34 (6)	29.6 ± 3.78 (6)	53**	+++
Lateral vestibular	24.5 ± 5.2 (6)	13 ± 2.94 (6)	47**	+++
Inferior colliculus	51.5 ± 6.66 (6)	24.5 ± 3.51 (6)	52**	+++
Med. geniculate n.	66.8 ± 2.93 (6)	28.7 ± 3.72 (6)	57**	++/+++
Thalamus	76.8 ± 5.34 (6)	39.3 ± 3.56 (6)	49**	+++
Caudate-Putamen	97.4 ± 8.26 (6)	94.6 ± 7.23 (6)	-	0
Frontal cortex	106.5 ± 12.2 (6)	103.5 ± 8.58 (6)	-	0

Values represent mean ± SD of neuronal cell counts made within fields of 500 mm x 500mm in tissue sections at 250 x magnification. n, number of animals per group. *$p<0.05$, **$p<0.001$, significant difference between pyrithiamine-treated and pair-fed control groups by Student's t test (two-tailed). The degree of gliosis is indicated by: + (mild), ++ (moderate), +++ (marked). Key: Med. geniculate n. (medial geniculate nucleus).

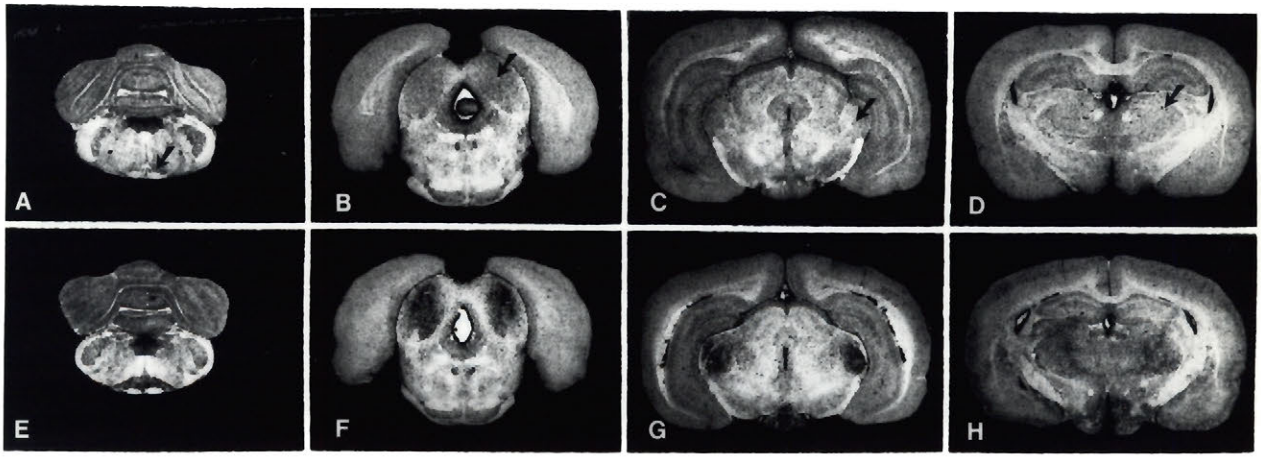


Figure 1- Representative autoradiograms of ^{3}H-PK11195 binding sites in pair-fed control (top row) and symptomatic pyrithiamine-treated (bottom row) animals. Shown are densities of ^{3}H-PK11195 binding sites in the inferior olive (A, E), inferior colliculus (B, F), medial geniculate nucleus (C, G), and thalamus (D, H), as indicated by arrows.

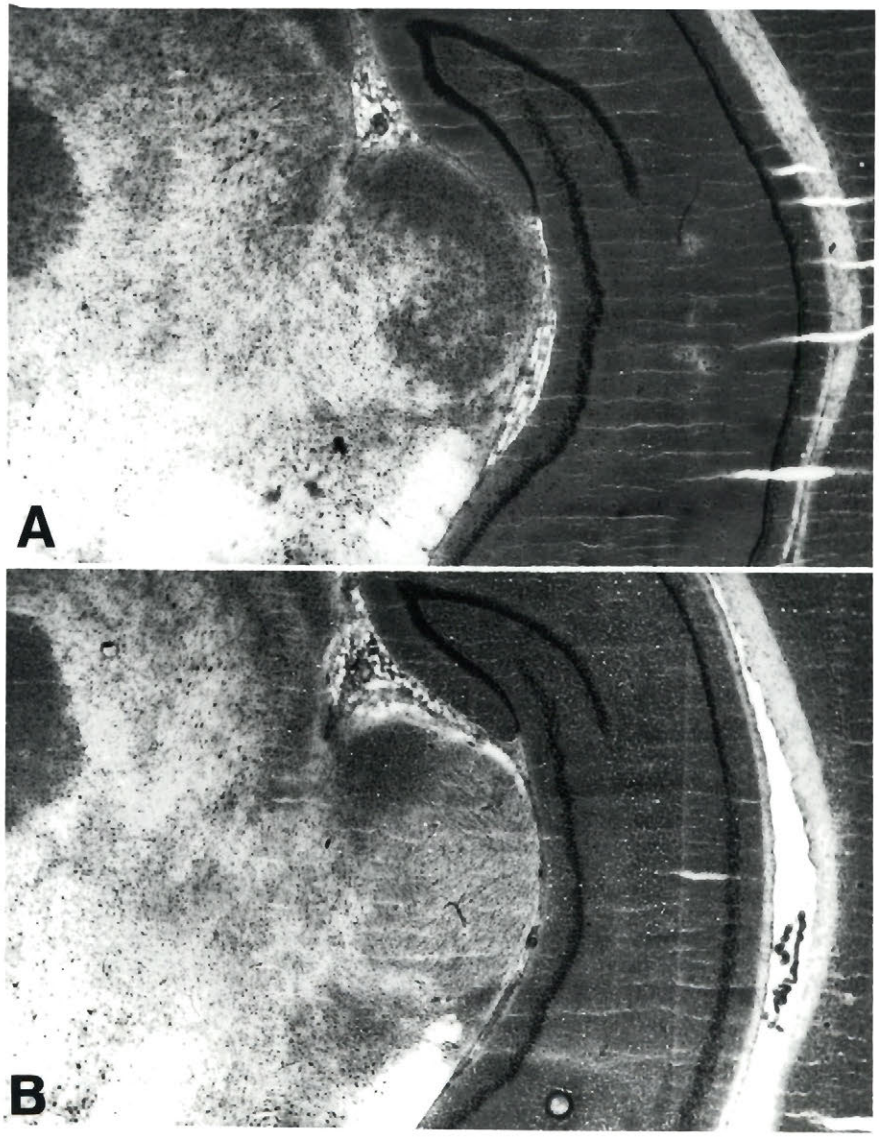


Figure 2- Characteristic appearance of the medial geniculate nucleus of the thalamus. Cresyl violet-stained coronal sections (20μm thick) of pair-fed control (A) and symptomatic pyrithiamine-treated (B) animals. Paleness of the neuropil is evident in the medial geniculate nucleus of the symptomatic pyrithiamine-treated animal (B).

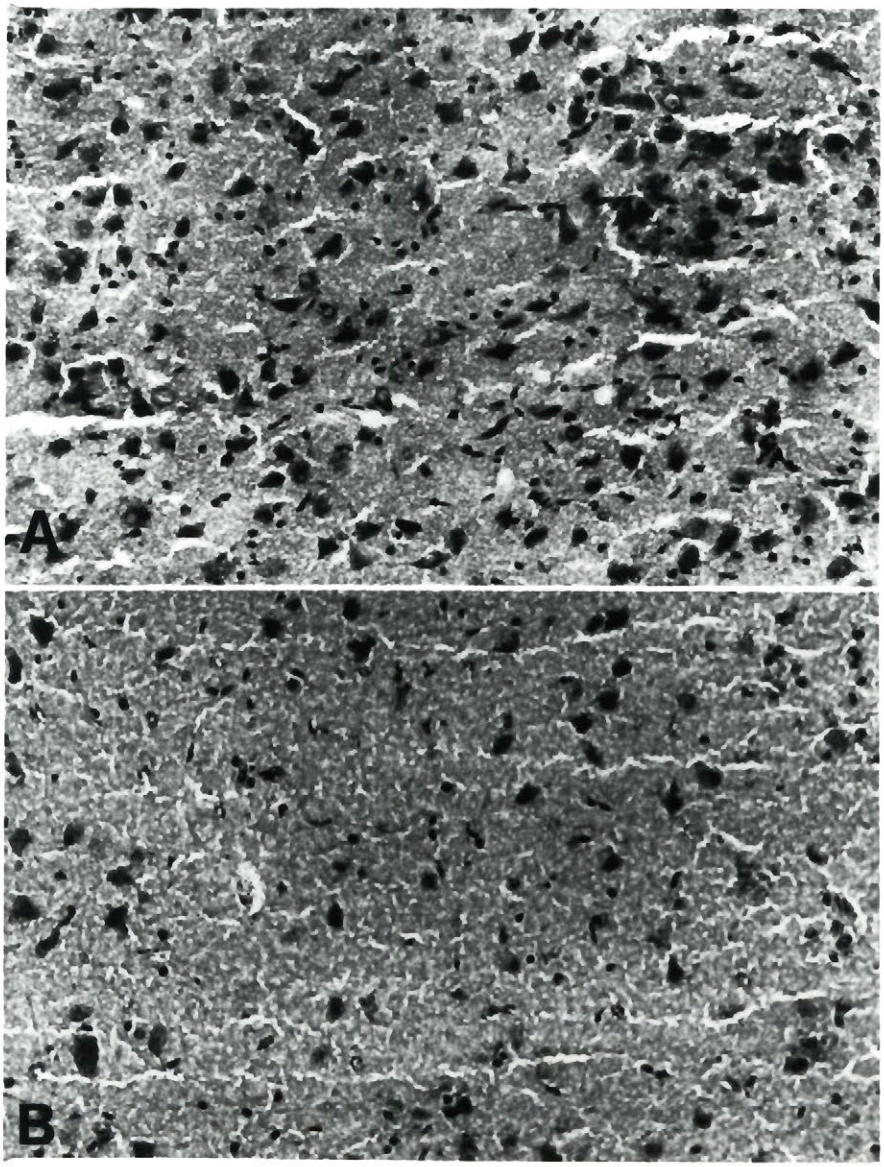


Figure 3- Characteristic appearance of the thalamus. Cresyl violet-stained coronal sections (20μm thick) of pair-fed control (A) and symptomatic pyrithiamine-treated (B) animals. Loss of neurons is evident in the symptomatic pyrithiamine-treated animal (B).

DISCUSSION

Results of the present study demonstrate increased densities of binding sites for the PTBR ligand ^{3}H-PK11195 in the brains of rats at the symptomatic stage of thiamine deficiency encephalopathy. Increases in binding site densities were particularly apparent in inferior colliculus, inferior olive and thalamus, brain structures that are ultimately the sites of selective neuronal damage in this experimental animal model of thiamine deficiency encephalopathy (Troncoso et al, 1981; Aikawa et al, 1984). Densities of ^{3}H-PK11195 binding sites in cerebral cortex, caudate-putamen and hippocampus, regions that are spared in thiamine deficiency, remained within normal limits.

Increased PTBRs resulting from thiamine deficiency are most likely the consequence of glial rather than neuronal changes. Previous studies have consistently shown that these receptors are localized on non-neuronal elements, particularly glial cells (Syapin and Skolnick, 1979; McCarthy and Harden, 1981; Tardy et al, 1981; Bender and Hertz, 1985, 1987). Histopathologic evaluations in the present study revealed significant reductions in neuronal cell counts as early as 7 days following the initiation of pyrithiamine treatment and prior to the appearance of neurological symptoms. Marked gliosis was not apparent until some 4 to 5 days later when animals showed marked neurological impairment. Significant increases of ^{3}H-PK11195 binding sites coincided with marked gliosis. Increased densities of PTBRs have previously been described in postmortem brain tissue from patients with neurodegenerative disorders such as Huntington's disease (Schoemaker et al, 1982) and Alzheimer's disease (Owen et al, 1983; Diorio et al, 1991). It was proposed that increases in densities of PTBRs reflected reactive gliosis accompanying neuronal loss in these conditions, and that the increases of PTBRs could provide a sensitive index of neuronal loss in these disorders (Benavides et al, 1987).

The present findings of increased densities of ^{3}H-PK11195 binding sites are consistent with the suggestion that neuronal cell loss in thiamine deficiency is the consequence of excitotoxic damage mediated by the N-methyl-D-aspartate (NMDA) receptor, similar to that proposed to explain neuronal loss in cerebral ischemia. The nature of the neuronal damage reported in pyrithiamine-induced thiamine deficiency resembles that observed in glutamate-induced excitotoxicity (Armstrong-James et al, 1988) and densities of PTBRs are increased in membrane preparations from rat striata following intrastriatal administration of excitotoxic compounds (Schoemaker et al, 1982; Owen et al, 1983; Benavides et al, 1987). In these studies increased PTBRs were found to parallel both the loss of neuronal marker enzymes as well as the appearance of reactive gliosis (Schoemaker et al, 1982; Benavides et al, 1987). Support for excitotoxic mechanisms in the pathogenesis of neuronal cell loss in thiamine deficiency encephalopathy is provided by recent reports of selective increases of glutamate release in vulnerable brain structures of symptomatic pyrithiamine-treated rats (Hazell and Hakim, 1992) and of significant neuroprotective effects of the NMDA receptor antagonist MK-801 in these animals (Langlais and Mair, 1990).

Pyrithiamine-induced thiamine deficiency in the rat affords an appropriate experimental animal model of the WKS in humans (Héroux and Butterworth, 1992). In both experimental thiamine deficiency and WKS, there is a predilection for neuronal loss in thalamic, midbrain and brainstem structures (Troncoso et al, 1981; Aikawa et al, 1984; Victor et al, 1989). Neuronal loss and concomitant gliosis are characteristic features of WKS in humans (Torvik et al, 1982; Harper, 1983; Victor et al, 1989). Harper (1983) reported that in chronic lesions, parenchymal elements were lost and reactive changes largely restricted to astrocytes were encountered. In the study by Torvik et al (1982) histologic changes in WKS patients included marked gliosis in medial thalamus. The WKS is difficult to diagnose in patients during life with up to 80% of cases of autopsy-proven WKS having been missed both in alcoholic patients (Harper, 1979) and in patients with

AIDS (Butterworth et al, 1991). ^{11}C-PK11195 is a widely available ligand for use in Positron Emission Tomography (PET). Assessment of PTBRs in brain using this PET ligand could offer a potentially useful diagnostic approach in WKS patients.

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Preface

In the previous chapter increased densities of binding sites for the "peripheral-type" benzodiazepine receptor (PTBR) ligand ^{3}H-PK11195 were found in selective brain regions of neurologically symptomatic, but not presymptomatic thiamine-deficient rats. Such increases were seen in regions such as the inferior olive, inferior colliculus and thalamus; histological studies of these regions revealed neuronal loss and concomitant marked gliosis in symtptomatic animals. Thus, increased densities of ^{3}H-PK11195 binding sites were found to coincide temporally with neurological signs of thiamine-deficiency, and spatially with the appearance of reactive gliosis. The PTBR has been localized to glial cells including astrocytes, microglia and macrophages. In the following study immunohistochemistry with antibodies to glial elements was used to study the glial reaction at different stages of thiamine-deficiency. In addition, an antibody to serum albumin was used to study the integrity of the blood-brain barrier.

CHAPTER 3

Increased ED-1 (macrophage lysosomal antigen) immunostaining and early breakdown of the blood-brain barrier in vulnerable brain regions in thiamine deficiency

ABSTRACT

Immunohistochemistry with antibodies to astrocytes (polyclonal glial fibrillary acidic protein; GFAP); and macrophages (monoclonal ED1), were used to examine the glial response in brains of rats at different stages of thiamine-deficiency. In addition, an antibody to serum albumin was used to study the integrity of the blood-brain barrier. The results of the study reveal early, region-selective, increases of immunostaining for ED-1 and serum albumin in brains of thiamine-deficient rats, prior to the appearance of neurological symptoms of thiamine deficiency or major histopathological lesions. Increased ED1-immunostaining, suggestive of an early macrophage response, and increased albumin-immunoreactivity suggestive of an early breakdown of the blood-brain barrier, was confined to regions including the inferior olive, inferior colliculus and medial thalamus; regions of the brain which ultimately manifest marked neuronal loss in thiamine deficiency. Histological studies of neurologically symptomatic thiamine-deficient rats, revealed significant neuronal loss, pallor of the neuropil and marked gliosis in vulnerable brain regions, while immunohistochemical studies of the same regions revealed marked increases of GFAP-immunostaining, suggestive of reactive gliosis. The findings of this study show evidence of an early, region-selective, macrophage response, prior to major histopathological lesions and the appearance of reactive astrocytes. In addition, we report increased permeability of the blood-brain barrier, which may underly the phenomenon of selective vulnerability of specific brain regions in thiamine deficiency.

INTRODUCTION

Pyrithiamine-induced thiamine deficiency in the rat results in bilaterally symmetrical lesions of diencephalic and brainstem nuclei that are similar in nature and distribution to lesions encountered in the Wernicke-Korsakoff Syndrome in humans (Troncoso et al., 1981). Previous reports suggest that the initial cellular changes in thiamine deficiency consist of glial modifications including edematous swelling of astrocytes, oligodendrocytes and myelin sheaths (Collins, 1967; Robertson et al., 1968). Other reports describe selective neuronal loss in inferior olive, inferior colliculus and thalamus (Troncoso et al., 1981; Vortmeyer and Colmant, 1988).

Neuronal loss in pyrithiamine-induced thiamine deficiency is accompanied by reactive gliosis and by increased densities of binding sites for ^{3}H-PK11195, a highly selective ligand for the "peripheral-type" benzodiazepine receptor (PTBR) (Leong et al., 1994), which has been localized to glial cells (Zavala et al., 1984; Bender and Hertz, 1985; Park et al , 1994). Reactive astrocytes show increased expression of Glial Fibrillary Acidic Protein (GFAP), the major subunit of astrocyte intermediate filaments, and GFAP immunohistochemistry affords a useful technique for the study of the astroglial response to neuronal injury (Eng, 1985). In addition, it is recognized that mononuclear phagocytes (macrophages and microglia) play an important role in the early glial response to brain injury (Perry et al., 1993). The ED1 antibody selectively labels a cytoplasmic antigen in mononuclear phagocytes, (i.e. monocytes and macrophages) (Dijkstra et al., 1985), and it has also been reported that activated and reactive (phagocytic) microglia express the ED1 antigen (Graeber et al., 1990). Previous studies suggest that cytokines released from infiltrating macrophages may contribute to the formation of the astroglial scar (Giulian, 1990).

Increased permeability of the blood-brain barrier may play a role in the pathogenesis of selective neuronal loss seen in thiamine-deficiency. Previous studies have revealed breakdown of the blood-brain barrier in selective brain regions of thiamine-deficient rats (Robertson and Manz, 1971; Manz and Robertson, 1972; Phillips and Cragg, 1984). In two recent studies increased permeability of the blood-brain barrier was seen at early stages of pyrithiamine-induced thiamine-deficiency in rats (Calingasan et al., 1995) and mice (Harata and Iwasaki, 1995).

In the study now described, immunohistochemistry with antibodies to ED1 (macrophage lysosomal antigen) and GFAP were employed in order to assess the responses of macrophages and astrocytes in thiamine deficiency, while an antibody to serum albumin was used to study the integrity of the blood-brain barrier. The aim was to relate changes to the time course for the appearance of histopathologic lesions in the brains of thiamine-deficient animals.

MATERIALS AND METHODS

Treatment Groups

Male Sprague-Dawley rats weighing 175-200 g were used for the experiments described. All animals were housed individually in the André-Viallet Clinical Research Centre's animal quarters under constant conditions of temperature, humidity, and light cycles. The following treatment groups were used for the studies described.

Group 1- Pair-fed controls

Rats (n=12) were fed a thiamine-deficient diet (ICN, Nutritional Biochemical, Cleveland, OH, U.S.A.) pair-fed to equal food consumption to that of rats in Groups 2 and 3. In addition, all rats received thiamine subcutaneously in a dose of 10 μg/100 g body weight/day.

Group 2- Presymptomatic thiamine deficient

Rats (n=6) were fed a thiamine-deficient diet and administered pyrithiamine subcutaneously (50 μg/100 g body weight/day) for 7 days. At this time rats showed no evidence of neurological abnormalities (normal gait, normal righting reflexes).

Group 3- Symptomatic thiamine deficient

Rats (n=6) were fed a thiamine-deficient diet and administered pyrithiamine subcutaneously (50 μg/100 g body weight/day). Neurological status was evaluated daily until loss of righting reflex was evident; this stage was generally between days 12 and 14.

Rats were decapitated, brains removed and frozen in isopentane chilled on dry ice, and then stored at -80°C.

For albumin studies, a separate group of animals which received the same thiamine-deficient regimen, were used. For sacrifice these animals were anaesthetized with pentobarbital (MTC Pharmaceutical, Ont., Canada), transcardially perfused with saline followed by perfusion fixation with 10% formalin. The brains were then removed and postfixed in formalin. Prior to sectioning, brains were cryoprotected in 30% sucrose in Tris HCl buffer (pH 7.4) containing sodium azide.

Brains were mounted onto microtome chucks, and sections of 20μm were cut using a IEC microtome cryostat at -18°C and were thaw-mounted onto gelatin-coated glass microscope slides.

Histology

Sections stained with cresyl violet using standard histological techniques were viewed by light microscopy. Studies of neurons and glial cells were made by a neuropathologist (L.O.) who was unaware of the treatment group. Observations were performed within fields measuring 500 x 500μm, in sections observed at 250x magnification.

Immunohistochemistry

Sections were fixed in 10% formalin for 5 min at room temperature, followed by a 5 min incubation in alcohol: H_2O_2 (99:1), to quench endogenous peroxidase activity. Sections were then rinsed in water, 50 mM Tris HCl, pH 7.4, and incubated in horse serum (1:10 in Tris HCl buffer) for 20 min to block nonspecific background. Sections were then incubated with primary antibodies for 60 min; glial fibrillary acidic protein (rabbit anti-cow GFAP, DAKO Ltd., Ont., Canada) at a dilution of 1:1000, ED1 monoclonal antibody (1:100 dilution; Serotec Ltd, Ont., Canada) and albumin (1:1000; rabbit anti-rat albumin, ICN Biochemicals, Ca., USA). Following incubation, sections were rinsed for 5 min with Tris HCl buffer and the secondary antibodies (1: 200; anti-rabbit or anti-mouse IgG, Vector Labs, Ca., USA) were applied for 15 min. After rinsing in buffer for 5 min, the avidin-biotin complex (1:100; Vector Ltd, Ont., Canada) was applied for 20 min. The reaction product was visualized using 3,3'-diaminobenzidine tetrahydrochloride-hydrogen peroxide (Sigma Chemical Co., Mo., USA). Sections were counterstained in Harris's haematoxylin, dehydrated, cleared, and mounted in Permount. Control slides were incubated without primary antibodies. Sections were then examined by a neuropathologist (L.O.). The degree of immunostaining with ED1, GFAP and serum albumin antibodies in

brain regions of thiamine-deficient animals was compared to that in corresponding brain regions of control animals. A four point grading system was used to indicate the degree of increased immunostaining in thiamine deficient animals compared to control animals: - (none), + (mild), ++ (moderate), +++ (marked). A 10-20% increase in the number of immunopositive cells within a field was regarded as a mild increase; 30-50% as a moderate increase; and >50% as a marked increase.

RESULTS

Daily treatment with pyrithiamine resulted, within 12-14 days, in neurological symptoms of thiamine deficiency such as loss of righting reflex, ataxia and opisthotonus. Pair-fed control animals showed no signs of neurological impairment.

Histology

Examination of histologic sections of presymptomatic thiamine- deficient animals (day 7) revealed no major histopathological changes in inferior olive, inferior colliculus and thalamus (Figure 1). However, at the symptomatic stage of thiamine deficiency (12 days of thiamine deficiency), histological examination of inferior olive, inferior colliculus and thalamus, revealed evidence of marked neuronal loss, pallor of the neuropil, necrosis, and marked gliosis. Histologic studies of nonvulnerable regions such as the striatum and frontal cortex showed no evidence of neuronal loss or gliosis.

Immunohistochemistry

Within the brain regions which appear to be most vulnerable to thiamine-deficiency, the pattern of immunohistochemical staining with GFAP and ED1 antibodies was found to be temporally different. At the presymptomatic stage of thiamine deficiency (day 7), ED1-positive cells were present in the inferior olive, inferior colliculus and medial thalamus (Table 1, Figure 2). In accordance with a previous report the ED1 antibody showed a

granular staining pattern within the cytoplasm of cells (Dijkstra et al., 1985). At higher power magnification of areas of increased ED1-immunstaining, individual cells of ED1-positivity could be seen (data not shown). There was a very mild increase in GFAP-immunostaining which was confined to the inferior olive and medial thalamus of presymptomatic thiamine-deficient animals compared to controls (Table 1).

At the symptomatic stage (12-14 days of thiamine deficiency) a marked increase in GFAP-immunostaining was seen in the inferior olive, inferior colliculus and medial thalamic regions (Table 1). Within the field of examination, GFAP-positive astrocytes appeared to be increased in both number and size (Figure 2F). In the more lateral areas of the thalamus the number of GFAP-positive cells was found to be only mildly increased. Increases of ED1-positive cells were maintained throughout the vulnerable brain regions of symptomatic thiamine-deficient animals compared to control animals (Table 1).

Examination of sections from presymptomatic thiamine-deficient animals also revealed focal increases of albumin-immunostaining in vulnerable brain regions including the inferior olive, inferior colliculus and thalamus (Table 1, Figures 3 and 4). In vulnerable brain regions of symptomatic thiamine-deficient animals, increased albumin-immunostaining was widespread and generalized (Figure 3). Focal increases of albumin staining were also seen in the cortex of these animals.

No immunostaining was seen in negative controls for any of the antibodies. No significant immunostaining for either ED1 or GFAP antibodies was observed in non-vulnerable brain regions such as striatum or frontal cortex of thiamine-deficient animals (Table 1).

Table 1- Degree of immunostaining with ED-1, GFAP and albumin antibodies in thiamine-deficient rats.

Experimental group (Brain region)		ED-1	GFAP	Albumin
Presymptomatic thiamine deficient				
Inferior olive		+++	-/+	++
Inferior colliculus		+++	-	++
Thalamus-	MGB	-	-	++
	Medial	+++	-/+	++
	Lateral	-	-	++
Striatum		-	-	-
Frontal cortex		-	-	-
Symptomatic thiamine deficient				
Inferior olive		++	+++	+++
Inferior colliculus		++	+++	+++
Thalamus-	MGB	+	++	+++
	Medial	++	+++	+++
	Lateral	+	+	+++
Striatum		-	-	-
Frontal cortex		-	-	+

Degree of immunostaining with antibodies to ED1, GFAP and albumin in thiamine deficient animals compared to control animals: - (none), + (mild), ++ (moderate), +++ (marked) increase of immunostaining. MGB, medial geniculate body.

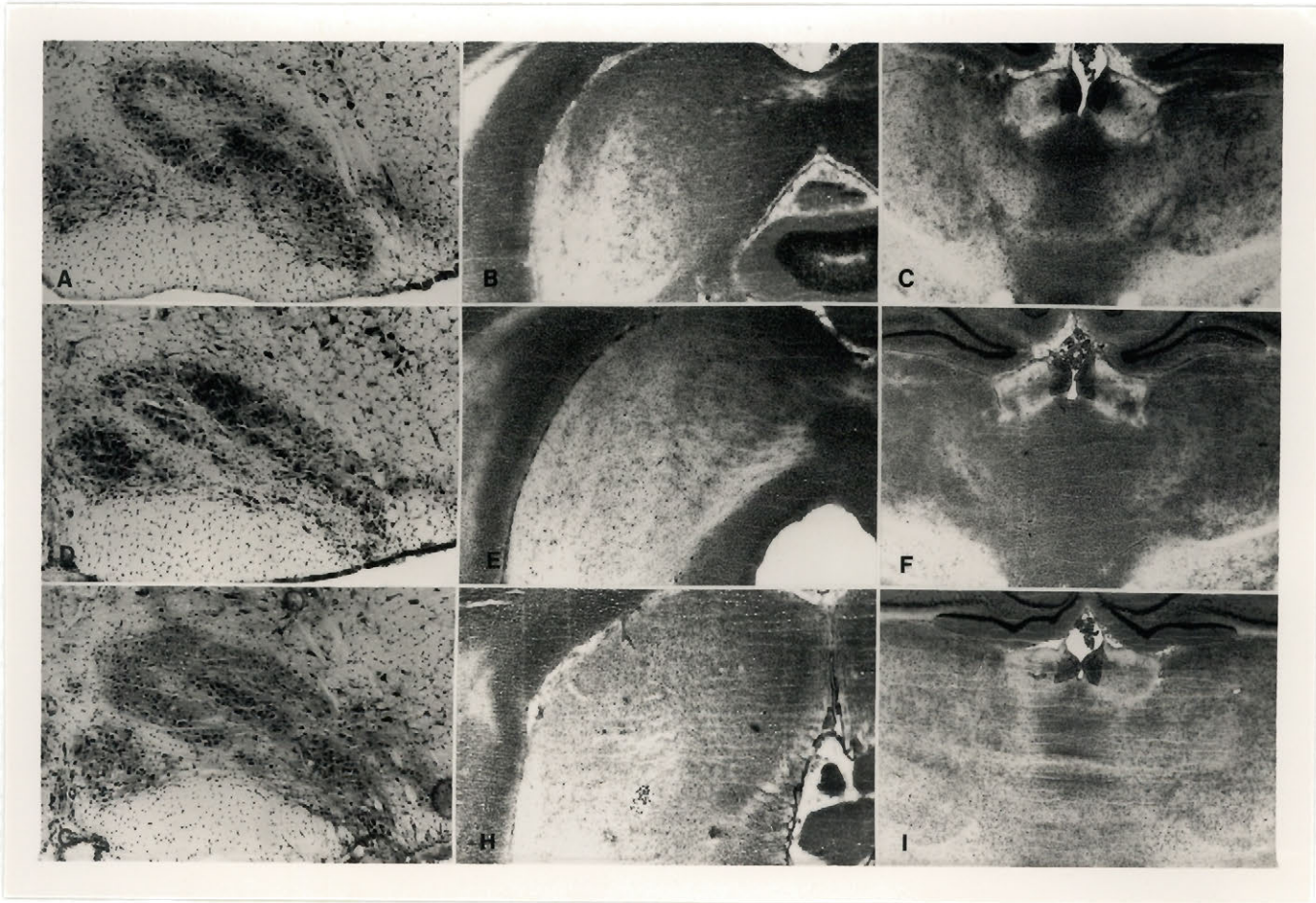

Figure 1- Photomicrographs showing cresyl-violet stained sections in control (top row), presymptomatic (second row) and symptomatic thiamine-deficient (bottom row) animals. Shown are sections of inferior olive (A, D, G), inferior colliculus (B, E, H) and thalamus (C, F, I). No major histopathological changes are seen in presymptomatic animals (D, E, F) compared to controls. Pallor of the neuropil and neuronal loss are seen in symptomatic thiamine-deficient animals (G, H, I). Haemorrhages are evident in the inferior colliculus of the symptomatic animal (H). Magnification: inferior olive (x100), inferior colliculus (x31.25) and thalamus (x25).

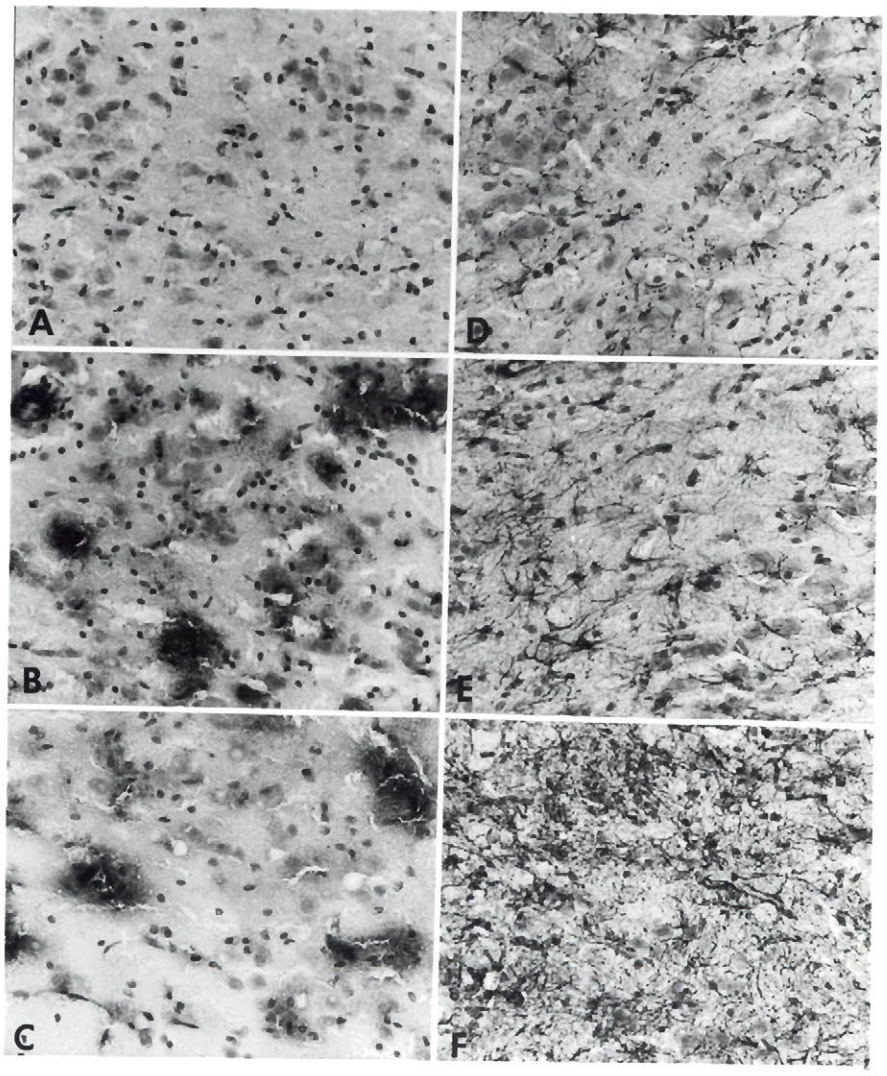

Figure 2- Photomicrographs showing ED1- (left panel) and GFAP- (right panel) immunostaining in the inferior olive of control (A, D), presymptomatic (B, E) and symptomatic (C, F) thiamine-deficient animals. Increased ED1-immunostaining can be seen in the inferior olive of presymptomatic (B) and symptomatic (C) thiamine-deficient animals compared to the control (A). Markedly increased GFAP-immunostaining is seen in the inferior olive of the symptomatic animal (F), but not the presymptomatic animal (E), compared to control (D). Magnification x400.

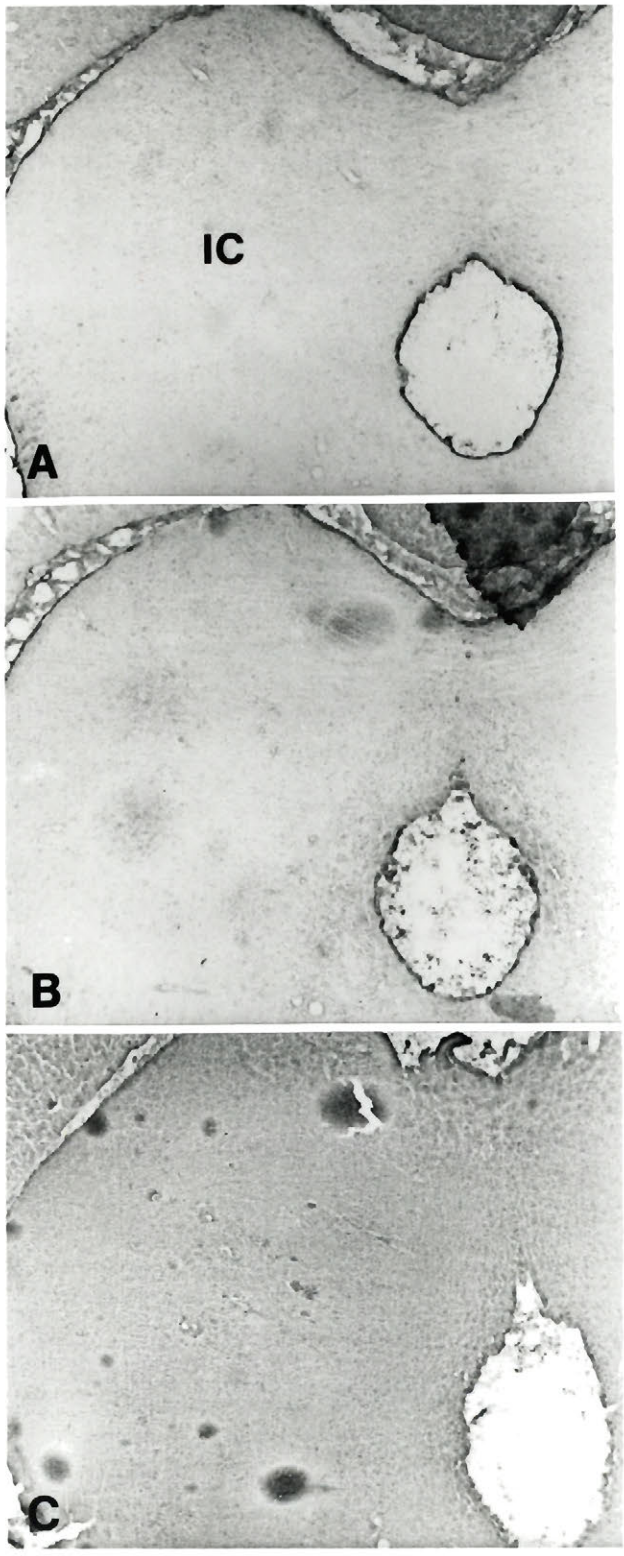

Figure 3- Photomicrographs showing albumin-immunostaining in the inferior colliculus (IC) of control (A), presymptomatic (B) and symptomatic (C) thiamine-deficient animals. Focal increases of albumin-immunostaining can be seen in the inferior colliculus of the presymptomatic (B) thiamine-deficient animal compared to the control (A). Increased albumin-immunostaining in the inferior colliculus of the symptomatic (C) thiamine deficient animal is more widespread and generalized. Magnification x 31.25.

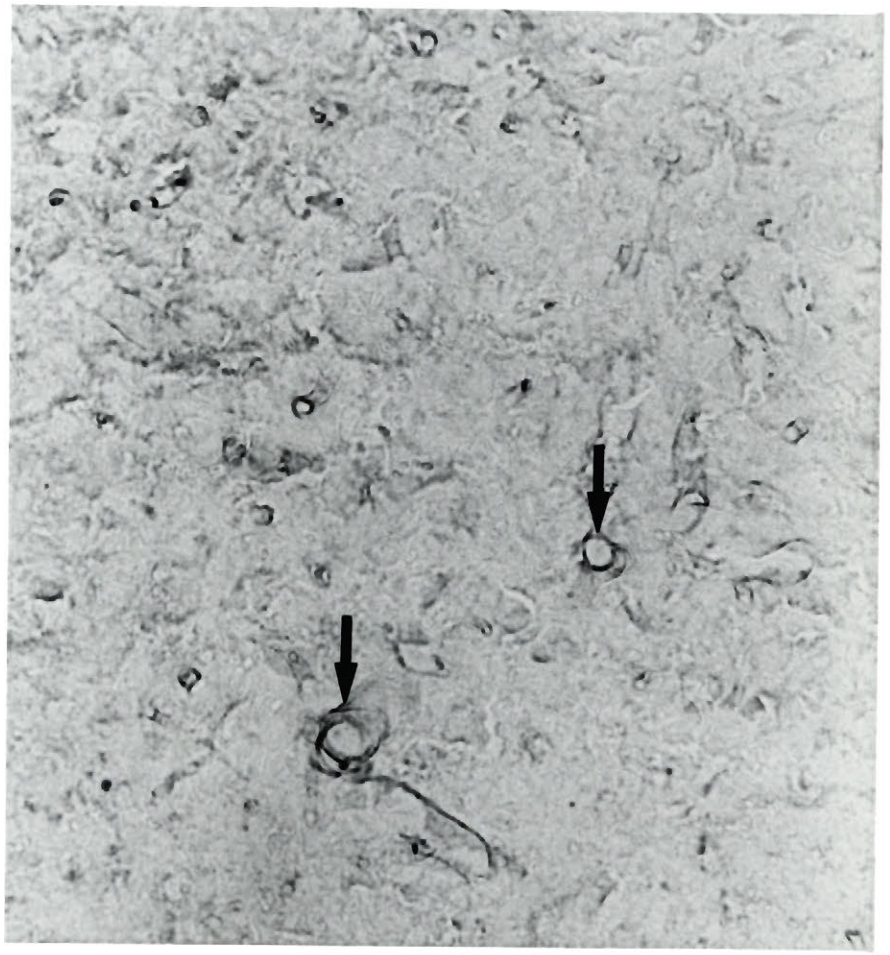

Figure 4- Photomicrographs showing a higher power view of increased albumin-immunostaining surrounding blood vessels (indicated by arrows) within an area of focal increased albumin-immunostaining (seen in Figure 3B) in the inferior colliculus of a presymptomatic thiamine-deficient animal. Magnification x 250.

DISCUSSION

The results of the present study reveal early, region-selective, increased ED1-immunostaining in vulnerable brain regions including the inferior olive, inferior colliculus and medial thalamus; regions of the brain which ultimately manifest significant neuronal loss in experimental thiamine deficiency (Troncoso et al., 1981) and the Wernicke-Korsakoff Syndrome in humans (Victor et al., 1989). At an early stage of thiamine deficiency, increased serum albumin- immunostaining, suggestive of an early breakdown of the blood-brain barrier, was also seen in vulnerable brain regions of thiamine-deficient rats coincident with the increased ED1-immunostaining, but prior to histological appearance of marked neuronal loss and tissue necrosis.

Previous studies have revealed breakdown of the blood-brain barrier in early stages of thiamine deficiency in both pyrithiamine-induced thiamine deficient mice (Harata and Iwasaki, 1995) and rats (Calingasan et al., 1995). In the latter study region-selective increases of IgG immunoreactivity were seen as early as 10 days of thiamine deficiency and prior to the appearance of neuropathological lesions. In the present study increased immunostaining for serum albumin was seen even earlier (as early as 7 days of thiamine deficiency). In a previous study of the blood-brain barrier in acute experimental allergic encephalomyelitis, albumin leakage was found to occur two days before IgG infiltration. It was suggested that the difference in permeability of the two molecules may have been due to differences in size or electrical charge (Juhler et al., 1986). The integrity of the blood-brain barrier has also been found to be compromised in human Wernicke's Encephalopathy. Magnetic Resonance studies have revealed damage of the blood-brain barrier in periventricular areas during the acute stage of WE (Schroth et al., 1991).

The ED1 antibody employed in the present study is a monoclonal antibody which selectively labels a cytoplasmic antigen in mononuclear phagocytes, (i.e. monocytes and

macrophages) and does not cross-react with endothelial cells. It has been shown that the ED1 antibody reacts with large, round oval cells and shows a granular staining pattern within the cytoplasm of cells (Dijkstra et al., 1985). Following neuronal injury, activated microglia and reactive (phagocytic) microglia, express the ED1 antigen (Graeber et al., 1990). Following CNS injury, activated microglia (the resident macrophages of the brain) as well as blood-borne macrophages appear at the site of injury in order to phagocytose and remove cellular debris (Perry and Gordon, 1991). Whether early increased ED1-immunostaining in vulnerable brain areas of thiamine-deficient rats results from an infiltration of blood-borne macrophages or activation of resident microglia, cannot be determined from the results of the present study. However, evidence of early breakdown of the blood-brain barrier in thiamine deficiency coincident with the increased ED1-immunostaining suggests that increased ED1-immunostaining in vulnerable brain regions is due in major part to infiltration of blood-borne macrophages in these areas of the brain.

A previous autoradiographic study revealed selective increases in densities of binding sites for ^{3}H-PK11195, a ligand with high selectivity for the "peripheral-type" benzodiazepine receptor (PTBR) in vulnerable brain regions of pyrithiamine-treated rats (Leong et al., 1994). The PTBR is localized on astrocytes, macrophages and microglia (Zavala et al., 1984; Bender and Hertz, 1985; Park et al , 1994). Increased densities of ^{3}H-PK11195 binding sites paralleled, both spatially and temporally, the appearance of reactive gliosis and it was therefore concluded that increased densities of ^{3}H-PK11195 binding sites were the result of reactive gliosis accompanying neuronal loss in the brains of these animals. The results of the present study add further credence to this interpretation since the appearance of increased densities of ^{3}H-PK11195 binding sites was coincident temporally with increases of GFAP-immunostaining, and did not coincide with the early increase of ED1-immunostaining. These findings contrast those observed in experimental focal

ischaemia, where increased densities of PTBRs appear to result from the presence of macrophages around the focal necrotic lesion (Myers et al., 1991).

It is conceivable that the phenomenon of reactive gliosis could be the consequence of the early sustained accumulation of macrophages that accompanies neuronal loss in thiamine deficiency since activated microglia may regulate astrocytic hyperplasia by microglial-released peptides (Giulian and Baker, 1985). Furthermore, increased concentrations of cytokines including interleukin-1 (IL-1), tumour necrosis factor-a (TNF-a), and interleukin-6 (IL-6) are encountered at the site of neuronal injury (Giulian and Lachman, 1985; Taupin et al., 1993) and astrocyte proliferation can be induced by these factors which are released by activated microglia (Giulian et al., 1988; Selmaj et al., 1990).

The present findings of the accumulation of macrophages prior to the appearance of major histopathologic lesions is not restricted to thiamine deficiency. Microglial activation precedes neuronal cell loss and astrocytic proliferation in the rat hippocampus following transient global forebrain ischaemia (Morioka et al., 1991). These results suggest that the early presence of reactive microglia are indicative of imminent neuronal damage. Similar findings have been observed in transient and permanent focal ischaemia, where increased immunostaining for microglia has been seen in the cerebral cortex of ischaemic animals at a time when neuronal loss was minimal (Morioka et al., 1993; Korematsu et al., 1994). Activation of microglia is a feature common to other types of neuronal injury having been described in neurodegenerative disorders such as Alzheimer's Disease (McGeer et al., 1987) and in the AIDS-Dementia Complex (Navia et al., 1986).

In the present study a macrophage response was seen early in thiamine deficiency, prior to major histopathological lesions and the appearance of reactive astrocytes, which suggests

that accumulation of macrophages may be the initial cellular event following disruption of the blood-brain barrier in thiamine deficiency.

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Preface

In the previous chapter immunohistochemical studies revealed early, region selective, increases of immunostaining for the macrophage marker ED1, and serum albumin, in presymptomatic thiamine-deficient animals. These findings were suggestive of increased permeability of the blood-brain barrier and infiltration of blood-borne macrophages in early thiamine deficiency. Studies of symptomatic thiamine-deficient animals revealed marked increases in GFAP-immunostaining, a marker for astrocytes, in vulnerable brain regions. This finding confirmed our previous suggestion that increased densites of ^{3}H-PK11195 binding sites coincided with the appearance of reactive gliosis. The following chapter describes autoradiographical studies with ^{3}H-PK11195 and histological studies, which were employed to study the potential neuroprotective effect of MK801 (a N-methyl-D-aspartate receptor antagonist) in thiamine deficiency.

CHAPTER 4

Failure of the N-methyl-D-aspartate receptor antagonist MK801 to protect against neuronal loss in thiamine deficiency

ABSTRACT

Histologic, immunohistochemical and quantitative receptor autoradiographic studies were used to study the neuroprotective effect of MK801, the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, in brain regions of rats made thiamine deficient by chronic (up to 14 days) treatment with pyrithiamine, a centrally acting thiamine antagonist. At preconvulsive stages of thiamine-deficiency, significant neuronal loss was seen in selective brain regions including the inferior olive, inferior colliculus, mammillary body and thalamus. Immunohistochemical studies with an antibody to astrocytes (polyclonal glial fibrillary acidic protein; GFAP) revealed that increased GFAP-immunostaining, indicative of reactive gliosis, accompanied the neuronal loss seen in vulnerable brain regions. Increased densities of ^{3}H-PK11195 binding sites, the highly selective ligand for the "peripheral-type" benzodiazepine receptor (PTBR), which has been localized to glial cells, were found to be spatially coincident with the location of reactive gliosis. Pretreatment of thiamine-deficient rats with MK801 offered no neuroprotective effect in any brain region examined. Histologic studies revealed significant neuronal loss in vulnerable brain regions of MK801-treated thiamine-deficient (PTM) animals of similar extent to that seen in saline vehicle-treated thiamine-deficient (PTV) animals. Marked neuronal loss was also seen in the thalamus of MK801-treated thiamine-deficient animals (PTM), and within the lesioned area there were no GFAP-positive astrocytes, which was reflected by the lack of ^{3}H-PK11195 binding in this area. At the dose and time of MK801 administration used in this study, it was found that MK801 offered no neuroprotective effect in nonconvulsing thiamine deficient animals. Results of the study also suggest that measurement of densities of PTBRs may offer a useful approach to the assessment of reactive gliosis following neuronal damage in the Wernicke-Korsakoff Syndrome in humans.

INTRODUCTION

Thiamine deficiency results in bilaterally symmetrical lesions of selective brain regions, including nuclei of the brainstem and diencephalon (Victor et al., 1989). The nature and distribution of lesions in the pyrithiamine-induced thiamine-deficient rat resemble those reported in the Wernicke-Korsakoff Syndrome in humans (Troncoso et al., 1981).

It has been suggested that neuronal cell loss in thiamine deficiency is the consequence of excitotoxic damage induced by glutamate and mediated by the N-methyl-D-aspartate (NMDA) receptor. The nature of neuronal damage observed in pyrithiamine-induced thiamine deficiency resembles that seen in glutamate-induced excitotoxicity (Armstrong-James et al., 1988) and recent reports have shown that extracellular concentrations of glutamate are selectively elevated in the thalamus of the thiamine-deficient rat (Hazell et al., 1993; Langlais and Zhang, 1993).

"Peripheral-type" benzodiazepine receptors (PTBRs) have been localized on glial cells (Zavala et al., 1984; Bender and Hertz, 1985; Park et al., 1994), and recent studies suggest that increased densities of PTBRs reflecting reactive gliosis may be used as an indirect index of neuronal damage in conditions of neuronal loss (Benavides et al., 1987). In a previous study, using quantitative receptor autoradiography, increased densities of binding sites for the PTBR ligand ^{3}H-PK11195 were seen in selective brain regions of thiamine-deficient rats (Leong et al., 1994). Increased densities of ^{3}H-PK11195 binding sites were observed in the inferior olive, inferior colliculus and thalamus; brain regions which showed histological evidence of neuronal loss and concomitant gliosis.

MK801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine hydrogen maleate] is a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist which has been shown to be effective in preventing ischaemia-induced neuronal loss (Gill et al., 1991;

Lin et al, 1993; Park et al, 1988; Swan and Meldrum, 1990). Previous neuropathologic studies suggest that the neuronal loss in the thalamus of the pyrithiamine-treated rat appears to be of the ischaemic type (Vortmeyer and Colmant, 1988). The present study was designed to evaluate the neuroprotective effect of MK801 in pyrithiamine-induced thiamine-deficiency. MK801 was administered from day 7, a time when animals show no neurological signs of thiamine deficiency (Héroux and Buttterworth, 1992), or major histopathological lesions (Leong et al., 1994). In parallel with histopathologic studies, quantitative receptor autoradiography was used to measure the densities of ^{3}H-PK11195 binding sites; and the concomitant glial reaction was assessed by immunohistochemistry using an antibody to glial fibrillary acidic protein (GFAP), the major subunit of astrocytic intermediate filaments (Eng, 1985).

MATERIALS AND METHODS

Treatment Groups

Male Sprague-Dawley rats weighing 175-200g were used for the experiments described. All animals were maintained, and experimental procedures performed, according to the guidelines of the Canadian Council on Animal Care. Animals were housed individually in the André-Viallet Clinical Research Centre's animal quarters under constant conditions of temperature, humidity, and light cycles. The following treatment groups were used for the studies described:

Brain sections were fixed in 2% glutaraldehyde and stained with cresyl violet using standard histological techniques and then viewed by light microscopy. Neuronal cell counts were made by a neuropathologist (L.O.) who was unaware of the treatment group. Measurements were made within fields measuring 500 x 500μm, in sections observed at 250x magnification.

The thalamus was examined at the level of Bregma -3.3mm according to the atlas of Paxinos and Watson (1986). For our studies the thalamus was divided into four quadrants; mediodorsal (MD), laterodorsal (LD), ventromedial (VM) and ventrolateral (VL) (Figure 1).

Immunohistochemistry

Sections were fixed in 10% formalin for 5 min at room temperature, followed by a 5 min incubation in alcohol: H_2O_2 (99:1), to quench endogenous peroxidase activity. Sections were then rinsed in water, 50 mM Tris HCl, pH 7.4, and incubated in horse serum (1:10 in Tris HCl buffer) for 20 min to block the nonspecific background. Sections were then incubated with primary antibody for 60 min; glial fibrillary acidic protein (GFAP polyclonal antibody, 1:1000 dilution; DAKO Ltd., Ontario, Canada). Following incubation, sections were rinsed for 5 min with buffer and the secondary antibody (1:200, biotinylated anti-rabbit IgG, Vector Labs, Ca., USA) was applied for 15 min. After rinsing in buffer for 5 min, the avidin-biotin complex (1:100; Vector Ltd, Ontario, Canada) was applied for 20 min. The reaction product was visualized using 3,3'-diaminobenzidine tetrahydrochloride-hydrogen peroxide (Sigma Chemical Co., St. Louis, Mo., USA). Sections were counterstained in Harris's haematoxylin, dehydrated, cleared, and mounted in Permount. Control slides were incubated without anti-GFAP antibody. Sections were then examined by a neuropathologist (L.O.). A four point grading system was used to indicate the degree

of increased immunostaining with the GFAP antibody in brain regions of thiamine-deficient animals compared to control animals: - (none), + (mild), ++ (moderate), +++ (marked).

Quantitative receptor autoradiography

For autoradiography studies, brain sections were incubated for 60 min at 25°C in Tris-HCl buffer (170 mM, pH 7.4) containing 1 nM ^{3}H-PK11195 (specific activity 86 Ci/mmol, New England Nuclear, Boston, MA, USA). Non-specific binding was assessed by incubating adjacent sections in the presence of 1 μM PK11195. The incubation was terminated by rinsing sections three times for 5 min in ice-cold incubation buffer. Sections were then dipped briefly in distilled water and dried rapidly under a stream of cold air.

Autoradiograms were prepared by apposing sections together with tissue-calibrated standards of known ^{3}H-concentrations (Amersham microscales) to ^{3}H-sensitive Hyperfilm (Amersham) for 4 weeks. Films were developed and tissue concentrations of ^{3}H-PK11195 were measured by quantitative densitometry analysis using a MCID computer-based densitometer and image analysis system (Imaging Research Inc., Ontario, Canada). The amount of ^{3}H-PK11195 bound to various brain regions was calculated from the specific activity of the ligand. Specific binding in each area was calculated by subtracting from the total binding the amount bound in corresponding regions in adjacent sections incubated for non-specific labeling.

Statistical Analysis

Data are presented as mean ± SD. Statistical comparisons of densities of ^{3}H-PK11195 binding sites and neuronal cell counts between animal groups were performed using two-way analysis of variance (ANOVA) to test for interaction between the groups and treatments. This was followed by multiple comparisons using one-way analysis of variance (ANOVA) and the Tukey-Kramer Multiple Comparisons test.

RESULTS

Daily administration of pyrithiamine to rats on a thiamine-deficient diet resulted, within 12-14 days, in neurological signs of early thiamine-deficiency including loss of righting reflex, ataxia and opisthotonus. Animals were sacrificed before any signs of convulsions which become apparent in later stages of thiamine-deficiency (Troncoso et al., 1981). In agreement with previous reports (Koek et al., 1988; Langlais and Mair, 1990; Kochhar et al., 1991; Loscher et al., 1991), the administration of MK801 resulted in behavioural effects characterized by initial hyperactivity and ataxia followed by sedation and decreased motor activity. Such effects were seen in both pair-fed control (CM) and thiamine-deficient animals (PTM).

Histological and immunohistochemical studies revealed significant neuronal loss (Table 1, Figure 2) and marked gliosis (Table 2, Figure 3) in selective brain regions of thiamine-deficient animals compared to controls (PTV cf CV). Such histopathological changes were apparent in the inferior olive, inferior colliculus, mammillary body, and thalamus.

Histopathological changes seen in MK801-treated thiamine-deficient (PTM) animals were characterized by marked neuronal loss in selective brain regions (Tables 1 and 2). Within the vulnerable brain regions of MK801-treated thiamine deficient animals, GFAP-positive astrocytes were seen in areas surrounding the lesions. Examination of the thalamus revealed extensive marked neuronal los in the medial thalamus (Figure 2C). Immunohistochemical studies revealed the absence of GFAP-positive astrocytes within the thalamic lesions of these animals (Figures 3C, F).

Studies of the striatum and frontal cortex revealed no histopathological differences between thiamine-deficient (PTV and PTM) animals compared to their respective controls (CV and CM).

Autoradiographic studies revealed that densities of ^{3}H-PK11195 binding sites were of similar magnitude in the two control groups (CV and CM) (Table 3). Densities of ^{3}H-PK11195 binding sites were, however, significantly increased in selective brain regions of vehicle-treated thiamine-deficient animals (PTV) compared to pair-fed controls (CV). Significant increases in densities of ^{3}H-PK11195 binding sites were seen in the inferior olive (116% increase, $p<0.001$), inferior colliculus (174% increase, $p<0.001$) and the thalamus (up to 150% increase, $p<0.001$), as shown in Table 3 and Figure 4.

Increases in densities of ^{3}H-PK11195 binding sites of a similar magnitude were observed in MK801-treated thiamine-deficient animals (PTM) in selective regions, including the inferior olive (148% increase, $p<0.001$) and inferior colliculus (128% increase, $p<0.001$) when compared to the MK801-treated control animals (CM) (Table 3). However, in the medial areas of the thalamus the densities of ^{3}H-PK11195 binding sites in MK801-treated thiamine-deficient (PTM) animals were lower, although only significantly ($p<0.05$) lower in the laterodorsal thalamus of PTM animals, than in vehicle-treated thiamine-deficient (PTV) animals (Table 3).

Table 2- GFAP- immunostaining in vehicle-treated and MK801-treated thiamine deficient animals compared to control animals

Experimental group (Brain region)		GFAP
Vehicle-treated (PTV vs CV)		
Inferior olive		+++
Inferior colliculus		+++
Mammillary nucleus		+++
Thalamus-	MGB	++
	MD	+++
	VM	++
	LD	++
	VL	+
Striatum		-
Frontal cortex		-
MK801-treated (PTM vs CM)		
Inferior olive		++ (Perilesional)
Inferior colliculus		++ (Perilesional)
Mammillary nucleus		++ (Perilesional)
Thalamus-	MGB	++ (Perilesional)
	MD	Lesions
	VM	Lesions
	LD	Lesions
	VL	+
Striatum		-
Frontal cortex		-

Table 3- Effects of MK801 or vehicle treatment on densities of ^{3}H-PK11195 binding sites in brain regions of pyrithiamine-treated and pairfed control animals

Brain region	^{3}H-PK11195 binding (pmol/g tissue) Vehicle-treated	MK801-treated
(a) Pair-fed controls	(CV)	(CM)
Cerebral cortex		
Motor cortex	47.6 ± 8.76	41.3 ± 10.7
Somatosensory cortex	55.1 ± 9.59	49.7 ± 11.9
Frontal cortex	61.3 ± 7.92	61.5 ± 7.68
Striatum	29.0 ± 8.30	32.5 ± 13.5
Limbic		
Hippocampus-CA1	44.6 ± 8.47	43.2 ± 11.5
CA3	44.3 ± 5.35	42.1 ± 8.38
Thalamus		
Mediodorsal	44.7 ± 9.69	46.5 ± 10.3
Laterodorsal	37.0 ± 6.39	41.0 ± 10.6
Ventromedial	37.5 ± 11.2	46.0 ± 11.0
Ventrolateral	36.4 ± 4.90	48.8 ± 6.7
Medial geniculate n.	54.0 ± 5.29	48.0 ± 11.6
Hypothalamus		
Dorsomedial	63.6 ± 11.9	74.4 ± 8.06
Ventromedial	60.4 ± 14.0	73.3 ± 14.2
Mammillary n.	84.9 ± 13.2	79.1 ± 14.1
Midbrain		
Inferior colliculus	77.8 ± 16.4	72.9 ± 7.6
Pons and Medulla		
Inferior olive	83.1 ± 6.09	72.3 ± 11.7
Lateral vestibular n.	51.8 ± 13.5	51.3 ± 13.6
Medial vestibular n.	74.5 ± 6.39	85.9 ± 17.6
Cerebellum	77.9 ± 8.99	65.8 ± 13.4
(b) PT-treated	(PTV)	(PTM)
Cerebral cortex		
Motor cortex	51.3 ± 8.50	55.4 ± 8.84
Somatosensory cortex	59.4 ± 9.11	52.1 ± 10.7
Frontal cortex	62.5 ± 12.4	59.1 ± 8.90
Striatum	29.3 ± 5.47	29.3 ± 2.86
Limbic		
Hippocampus-CA1	50.0 ± 8.51	49.5 ± 10.5
CA3	57.1 ± 11.0	52.1 ± 7.45
Thalamus		
Mediodorsal	80.3 ± 15.2[a]	60.9 ± 16.4
Laterodorsal	82.4 ± 15.2[a]	55.4 ± 12.5[b]
Ventromedial	79.3 ± 15.7[a]	66.2 ± 14.1
Ventrolateral	90.7 ± 20.3[a]	77.0 ± 15.4[a]
Medial geniculate n.	134.2 ± 32.4[a]	127.6 ± 26.1[a]
Hypothalamus		
Dorsomedial	89.4 ± 21.6[a]	114.0 ± 11.9[a]
Ventromedial	73.8 ± 16.9	95.1 ± 15.3
Mammillary n.	145.1 ± 20.3	172.0 ± 47.8[a]
Midbrain		
Inferior colliculus	213.6 ± 36.3[a]	180.2 ± 15.6[a]
Pons and Medulla		
Inferior olive	179.3 ± 19.9[a]	180.6 ± 34.8[a]
Lateral vestibular n.	75.3 ± 27.6	78.2 ± 21.6
Medial vestibular n.	91.6 ± 19.9	97.3 ± 15.1
Cerebellum	76.2 ± 23.3	76.3 ± 16.4

Values represent mean ± SD (pmol/g of tissue) specific binding of ^{3}H-PK11195. n=6 animals per group. [a]$p<0.05$ significant difference between PT-treated and controls. [b]$p<0.05$ significant difference between MK801-treated and vehicle-treated thiamine deficient groups. Statistical analysis by ANOVA and Tukey-Kramer Multiple Comparisons test.

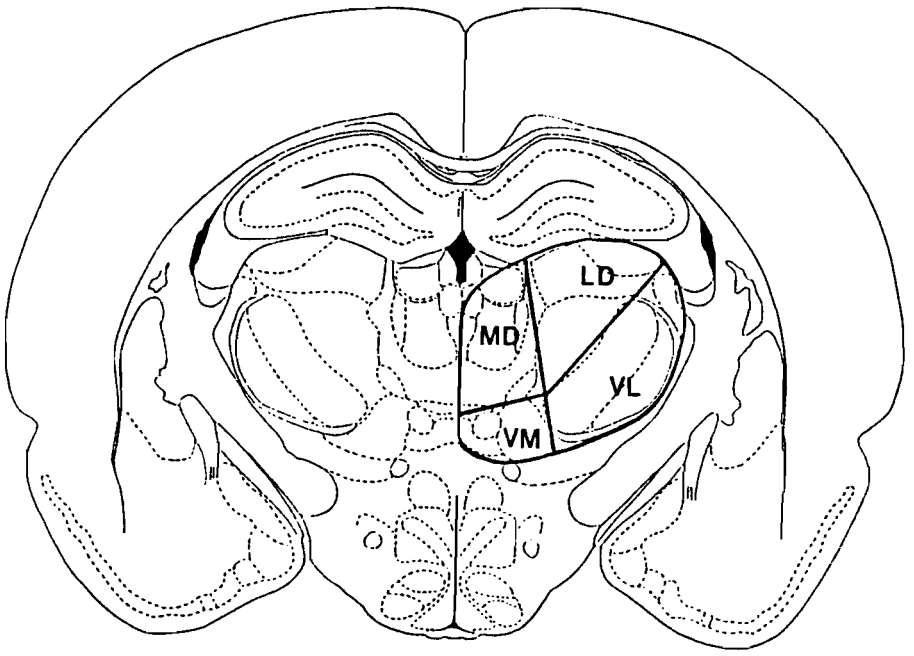

Figure 1- Line drawing showing the four quadrants of the thalamus: MD (mediodorsal), LD, (laterodorsal), VM (ventromedial) and VL (ventrolateral). Based on the atlas of Paxinos and Watson (1986).

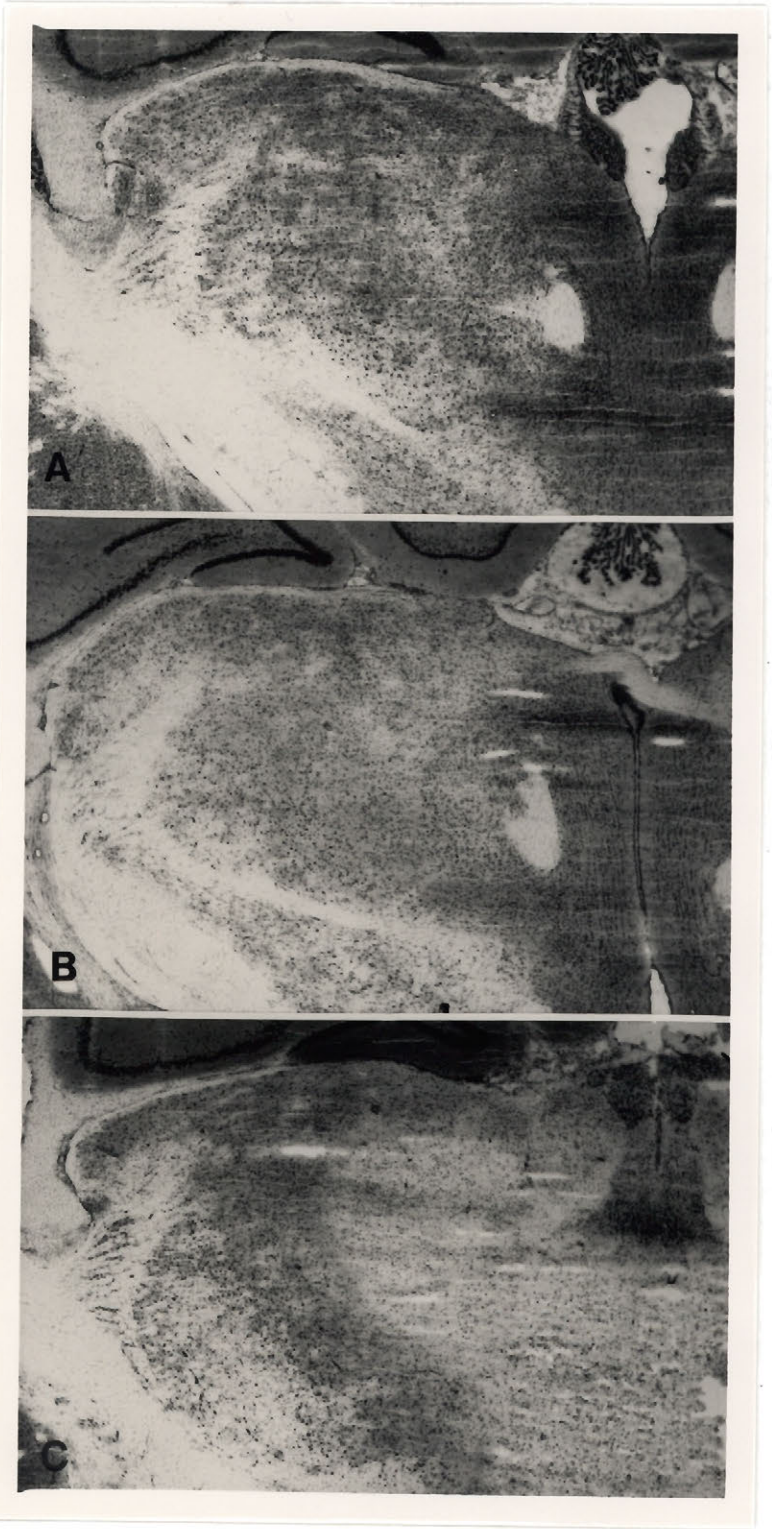

Figure 2- Photomicrographs showing cresyl-violet stained sections of the thalamus of control (A), vehicle-treated thiamine-deficient, PTV (B) and MK801-treated thiamine-deficient animals, PTM (C). Pallor of the neuropil and neuronal loss are seen in the thalamus of thiamine-deficient animal (B). Lesions are evident in the medial thalamus of MK801-treated thiamine-deficient animal (C). Magnification x25.

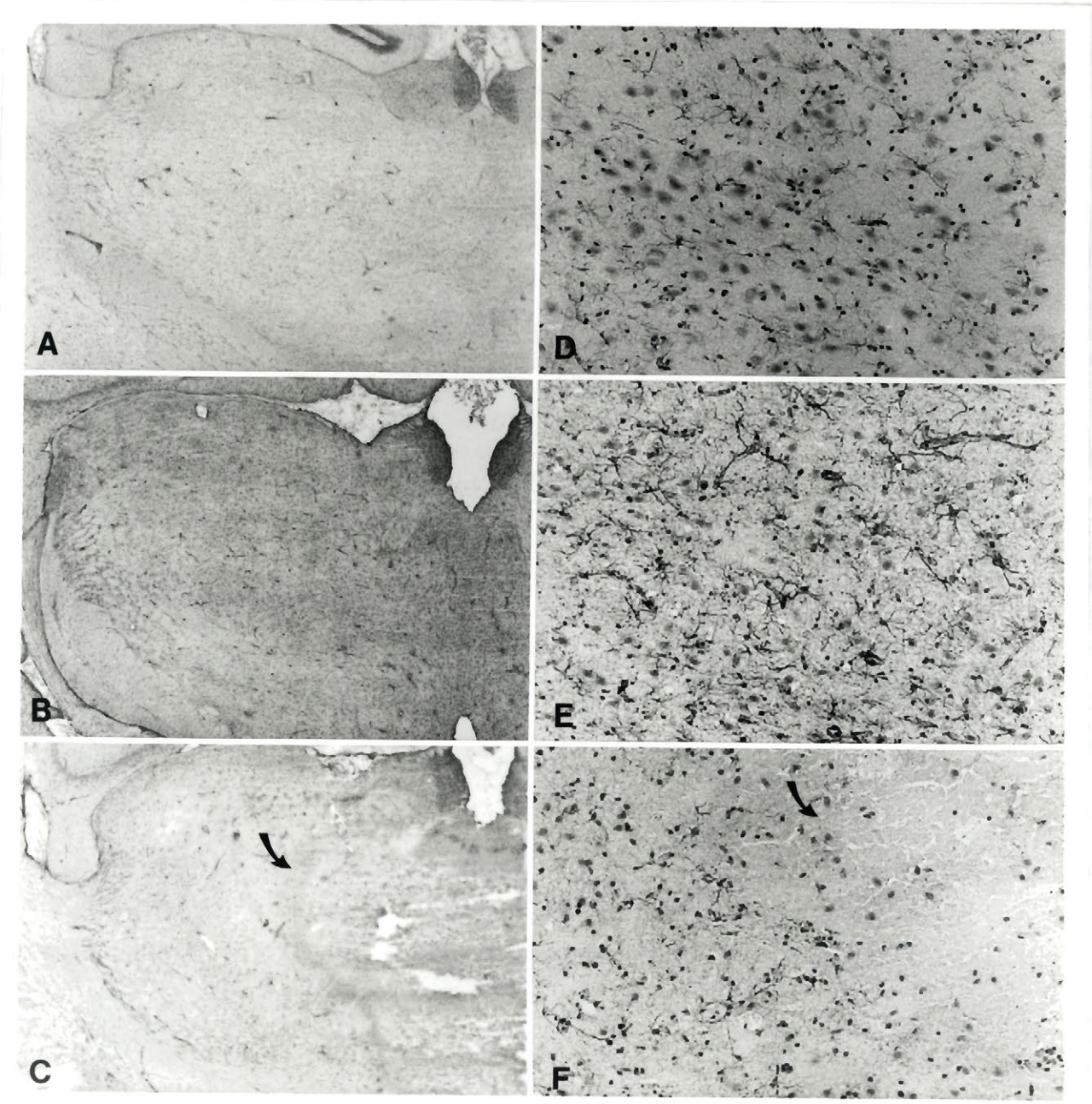

Figure 3- Photomicrographs showing GFAP-immunostaining in the thalamus of control (A, D), vehicle-treated thiamine-deficient (B, E) and MK801-treated thiamine-deficient (C, F) animals. Increased GFAP-immunostaining can be seen in the thalamus of the vehicle-treated (B, E) thiamine-deficient animal compared to the control (A, D). Increased GFAP-immunostaining is also seen in perilesional areas of the MK801-treated thiamine-deficient animal (C, F). Magnification: low power x25 (A, B, C); high power x250 (D, E, F).

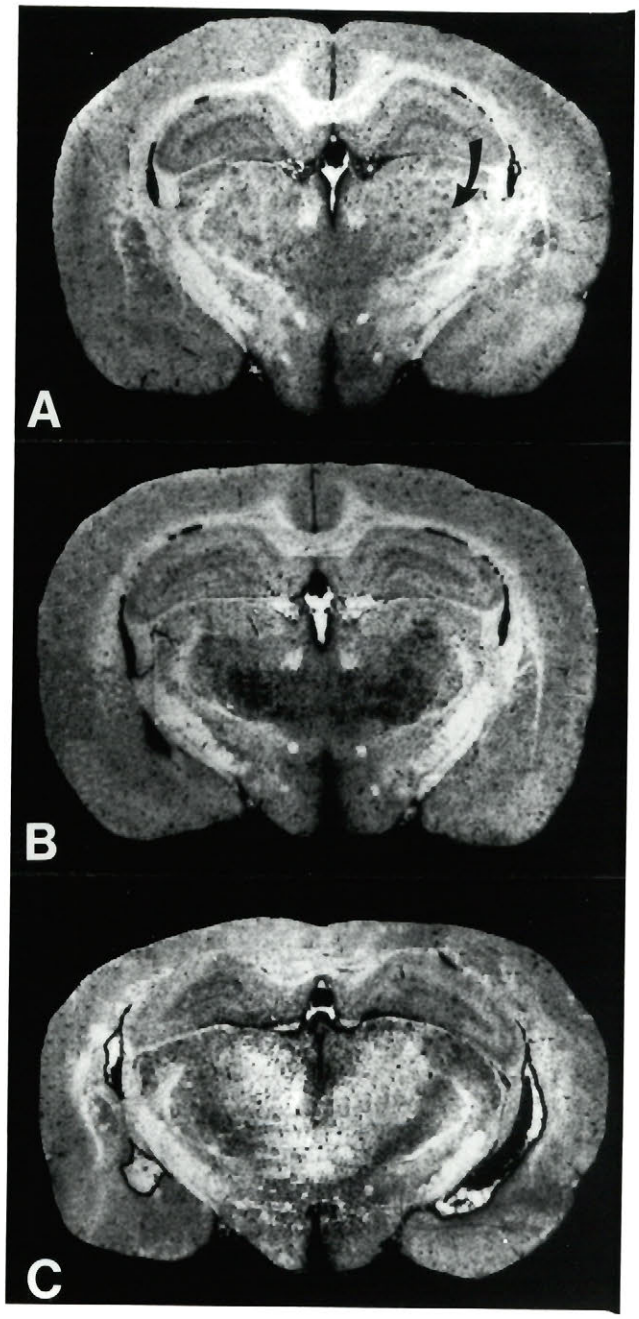

Figure 4- Representative autoradiograms of ^{3}H-PK11195 binding sites in thalamus of control (A), vehicle-treated thiamine-deficient (B) and MK801-treated thiamine-deficient (C) animals. Increased densities of ^{3}H-PK11195 binding sites are seen throughout the thalamus of the vehicle-treated thiamine-deficient animal (B), and in the lateral thalamus of the MK801-treated thiamine-deficient animal (C).

DISCUSSION

In accordance with our previous report (Leong et al., 1994), results of the present study demonstrate that pyrithiamine-induced thiamine-deficiency results in neuronal loss, reactive gliosis and increased densities of ^{3}H-PK11195 binding sites in selective brain regions including inferior olive, inferior colliculus, mammillary body and thalamus; regions of brain that ultimately manifest brain lesions in thiamine deficiency (Troncoso et al., 1981). Brain regions which are not vulnerable to thiamine deficiency, such as the striatum and frontal cortex, did not manifest significant neuronal loss, nor reactive gliosis, nor changes in the densities of ^{3}H-PK11195 binding sites.

However, the results of the study also show that treatment of preconvulsive thiamine-deficient animals with MK801 offered no neuroprotection in any brain regions examined. Histological and immunohistochemical studies revealed neuronal loss and extensive lesions in vulnerable brain regions of MK801-treated thiamine-deficient animals. The absence of a glial reaction within the lesions of the thalamus was reflected by the lower density of ^{3}H-PK11195 binding sites in the thalamus of MK801-treated thiamine-deficient animals (PTM) compared to vehicle-treated thiamine-deficient (PTV) animals. Thus, increased densities of ^{3}H-PK11195 binding sites are seen in areas where gliosis is present. These findings suggest that changes in the densities of PTBR binding sites could afford a useful topographic indirect index of neuronal loss in thiamine-deficiency. Increased ^{3}H-PK11195 binding sites have previously been suggested as indicators of neuronal loss in cerebral ischaemia (Benavides et al., 1987, 1988; Price et al., 1990). Pyrithiamine-induced thiamine-deficiency in the rat affords a useful experimental animal model of Wernicke's Encephalopathy in humans (Troncoso et al., 1981; Héroux and Butterworth, 1992). Thus, future studies with the Positron Emission Tomography (PET) ligand ^{11}C-PK11195, a ligand currently used in the radiologic assessment of human gliomas (Junck et al., 1989)

could provide a potentially useful approach to the assessment of neuronal damage in patients with Wernicke's Encephalopathy.

In common with Wernicke's Encephalopathy, pyrithiamine-induced thiamine-deficiency in the rat results in two distinct types of neuronal lesions (Torvik, 1985; Vortmeyer and Colmant, 1988). In both the human condition and rat model, thalamic lesions are characterized by the presence of shrunken neurons with eosinophilic cytoplasm, closely resembling ischaemic neuronal necrosis. Lesions of inferior colliculus and vestibular nuclei, on the other hand, are not of the ischaemic type; rather, they are characterized by a bulbous spongiform appearance of the neuropil with accompanying pallor and edematous damage to neurons (Vortmeyer and Colmant, 1988). There is experimental evidence to suggest that ischaemic neuronal loss may result from N-methyl-D-aspartate (NMDA)-receptor mediated glutamate excitotoxicity. Evidence in favour of excitotoxic mechanisms in ischaemic damage include the demonstration of increased extracellular concentrations of glutamate in brain structures that are ultimately damaged by ischaemia (Baker et al., 1991; Shimada et al., 1993) as well as by studies demonstrating a neuroprotective effect of the NMDA-receptor antagonist MK801 in this condition (Gill et al., 1991; Lin et al., 1993; Park et al., 1988; Swan and Meldrum, 1990). However, there are also reports that MK801 fails to protect against ischaemic-induced neuronal damage (Buchan and Pulsinelli, 1991; Fleischer et al., 1989; Lanier et al., 1990; Nellgard et al., 1991), or that the neuroprotective effects of MK801 are mediated by a hypothermic mechanism (Buchan and Pulsinelli, 1990; Corbett et al., 1990).

In pyrithiamine-induced thiamine-deficiency, increased extracellular glutamate concentrations have been found in the thalamus (Hazell et al., 1993; Langlais and Zhang, 1993), raising the possibility that the thalamic "ischaemic-type" lesions in pyrithiamine-induced thiamine-deficiency are the result of NMDA-receptor mediated excitotoxic damage.

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Preface

The previous chapters have demonstrated the usefulness of ^{3}H-PK11195 as an indirect marker of neuronal loss in thiamine deficiency. In the following study autoradiography using radioligands for central (neuronal) and "peripheral-type" (glial) benzodiazepine receptors was used to study the regional distribution of changes in densities of these binding sites in relation to neuronal loss and reactive gliosis. Both the PTBR ligand (^{11}C-PK11195) and the central benzodiazepine receptor ligand (^{11}C-Ro 15 1788) have been used in Positron Emission Tomography (PET) studies of disorders of the central nervous system in humans. Non-invasive diagnostic techniques such as PET may offer a potential diagnostic aid for WKS, a condition that is difficult to diagnose during life.

CHAPTER 5

Quantitative receptor autoradiography using selective radioligands for central and peripheral-type benzodiazepine receptors in experimental Wernicke's encephalopathy: Implications for PET imaging

ABSTRACT

Wernicke's encephalopathy (WE) is difficult to diagnose during life, with up to 80% of cases being missed by routine neurological evaluation in both alcoholics and AIDS patients. Therefore, there is a need for non-invasive diagnostic procedures. Using the pyrithiamine-treated rat, an animal model of WE, we have studied, using radioligands for central (neuronal) and "peripheral-type" (glial) benzodiazepine receptors, the regional distribution of changes in the densities of these receptors in relation to the degree of neuronal loss and the degree of reactive gliosis accompanying neuronal loss. Histological studies revealed neuronal loss in selective regions including the thalamus, inferior colliculus, inferior olivary nucleus, and mammillary body. Autoradiographic studies demonstrated increases in densities of ^{3}H-PK11195 binding sites which closely paralleled the topographic distribution of neuronal cell loss and reactive gliosis. In contrast, ^{3}H-Ro 15 1788, showed poor spatial correlation with the neuronal loss seen in pyrithiamine-induced thiamine deficiency. The PET ligand ^{11}C-PK11195 may be useful for the assessment of thiamine deficiency-induced brain damage in human alcoholics.

INTRODUCTION

Wernicke's encephalopathy (WE) is a neuropsychiatric condition resulting from thiamine deficiency. Although most commonly encountered in chronic alcoholics (Harper, 1983), it has also been described in other disorders such as gastrointestinal carcinoma, hyperemesis gravidarum and AIDS (Butterworth et al., 1991). Thiamine deficiency in alcoholics results from inadequate dietary intake, impaired gastrointestinal absorption and depletion of liver and brain stores of the vitamin (Butterworth et al., 1995).

WE is difficult to diagnose and up to 80% of cases are missed by routine clinical neurological evaluation (Harper, 1983; Butterworth, 1991). Recent advances in the use of non-invasive procedures may prove to be useful diagnostic aids. Computed Tomographic (CT) studies of acute WE reveal areas of decreased density in diencephalic structures (McDowell and LeBlanc, 1984; Mensing et al., 1984) and Magnetic Resonance Imaging (MRI) reveals T2 signal hyperintensity in periventricular regions (Gallucci et al., 1990; Yokote et al., 1991) as well as mammillary body atrophy (Charness and DeLaPaz, 1987).

Recent studies using Positron Emission Tomography (PET) have assessed the effects of alcohol on cerebral blood flow, glucose metabolism and receptor binding (Farde et al., 1994). However, few studies have addressed these issues in alcoholics with WE. As part of a series of studies to evaluate the suitability of various radioligands for the PET diagnosis and assessment of neuronal cell loss in WE, we undertook to measure, using quantitative receptor autoradiography, the integrity of benzodiazepine binding sites in brain. Two radioligands were chosen, namely ^{3}H-PK11195, a ligand with high selectivity for the "peripheral-type" benzodiazepine receptor, predominantly found in glia; and ^{3}H-Ro15 1788, a ligand with high selectivity for the GABA-receptor related benzodiazepine receptor complex, which is localized to neurones. Both ^{11}C-PK11195 and ^{11}C-Ro15 1788 have previously been used in PET assessment of metabolic and degenerative disorders of the

central nervous system in humans (Holthoff et al., 1993; Myers, 1993). Evaluation of these ligands was performed in an animal model of WE, the pyrithiamine-treated rat (Troncoso et al., 1981; Héroux and Butterworth, 1992). Given the neuronal localization of the central benzodiazepine receptor, and the glial localization of the "peripheral-type" benzodiazepine receptor, the aim of the present study was to assess whether changes in the densities of these binding sites would be useful indicators of neuronal loss and concomitant reactive gliosis, respectively, in selective brain regions of thiamine-deficient animals.

MATERIALS AND METHODS

Treatment Groups

Male Sprague-Dawley rats weighing 175-200 g were used for the experiments described. All animals were housed individually in the André-Viallet Clinical Research Centre's animal quarters under constant conditions of temperature, humidity, and light cycles. The following treatment groups were used for the studies described:

Group 1- Pair-fed control animals

Rats (n=6) were fed a thiamine-deficient diet (ICN, Nutritional Biochemical, Cleveland, OH, U.S.A.) pair-fed to equal food consumption to that of rats in Group 2. In addition, all rats received thiamine subcutaneously in a dose of 10 µg/100 g body weight/day.

Group 2- Pyrithiamine-treated animals

Rats (n=6) were fed a thiamine-deficient diet and administered pyrithiamine subcutaneously (50 µg in 0.2 ml of saline/100 g body weight/day). Neurological status was evaluated daily (Butterworth and Héroux, 1989) until loss of righting reflex was evident; this stage was generally between days 12 and 14. At this time, rats were fed a thiamine-supplemented diet (vitamin B Complex Test diet, ICN Nutritional Biochemical) in addition

to being administered thiamine (2 mg in 0.2 ml of saline/ 100 mg body weight/ day) for three consecutive days.

Quantitative receptor autoradiography

Rats were decapitated and the brains were removed rapidly and frozen in isopentane chilled on dry ice. Frozen brains were mounted onto microtome chucks. Brain sections (20 μm thick) were cut using a Microtome cryostat at -18°C and were thaw-mounted onto gelatin-coated glass microscope slides. Brain sections were kept at -80°C prior to use in autoradiographic experiments.

^{3}H-PK11195 autoradiography

For autoradiographic studies, brain sections were incubated for 60 min at 25°C in Tris-HCl buffer (170 mM, pH 7.4) containing 1 nM ^{3}H-PK11195 (specific activity 86 Ci/mmol, New England Nuclear, Boston, MA, USA). Non-specific binding was assessed by incubating adjacent sections in the presence of 1 μM PK11195. The incubation was terminated by rinsing sections three times for 5 min in ice-cold incubation buffer. Sections were then dipped briefly in distilled water and dried rapidly under a stream of cold air.

^{3}H-Ro 15-1788 autoradiography

Brain sections were incubated for 90 min at 4°C in Tris-HCl buffer (170 mM, pH 7.4) containing 2 nM ^{3}H-Ro 15-1788 (specific activity 76.8 Ci/ mmol, NEN). Non-specific binding was defined by incubating adjacent sections in the presence of 1 mM clonazepam. The incubation was terminated by rinsing sections twice for 30 sec in ice-cold incubation buffer. Sections were then dipped in cold distilled water and dried under a stream of cold air.

Autoradiograms for both ligands were prepared by apposing sections together with tissue-calibrated standards of known ^{3}H-concentrations (Amersham microscales) to ^{3}H-sensitive Hyperfilm (Amersham) for 4 weeks. Films were developed and tissue concentrations of ^{3}H-PK11195 and ^{3}H-Ro15-1788 were measured by quantitative densitometry analysis using a MCID computer-based densitometer and image analysis system (Imaging Research Inc., Ont., Canada). The amount of ^{3}H-PK11195 and ^{3}H-Ro15-1788 bound to various brain regions was calculated from the specific activity of the ligand. Specific binding in each area was calculated by subtracting from the total binding the amount bound in corresponding regions in adjacent sections incubated for non-specific labeling.

Histological Analysis

Sections adjacent to those used for autoradiography were stained with cresyl violet using standard histological techniques and then viewed by light microscopy. Neuronal cell counts were made by a neuropathologist (L.O.) who was unaware of the treatment group. Measurements were made within fields measuring 500 x 500 μm, in sections observed at 250x magnification.

RESULTS

In agreement with previous reports, daily administration of pyrithiamine resulted, within 12-14 days, in neurological symptoms of thiamine deficiency such as loss of righting reflex and opisthotonus (Héroux and Butterworth, 1988). Following three days of thiamine replenishment, all animals had regained their righting reflex.

Autoradiographic studies

Assessment of the densities of ^{3}H-PK11195 binding sites revealed significant differences between pyrithiamine-treated animals and their pair-fed controls. Region-selective increases in the densities of ^{3}H-PK11195 binding sites were seen in the inferior olive

(105% increase, $p<0.001$), inferior colliculus (227% increase, $p<0.001$), mammillary body (111% increase, $p<0.001$) and the medial geniculate body of the thalamus (347% increase, $p<0.001$) of pyrithiamine-treated animals compared to pair-fed controls (Table 1, Figure 1). More modest but significant increases were also seen in lateral vestibular nucleus, and medial dorsal and ventroposterior nuclei of the thalamus (Table 1). There were no significant differences in the densities of ^{3}H-PK11195 binding sites in cerebral cortex, hippocampus, or striatum.

Autoradiographic assessment of the densities of ^{3}H-Ro15 1788 binding sites revealed significant decreases confined to an area within the inferior colliculus (Figure 2, Table 2). In other brain vulnerable regions, there were no differences in the densities of ^{3}H-Ro15 1788 binding sites, between pyrithiamine-treated animals and pair-fed controls.

Histopathologic studies

Examination of histologic sections from pyrithiamine-treated animals revealed evidence of neuronal loss and concomitant gliosis. Reductions in neuronal cell counts were seen in the inferior olive, inferior colliculus and thalamus (Table 3). There was evidence of marked gliosis within these brain regions. Histological studies also revealed the presence of tissue necrosis which was most severe in the inferior colliculus, and also present in the inferior olive, thalamus and mammillary bodies.

Table 1- Densities of ^{3}H-PK11195 binding sites in brain regions of pyrithiamine (PT)-treated and pair-fed (PF) control rats.

Brain region	^{3}H-PK11195 binding (pmol/g tissue) PF-control	PT-treated
Cerebral cortex		
Motor cortex	43.6 ± 8.25	61.0 ± 10.6
Somatosensory cortex	44.4 ± 4.29	56.8 ± 8.67
Frontal cortex	52.2 ± 7.32	55.3 ± 8.13
Striatum	22.9 ± 3.33	26.7 ± 3.48
Limbic		
Hippocampus-CA1	40.7 ± 11.4	47.1 ± 8.22
CA3	31.2 ± 5.04	44.5 ± 6.99
Thalamus		
Medial dorsal nucleus	61.3 ± 6.3	133 ± 27.6*
Medial geniculate nucleus	46.8 ± 6.41	210 ± 12.1**
Ventral posterior nucleus	39.6 ± 5.29	116 ± 31.8*
Hypothalamus		
Dorsomedial nucleus	79.0 ± 5.69	89.9 ± 9.56
Ventromedial nucleus	81.5 ± 6.91	101.5 ± 10.6
Mammillary nucleus	105 ± 9.04	222 ± 17.5**
Midbrain		
Inferior colliculus	71.9 ± 6.94	232 ± 12.1**
Substantia nigra	52.6 ± 4.42	54.7 ± 4.95
Pons and Medulla		
Inferior olivary nucleus	97.0 ± 7.6	199 ± 13.7**
Lateral vestibular nucleus	68 ± 5.37	106 ± 16.1*
Medial vestibular nucleus	98.7 ± 9.56	124 ± 25.4
Facial nucleus	96.7 ± 16.9	101 ± 13.8
Raphe nucleus	60.5 ± 11.0	67.5 ± 8.08
Cerebellar Cortex	78.9 ± 3.37	78.7 ± 10.0

Values are means ± S.E.M., n=6 animals per group. *$p < 0.05$, **$p < 0.001$ PT-treated compared to PF controls by Student's t test.

Table 2- Densities of ^{3}H-Ro15-1788 binding sites in brain regions of pyrithiamine (PT)-treated and pair-fed (PF) control rats.

Brain region	^{3}H-Ro15-1788 binding (pmol/g tissue) PF-control	PT-treated
Cerebral cortex		
Motor cortex	345 ± 19.4	334 ± 22.0
Somatosensory cortex	361 ± 18.5	343 ± 21.9
Frontal cortex	381 ± 20.2	358 ± 23.0
Striatum	140 ± 13.3	149 ± 12.6
Limbic		
Hippocampus-CA1	361 ± 20.5	334 ± 17.9
CA3	314 ± 16.6	307 ± 13.0
Thalamus		
Medial dorsal nucleus	178 ± 10.2	199 ± 6.9
Medial geniculate nucleus	211 ± 25.3	209 ± 22.1
Ventral posterior nucleus	150 ± 2.9	173 ± 10.1
Hypothalamus		
Dorsomedial nucleus	267 ± 8.8	270 ± 16.7
Ventromedial nucleus	293 ± 13.6	300 ± 21.0
Mammillary nucleus	280 ± 27.2	231 ± 22.2
Midbrain		
Inferior colliculus	309 ± 6.92	89 ± 5.85*
Substantia nigra	313 ± 36.1	302 ± 34.2
Pons and Medulla		
Inferior olivary nucleus	46 ± 3.92	36 ± 7.02
Lateral vestibular nucleus	118 ± 8.82	108 ± 10.1
Medial vestibular nucleus	204 ± 15.9	212 ± 22.3
Facial nucleus	164 ± 13.4	164 ± 20.3
Raphe nucleus	184 ± 18.5	172 ± 19.6
Cerebellar Cortex		
Molecular layer	287 ± 22.8	315 ± 22.3
Granular layer	175 ± 7.91	181 ± 7.3

Values are means ± S.E.M., n=6 animals per group. *p< 0.001; PT-treated compared to PF controls by Student's t test.

Table 3- Neuronal cell counts and degree of gliosis in pyrithiamine (PT)-treated and pair-fed (PF) control rats

Brain region	PF-control	PT-treated	% loss	Gliosis
Inferior olive	46 ± 5	21 ± 3	54	+++
Lateral vestibular n.	25 ± 1	12 ± 1	49	++
Inferior colliculus	59 ± 3	27 ± 4	53	+++
Mammillary n.	52 ± 7	27 ± 8	47	++
Thalamus-MGB	55 ± 7	22 ± 5	61	++/+++
MD	69 ± 14	39 ± 6	44	+++
Striatum	80 ± 8	80 ± 3	-	-
Frontal cortex	99 ± 7	97 ± 6	-	-

Values represent mean ± SD of neuronal counts made within fields of 500 x 500 μm in tissue sections at 250x magnification. The degree of gliosis: + (mild), ++ (moderate), +++ (marked). MGB, medial geniculate body; MD, medial dorsal nucleus of the thalamus.

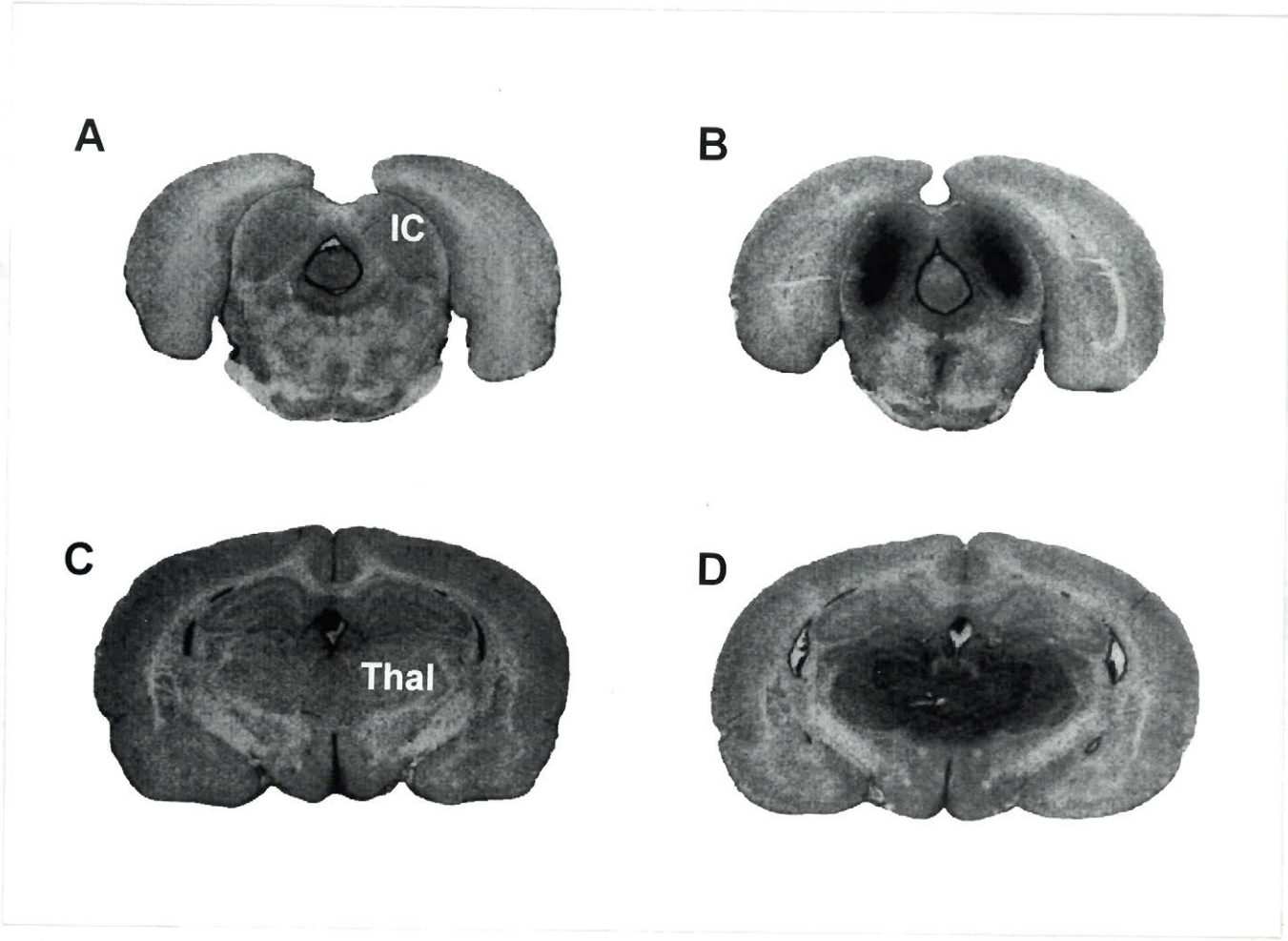

Figure 1- Representative autoradiograms of ^{3}H-PK11195 binding sites in pair-fed control (A, C) and thiamine-deficient (B, D) animals. Shown are densities of ^{3}H-PK11195 binding sites in the inferior colliculus (IC) and thalamus (Thal), as indicated.

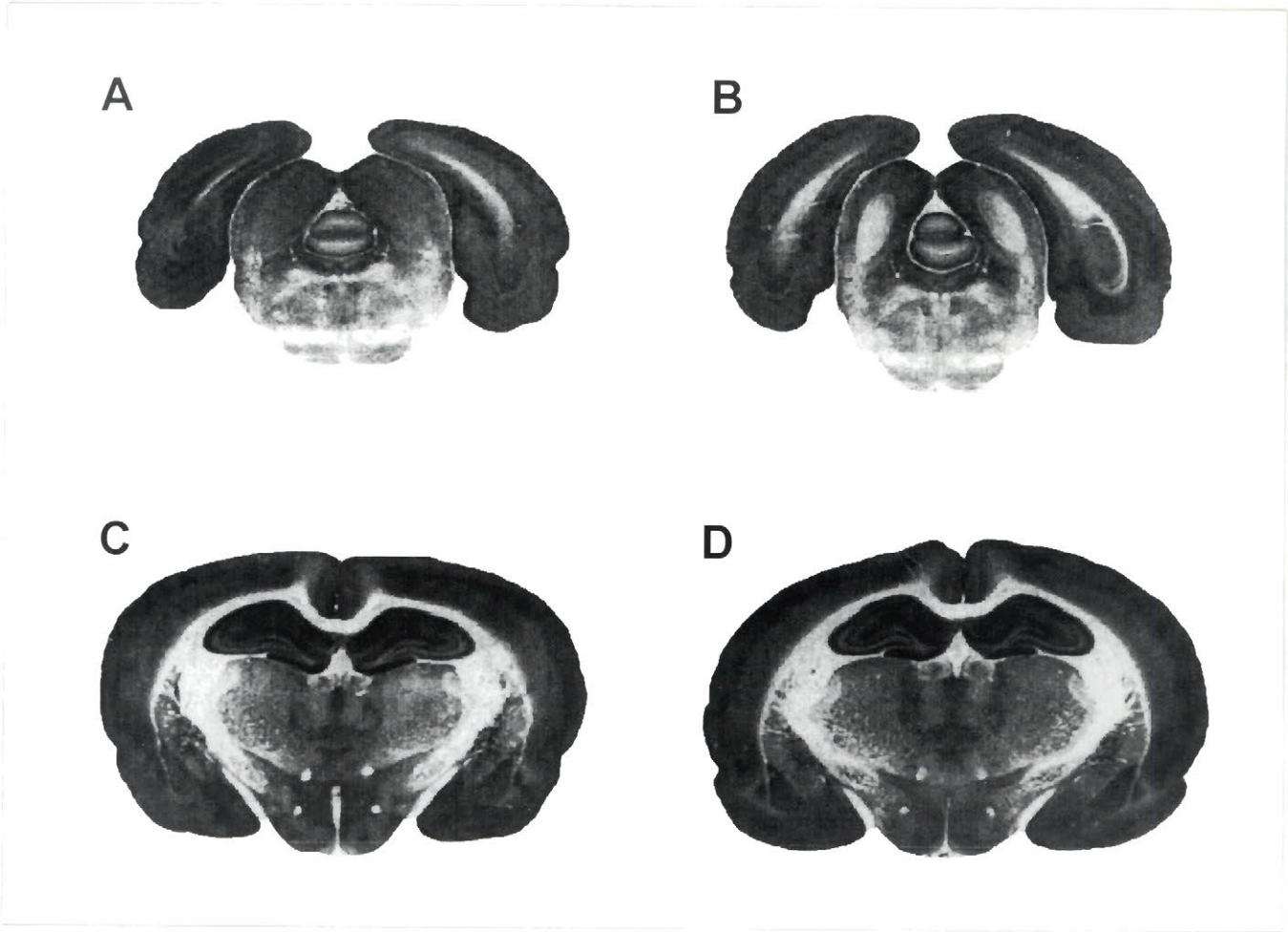

Figure 2- Representative autoradiograms of ^{3}H-Ro15-1788 binding sites in pair-fed control (A, C) and thiamine-deficient (B, D) animals. Shown are densities of ^{3}H-Ro15-1788 binding sites in the inferior colliculus (IC) and thalamus (Thal), as indicated in Figure 1.

DISCUSSION

The results of the present study reveal significant region-selective increases in densities of ^{3}H-PK11195 binding sites in brains of pyrithiamine-induced thiamine deficient animals. Increased densities of ^{3}H-PK11195 binding sites were evident in the medial-dorsal, ventroposterior and medial geniculate nuclei of the thalamus; as well as in mammillary bodies, inferior colliculus, inferior olivary nucleus and lateral vestibular nucleus; brain regions which manifest severe neuronal loss in pyrithiamine-induced thiamine deficiency in the rat (Troncoso et al., 1981; Heroux and Butterworth, 1992), as well as in human WE (Victor et al., 1989). ^{3}H-PK11195 is a radioligand with high selectivity for the "peripheral-type" benzodiazepine receptor which in brain, unlike the GABA-related central-type receptor, is predominantly non-neuronal in localization, being encountered primarily on the outer mitochondrial membrane of astrocytes (Syapin and Skolnick, 1979; Anholt et al., 1986) as well as on macrophages (Zavala et al., 1984). The increased densities of ^{3}H-PK11195 binding sites presently observed in vulnerable brain structures in experimental thiamine deficiency is probably the result of reactive gliosis in response to neuronal injury, since a previous report described increases in immunostaining for the astrocytic marker glial fibrillary acidic protein (GFAP) concomitant with the increased densities of ^{3}H-PK11195 binding sites (Leong et al., 1995).

In contrast to the findings of *increased* densities of ^{3}H-PK11195 binding sites in selective brain regions of pyrithiamine-induced thiamine deficient animals, densities of binding sites for ^{3}H-Ro15-1788, a radioligand with high selectivity for the GABA-related benzodiazepine receptor, located on neurons, were reduced in density. However, these changes were confined to inferior colliculus, only one of several brain structure manifesting severe neuronal loss in both experimental thiamine deficiency and in Wernicke's encephalopathy in humans. No significant decreases in ^{3}H-Ro15-1788 binding sites were evident in other vulnerable brain structures such as the thalamus, inferior olive or lateral

vestibular nucleus. The selective decrease in densities of ^{3}H-Ro15-1788 binding sites in inferior colliculus of pyrithiamine-treated rats despite comparable degrees of neuronal loss in inferior colliculus and thalamic and brainstem structures could be related to relatively low baseline densities of ^{3}H-Ro15-1788 binding sites in these brain regions. Previous studies in autopsied brain tissue from non-Wernicke alcoholic patients did not reveal significant reductions of ^{3}H-Ro15-1788 binding site densities (Butterworth et al., 1988). However, the integrity of these sites has not been studied previously in autopsied brain tissue from alcoholic patients wiht WE. Pyrithiamine-induced thiamine deficiency does not result in alterations of the densities of binding sites for the $GABA_A$-receptor ligand ^{3}H-muscimol (Héroux and Butterworth, 1988).

The findings of the present study could have important implications for the design of non-invasive approaches to the diagnosis and assessment of the degree of neuronal damage in chronic alcoholics. WE in humans is difficult to diagnose and cases are frequently missed by routine clinical neurologic evaluation in both alcoholic and non-alcoholic patients (Torvik et al., 1982; Harper, 1983; Butterworth, 1990). The classical triad of symptoms (ophthalmoplegia, ataxia, global confusional state) may occur in only a minority of WE patients (Victor et al., 1989) and, in a necropsy study by Harper (1983), 80% of WE cases identified postmortem had not been clinically diagnosed during life; retrospective analysis of the clinical data revealed that only 16% showed the classical triad of symptoms (Harper et al., 1986). Diagnosis of WE has recently improved with the advent of non-invasive diagnostic procedures such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI). CT studies of acute WE reveal areas of decreased density in diencephalic regions (McDowell and LeBlanc 1984; Mensing et al., 1984). MRI, on the other hand, reveals T2 signal hyperintensity in areas around the 3rd ventricle, aqueduct and 4th ventricle, which correlate with subsequently defined pathologic lesions in WE (Gallucci et al., 1990; Yokote et al., 1991). In chronic WE, neuropathologic studies frequently

demonstrate shrinkage of mammillary bodies (Victor et al., 1989) and MRI has consistently been used to detect mammillary body atrophy in WE (Charness and DeLaPaz, 1987; Yokote et al., 1991). However, MRI evidence of mammillary body atrophy has also been reported in non-WE alcoholics (Pfefferbaum and Rosenbloom, 1993).

The advent of Positron Emission Tomography (PET) affords a potentially alternative (or additional) non-invasive technique for the diagnosis and assessment of neuronal cell loss in WE based on selective metabolic and neurotransmitter changes due to thiamine deficiency. Autoradiographic studies using ^{14}C-deoxyglucose have shown that pyrithiamine-induced thiamine deficiency in the rat leads to reduced local cerebral glucose utilization (Hakim and Pappius, 1983). Subsequent PET studies using ^{18}F-fluoro-2-deoxy-D-glucose in alcoholic revealed a 22% decrease of brain glucose utilization in alcoholic Korsakoff patients (Kessler et al., 1984). PET studies using either ^{11}C-PK11195 or ^{11}C-Ro15-1788 are increasingly finding application in the assessment of neuronal loss in several neurologic and neuropsychiatric disorders in humans. ^{11}C-Ro15-1788 has been used for the evaluation of patients with Huntington's Disease (Holthoff et al., 1993), epilepsy (Savic et al., 1993; Prevett et al., 1995), as well as in non-WE alcoholics (Pauli et al., 1992; Litton et al., 1993; Farde et al., 1994). In the latter study, no significant alterations in densities of ^{11}C-Ro15-1788 binding sites were observed, leading the authors to conclude that GABA-related benzodiazepine receptors are not involved in alcoholic brain damage (Farde et al., 1994). Results of the present study demonstrate that changes in the densities of ^{3}H-Ro15-1788 binding sites do not accurately reflect the extent of brain lesions in experimental WE suggesting that PET studies using ^{11}C-Ro15-1788 may be of limited value in the assessment of brain damage in human alcoholics with WE.

Previous studies have demonstrated that neuronal loss resulting from other metabolic or degenerative conditions such as cerebral ischaemia (Myers et al., 1991), Huntington's

Disease (Schoemaker et al., 1982) and Alzheimer's Disease (Diorio et al., 1991) are also accompanied by significant increases in densities of ^{3}H-PK11195 binding sites, and consequently it has been suggested that alterations in the densities of these sites could offer a potentially useful indirect approach to the assessment of neuronal cell loss in these conditions (Benavides et al., 1987). ^{11}C-PK11195 is a PET ligand which has been used for the imaging of human gliomas (Junck et al., 1989; Pappata et al., 1991), as well as in studies of stroke patients (Myers et al., 1993). Results of the present study suggest that ^{11}C-PK11195 could afford a useful PET ligand for the assessment of the extent of gliosis accompanying neuronal cell loss from diencephalic and brainstem structures, which when used in conjunction with MRI could prove to be useful in the diagnosis of WE in humans.

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CHAPTER 6

General discussion

Thiamine deficiency results in the Wernicke-Korsakoff Syndrome (WKS) which is characterized neuropathologically by selective lesions in regions of the brainstem and diencephalon (Victor et al., 1989), and the pyrithiamine-induced thiamine-deficient rat affords a useful experimental animal model of Wernicke's Encephalopathy (WE) in humans (Troncoso et al., 1981; Héroux and Butterworth, 1992). The pathology of thiamine deficiency disorders has been described extensively, but the mechanism(s) responsible for the selective vulnerability of certain brain regions remains to be elucidated. This discussion of the results contained within this thesis is comprised of two sections. Firstly, immunohistochemical studies using antibodies to serum albumin and glial elements (astrocytes and macrophages/microglia), were employed to study the integrity of the blood-brain barrier, and the glial reaction, respectively, in thiamine deficiency. The results of these studies may contribute to the elucidation of events leading to neuronal loss in thiamine deficiency disorders. This collection of studies is also concerned with the topographical distribution of lesions in brain regions of pyrithiamine-induced thiamine-deficient rats, which has been assessed by using quantitative receptor autoradiography (QRA) with ^{3}H-PK11195, a selective ligand for the "peripheral-type" benzodiazepine receptor (PTBR). The results of these studies may have implications for non-invasive techniques as diagnostic aids in WE in humans.

Pathogenesis of neuronal loss in thiamine deficiency encephalopathy

The work in this thesis attempts to elucidate the sequence of events culminating in neuronal loss in thiamine deficiency encephalopathy. Thiamine is a cofactor for enzymes involved in cerebral glucose oxidation. Thus the primary cause of neuronal loss in thiamine-deficiency disorders may be decreased cerebral energy metabolism. Limiting the availability of the necessary cofactor for citric acid cycle enzymes, such as PHHC and α-KGDH, leads to reduced generation of high energy phosphates. Compromising the energy status of the brain in such a way may have a number of deleterious consequences (Erecinska and Silver,

1989). Decreased activities of citric acid cycle enzymes may lead to increased glycolysis, in an attempt to restore the cellular energy status (the Crabtree Effect), which in turn may result in accumulation of lactate and H^+ (Hakim and Pappius, 1983; Hakim, 1984). Lactic acidosis and low pH may contribute to endothelial cell damage and increased permeability of the blood-brain barrier (Klatzo, 1983; Palijarvi et al., 1983; Kuroiwa et al., 1985; Nagy et al., 1985).

Previous studies have revealed breakdown of the blood-brain barrier in thiamine deficient animals (Harata and Iwasaki, 1995; Calingasan et al., 1995) and acute WE (Schroth et al., 1991). Calingasan et al. (1995) described region-selective increases of IgG-immunoreactivity as early as 10 days of thiamine deficiency and prior to the appearance of neuropathological lesions. The study described in Chapter 3 revealed early increased immunostaining for serum albumin in presymptomatic animals as early as 7 days of thiamine deficiency; suggestive of increased permeability of the blood-brain barrier, prior to major histopathological lesions and neurological symptoms of thiamine deficiency. Albumin leakage has been found to precede IgG infiltration in acute experimental allergic encephalomyelitis; this difference in permeability of the two molecules may have been due to differences in size or electrical charge (Juhler et al., 1986).

Extravasation of plasma proteins into the brain may occur by a number of routes including, endothelial cell membrane damage, diffusion through disrupted tight junctions, formation of transendothelial channels, or stimulation of pinocytotic vesicles and transport across the capillary endothelium (Westergaard et al., 1976; Nagy et al., 1985). Cerebral capillary endothelium normally contain very few pinocytotic vesicles, but it has been suggested that increased permeability of the blood-brain barrier occurs through enhanced numbers of pinocytotic vesicles in certain conditions including ischaemia (Westergaard et al., 1977; Petito et al., 1982) and seizures (Petito et al., 1977; Westergaard et al., 1978). The

vesicular transport mechanism is an active process and therefore may not occur during conditions of impaired energy metabolism (Greenwood et al., 1989). However, enhanced pinocytotic transport for horseradish peroxidase has been reported (Manz and Robertson, 1972); as well as dilated interendothelial junctions (Horita et al., 1983) in thiamine-deficient rats.

Results of immunohistochemical studies described in Chapter 2 reveal a macrophage response in early stages of thiamine deficiency. Region-selective increases of ED1-immunostaining were seen in vulnerable brain regions of presymptomatic thiamine-deficient animals, including the inferior olive, inferior colliculus and medial thalamus. This increase in ED1-immunostaining was found to precede major histopathological lesions and neurological signs of thiamine-deficiency. The ED1 antibody selectively labels a cytoplasmic antigen in mononuclear phagocytes, (i.e. monocytes and macrophages) (Dijkstra et al., 1985), as well as activated microglia and reactive (phagocytic) microglia (Graeber et al., 1990). Following CNS injury, activated microglia (the resident macrophages of the brain) as well as blood-borne macrophages appear at the site of injury in order to phagocytose and remove cellular debris (Perry and Gordon, 1991). Thus, increased ED1-immunostaining in vulnerable brain regions of thiamine-deficient rats could result from an infiltration of blood-borne macrophages or activation of resident microglia. Evidence of increased serum albumin-immunostaining in early thiamine deficiency, indicative of increased permeability of the blood-brain barrier, suggests that increased ED1-immunostaining in vulnerable brain regions may be due in major part to infiltration of blood-borne macrophages in these areas of the brain. However, microglial activation may also occur without obvious neuropathological changes (Gehrmann et al., 1995).

The macrophage response seen early in thiamine deficiency, prior to the appearance of reactive astrocytes, suggest that accumulation of macrophages is the initial cellular event

following increased permeability of the blood-brain barrier in thiamine deficiency. Reactive gliosis may be the consequence of the early sustained accumulation of macrophages, since activated microglia are capable of releasing cytokines that induce astrocytic proliferation (Giulian and Baker, 1985; Giulian et al., 1988; Selmaj et al., 1990), and increased concentrations of cytokines including interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6) have been found at sites of neuronal injury (Giulian and Lachman, 1985; Taupin et al., 1993).

It has previously been suggested that the early presence of reactive microglia could be indicative of imminent neuronal damage (Morioka et al., 1991). Microglial activation has been reported to precede neuronal cell loss and astrocytic proliferation in transient global forebrain ischaemia (Morioka et al., 1991), as well as in transient and permanent focal ischaemia, (Morioka et al., 1993; Korematsu et al., 1994). Activation of microglia is a feature common to other types of neuronal injury, having been described in neurodegenerative disorders such as Alzheimer's Disease (McGeer et al., 1988) and in the AIDS-Dementia Complex (Navia et al., 1986). The macrophage response in early stages of thiamine deficiency may also play a role in the pathogenesis of neuronal cell death in this condition.

In addition to their role as phagocytes, both microglia and invading macrophages are capable of releasing cytotoxic substances such as proteases, reactive oxygen intermediates, reactive nitrogen intermediates, arachidonic acid metabolites and cytokines; suggestive of a role in tissue damage (Banati et al., 1993; Gehrmann et al., 1995). Reactive oxygen intermediates (Colton and Gilbert, 1987, Sonderer et al., 1987) and nitric oxide (Green et al., 1991) released from macrophages can lead to neuronal cell death (Beckman, 1991; Dawson et al., 1991; Thery et al., 1991). Cytokines such as IL-1 and TNF-α not only play a role in scar formation and wound healing, but may also play a role in cytotoxicity.

These cytokines are produced by peripheral circulating macrophages, and in the brain the main source of IL-1 are microglia, although it is also produced by astrocytes and neurones (Merrill, 1992; Rothwell and Relton, 1993). A recent study has suggested a role for IL-1 in neuronal cell death (Relton and Rothwell, 1992); a recombinant IL-1 receptor antagonist (IL-1ra) was found to prevent neuronal damage resulting from experimental cerebral ischaemia, and intrastriatal injection of a NMDA-receptor agonist. Interleukin-1 is a potent inducer of arachidonic acid, stimulates production of nitric oxide, causes increases in levels of intracellular calcium and also causes breakdown of the blood-brain barrier (Martin and Resch, 1988; Beasley et al., 1991; Di Julio et al., 1991; Quagliarello et al., 1991). Interleukin-6 and TNF-α are also produced by glial cells, and potentiate many of the effects of IL-1 (Helle et al., 1988; Quagliarello et al., 1991; Benveniste, 1992).

Brain macrophages have also been shown to release large amounts of glutamate *in vitro* that are neurotoxic to cerebellar granule cells (Piani et al., 1991). It has also been found that activated microglia release a neurotoxin, which is distinct from glutamate, but whose actions also appear to be mediated by the NMDA receptor (Vaca and Wendt, 1991). Activated microglia express considerable amounts of amyloid precursor protein (APP) mRNA as well as L-APP mRNA, a novel alternative splice isoform of APP mRNA (Konig et al., 1992; Sandbrink et al., 1993), and a recent communication described increased expression of APP in vulnerable brain regions early in the progression of pyrithiamine-induced thiamine deficiency (Calingasan et al., 1994), suggesting an additional role for microglial-associated mechanisms in the pathogenesis of neuronal cell death in thiamine deficiency.

Therapies aimed at interfering with microglial-induced cytotoxicity may be of value in the early stages of thiamine deficiency disorders. Chloroquine is able to block the activity of mononuclear phagocytes and has been found to improve functional recovery from

ischaemic injury to the spinal cord (Giulian and Robertson, 1990). Agents such as lipocortin-1 and IL-1 receptor antagonist (IL-1ra) can interfere with the action of IL-1, and have been found to prevent ischaemic-induced neuronal damage (Relton et al., 1991; Relton and Rothwell, 1992).

Microdialysis studies have reported increased levels of glutamate in thiamine deficient rats (Hazell et al., 1993; Langlais and Zhang, 1993), and histopathological studies have indicated that lesions in thiamine deficiency resemble those seen in ischaemia (Armstrong-James, 1988; Torvik, 1985; Vortmeyer and Colmant, 1988); such findings have suggested a role for glutamate excitotoxicity in thiamine deficiency. Excitotoxicity results from receptor overstimulation caused by elevated levels of glutamate which may result from excessive release or decreased uptake of the neurotransmitter (Benveniste et al., 1984; Rothman, 1984; Choi, 1988; Choi et al., 1989).

It has been suggested that the status of cellular energy metabolism may be involved in excitotoxicity (Choi, 1988; Novelli et al., 1988; Henneberry et al., 1989; Beal et al., 1993). Impairment of neuronal energy metabolism and decreased production of ATP may have several deleterious consequences (Rothman et al., 1987; Choi, 1988; Siesjo, 1988b). High-affinity reuptake of glutamate into neurones and glia, an energy requiring process (Logan and Snyder, 1972; Schousboe et al., 1977), may be impaired or actually reversed (Kauppinen et al., 1988; Szatkowski et al., 1990; Madl and Burgesser, 1993). During conditions of compromised energy metabolism decreased function of the Na^+/K^+ ATPase will lead to a deterioration of the membrane potential and prevent repolarization (Erecinska and Dagani, 1990). A reduced membrane potential may result in calcium influx by opening of voltage-sensitive Ca^{2+} channels (VSCC), and agonist-operated calcium channels (AOCC) by reducing the voltage-dependent Mg^{2+} block of the NMDA receptor allowing the influx of Na^+ and Ca^{2+}. In addition, high levels of Ca^{2+} can inhibit the activity of the

Na+/K+ pump, resulting in further disruption of the ionic gradients required for normal neuronal functioning (Yingst, 1988). The loss of the Na^+ gradient and membrane potential may also reverse the direction of the Na^+/Ca^{2+} antiport system which normally removes Ca^{2+} from the cell (Rothman et al., 1987; Choi, 1988; Siesjo, 1988b). The extrusion and sequestration of Ca^{2+} are energy requiring processes. (Carafoli, 1987; Blaustein, 1988), thus energy impairment may result in reduced extrusion of Ca^{2+} by the calcium-activated ATPase, and reduced sequestration by endoplasmic reticulum. At high concentrations of intracellular Ca^{2+}, mitochondria take up Ca^{2+} from the cytosol in preference to producing ATP (Carafoli, 1987; Beal et al., 1993).

In a study of the potential neuroprotective effect of NMDA-receptor antagonist MK801 in thiamine deficient animals prior to convulsions (Chapter 4), histological and immunohistochemical studies revealed neuronal loss and extensive lesions in vulnerable brain regions of MK801-treated thiamine-deficient animals. In contrast, in a study by Langlais and Mair (1990), MK-801 was found to significantly attenuate the thalamic neuronal loss in late stage (convulsing) thiamine-deficient animals (Langlais and Mair, 1990). MK801 has potent anticonvulsant properties (Clineschmidt et al., 1982) and prevented seizures associated with late stage pyrithiamine-treatment of rats (Langlais and Mair, 1990). Convulsions per se may result in thalamic lesions, and excitotoxic mechanisms have been proposed to explain neuronal loss in status epilepticus (Collins and Olney, 1982, Simon et al., 1984; Rothman and Olney, 1986, 1987). The extent to which the neuroprotective effect of MK801 in pyrithiamine-treated rats was the result of its anticonvulsant action has not been established. The results of the study in Chapter 4 did not support a neuroprotective role for MK801-treatment in non-convulsing pyrithiamine-treated thiamine deficient animals. However, MK801 treatment was initiated from day 7 of thiamine deficiency, which is temporally coincident with a macrophage response in vulnerable brain regions of thiamine deficient animals (Chapter 3). It remains to be

elucidated whether earlier treatment with MK801 would be beneficial in protecting against neuronal loss in thiamine deficiency. There are reports that MK801 fails to protect against ischaemic-induced neuronal damage (Buchan and Pulsinelli, 1991; Fleischer et al., 1989; Lanier et al., 1990; Nellgard et al., 1991) and that the neuroprotective effects of MK801 are mediated by a hypothermic mechanism (Buchan and Pulsinelli, 1990; Corbett et al., 1990). In addition to acting via the NMDA receptor, glutamate neurotransmission can modulate intracellular calcium through the inositol phosphate-coupled ACPD receptor (Cotman and Monaghan, 1989; Monaghan et al., 1989), and also sustained stimulation of phospholipase C by glutamate causes IP_3-triggered release of Ca^{2+} from the endoplasmic reticulum (Siesjo and Bengtsson, 1989). The lack of a neuroprotective effect by MK801 might possibly be due to the multiple routes of Ca^{2+} entry and multitude of deleterious effects of elevated levels of calcium

Detection of brain damage in thiamine deficiency encephalopathy

In a study of the topographic distribution of neuronal loss in thiamine-deficiency encephalopathy, results contained in Chapter 1 reveal that densities of ^{3}H-PK11195 binding sites in brain regions of presymptomatic pyrithiamine-treated thiamine deficient rats (animals showing no neurological signs of thiamine deficiency) were not significantly different from controls. At the symptomatic stage of thiamine deficiency, however, densities of ^{3}H-PK11195 binding sites were significantly increased in selective brain regions including the inferior olive, inferior colliculus and thalamus; regions of the brain that ultimately manifest significant neuronal loss in experimental thiamine-deficiency (Troncoso et al., 1981) and in the WKS in humans (Victor et al., 1989) In brain regions that are spared in thiamine-deficiency encephalopathy, including the cerebral cortex, striatum and hippocampus, there were no significant differences in the densities of ^{3}H-PK11195 binding sites between thiamine-deficient animals and controls.

In brain PTBRs are predominantly found on non-neuronal cells (McCarthy and Harden, 1981; Marangos et al., 1982; Sher and Machen, 1984; Schoemaker et al., 1982), including astrocytes (Bender and Hertz, 1985a; Tardy et al., 1981), microglia (Park et al., 1994) and cells of monocytic lineage, including macrophages (Zavala et al., 1984, 1987; Benavides et al., 1989; Ferrarese et al., 1990). The results of histopathologic studies described in Chapter 1 suggest that increased densities of PTBRs are most likely the consequence of glial changes. While histopathologic examination of vulnerable brain regions of presymptomatic animals revealed only mild gliosis, similar studies of symptomatic animals revealed marked gliosis. Thus, significant increases in densities of ^{3}H-PK11195 binding sites were both spatially and temporally coincident with the appearance of reactive gliosis, which suggested that increased densities of ^{3}H-PK11195 binding sites were the result of reactive gliosis accompanying neuronal loss in the brains of thiamine-deficient animals. In support of this suggestion, the results of the study in Chapter 2 revealed that increased densities of ^{3}H-PK11195 binding sites in vulnerable brain regions, were coincident with increases of immunostaining for the astrocytic marker glial fibrillary acidic protein (GFAP) in symptomatic animals, but were not coincident with the early increased ED1-immunostaining for macrophages seen in presymptomatic animals. These findings were in contrast with those observed in experimental focal ischaemia, where increased densities of PTBRs coincided with macrophages surrounding focal necrotic lesions (Myers et al., 1991a). This discrepancy may be a reflection of the difference in age of the lesions and chronicity of the conditions.

Densities of PTBRs are increased in membrane preparations from rat striata following intrastriatal injections of excitotoxic compounds (Schoemaker et al, 1982; Owen et al, 1983; Benavides et al, 1987). In these studies, increased PTBRs were found to parallel both the loss of neuronal marker enzymes as well as the appearance of reactive gliosis (Schoemaker et al, 1982; Benavides et al, 1987). Increased densities of PTBRs have also

been described in postmortem brain tissue from patients with neurodegenerative disorders such as Huntington's disease (Schoemaker et al, 1982) and Alzheimer's disease (Owen et al, 1983; Diorio et al, 1991). These findings suggested that increased densities of PTBRs reflect reactive gliosis accompanying neuronal loss, and that increased densities of PTBRs can be used as a sensitive index of neuronal loss in these disorders (Benavides et al, 1987). The results of our studies suggest that ^{3}H-PK11195 is also a good indirect marker of neuronal loss in experimental thiamine-deficiency encephalopathy.

It has also been suggested that measurements of the densities of PTBRs can be used to evaluate the effectiveness of potential neuroprotective agents (Benavides et al., 1989; Demerle-Pallardy et al., 1991). In the study of the effect of the NMDA-receptor antagonist MK801 in thiamine defiiciency (Chapter 4) histological and immunohistochemical studies revealed neuronal loss in selective brain regions, and an absence of a glial reaction within the lesions of the thalamus of MK801-treated thiamine-deficient animals. Coincident with the lack of a glial reaction, densities of ^{3}H-PK11195 binding sites were significantly lower in the thalamus of MK801-treated thiamine deficient animals. Thus, increased densities of ^{3}H-PK11195 binding sites were seen in areas where gliosis was present. Results of the study confirmed that measurement of densities of PTBR binding sites could afford a useful topographic index of reactive gliosis in thiamine deficiency.

The regional distribution of densities of central benzodiazepine (neuronal) and PTBR (glial) binding sites in relation to neuronal loss was also studied by autoradiography (Chapter 5). In accordance with the study described in Chapter 1, increased densities of ^{3}H-PK11195 binding sites were evident in selective brain regions. In contrast, densities of ^{3}H-Ro15-1788 binding sites, a radioligand with high selectivity for the GABA-related benzodiazepine receptor (localized on neurones) were reduced in density. However, these changes were confined to inferior colliculus, only one of several brain structures

manifesting neuronal loss in thiamine-deficiency. Densities of ^{3}H-Ro15-1788 binding sites were not significantly decreased in other vulnerable brain structures such as the inferior olive or thalamus. The selective decrease in densities of ^{3}H-Ro15-1788 binding sites in inferior colliculus of pyrithiamine-treated rats, despite comparable degrees of neuronal loss in inferior colliculus and diencephalic and brainstem structures, could be related to relatively low baseline densities of ^{3}H-Ro15-1788 binding sites in these other brain regions. Previous studies in autopsied brain tissue from non-Wernicke alcoholic patients did not reveal significant reductions of densities of ^{3}H-Ro15-1788 binding sites (Butterworth et al., 1988). However, the integrity of these sites has not been studied previously in autopsied brain tissue from alcoholic patients with WE. Binding studies using ^{3}H-muscimol have not revealed any alterations of densities of $GABA_A$-receptor binding sites in thiamine-deficient animals (Héroux and Butterworth, 1988)

The results of QRA studies showing increased densities of ^{3}H-PK11195 binding sites in thiamine deficiency could have important implications for non-invasive approaches to the diagnosis of WE, as well as assessment of neuronal damage in this condition. WE in humans is difficult to diagnose and cases are frequently missed by routine clinical neurologic evaluation in both alcoholic and non-alcoholic patients (Torvik et al., 1982; Harper, 1983; Butterworth et al., 1991). The classical clinical triad of symptoms (ophthalmoplegia, ataxia, global confusional state) may occur in only a minority of WE patients (Victor et al., 1989), and in a necropsy study by Harper (1983) 80% of WE cases identified postmortem had not been clinically diagnosed during life; retrospective analysis of the clinical data revealed that only 16% of patients showed the classical triad of symptoms (Harper et al., 1986).

Diagnosis of WE has recently improved with the advent of non-invasive diagnostic procedures such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI).

CT studies (McDowell and LeBlanc 1984; Mensing et al., 1984; Kitaguchi et al., 1987) and MRI studies (Charness and DeLaPaz, 1987; Gallucci et al., 1990; Yokote et al., 1991; D'Aprile et al., 1994) have revealed changes in selective brain regions of WE patients. PET may also be a useful non-invasive technique for the diagnosis and assessment of neuronal cell loss in WE. PET studies using the PTBR ligand ^{11}C-PK11195, and the central benzodiazepine receptor ligand ^{11}C-Ro15-1788, have been used in PET assessment of neuronal loss in several neurologic and neuropsychiatric disorders in humans. ^{11}C-Ro15-1788 has been used for the evaluation of patients with Huntington's Disease (Holthoff et al., 1993), epilepsy (Savic et al., 1993, 1994; Prevett et al., 1995), as well as in non-WE alcoholics (Pauli et al., 1992; Litton et al., 1993; Farde et al., 1994). In the latter study, no significant alterations in densities of ^{11}C-Ro15-1788 binding sites were observed, leading the authors to conclude that GABA-related benzodiazepine receptors are not involved in alcoholic brain damage (Farde et al., 1994). The results described in Chapter 5 revealed that changes in the densities of binding sites for ^{3}H-Ro15-1788, a ligand for the central benzodiazepine receptor, did not accurately reflect the extent of brain lesions in thiamine-deficient rats, suggesting that PET studies using ^{11}C-Ro15-1788 may be of limited value in the assessment of brain damage in human WE. The PTBR ligand ^{11}C-PK11195 is available for use in PET and has been used for the imaging of human gliomas (Starosta-Rubenstein et al., 1987; Black et al., 1989, 1990; Junck et al., 1989, 1991; Pappata et al., 1991); as well as in studies of stroke patients (Junck et al., 1990; Myers et al., 1993). Results from studies presented in this thesis suggest that ^{11}C-PK11195 could afford a useful PET ligand for the assessment of reactive gliosis in diencephalic and brainstem structures, which when used in conjunction with MRI could prove to be useful in the diagnosis of WE, and assessment of brain damage in WE in humans.

The WKS is a treatable condition, and early detection and treatment could be of great value in improving the prognosis of this condition, especially in view of the finding that 85% of patients who survive Wernicke's encephalopathy show symptoms of Korsakoff's syndrome, which is characterized by anterograde and retrograde amnesia (Victor et al., 1989). Korsakoff's syndrome has been reported to occur without an antecedent episode of WE (Harper, 1983; Victor et al., 1989). It has been suggested that subclinical episodes of WE may culminate in Korsakoff's syndrome (Harper, 1979; Bowden, 1990). Neuropathological studies of Korsakoff patietnts have revealed lesions of the anterior thalamus (Mair et al., 1979; Halliday et al., 1994), and recent studies suggest that a circuit involving the mammillary bodies, mamillo-thalamic tract and the anterior thalamus is critical in memory formation (Kopelman, 1995).

In summary, together the studies described above represent an examination of the pathogenesis and detection of neuronal loss in thiamine deficiency encephalopathy. Immunohistochemical studies revealed a macrophage response and increased permeability of the blood-brain barrier in the early stages of thiamine deficiency, and such events were found to precede the appearance of major neuropathological lesions and the onset of neurological symptoms of thiamine deficiency. These findings may contribute to the elucidation of events culminating in neuronal loss in thiamine deficiency. In addition, the topographic distribution of brain lesions was assessed by using a selective ligand for the "peripheral-type" benzodiazepine receptor. Autoradiographic measurement of increased densities of these receptor sites were found to coincide both spatially and temporally with histological and immunohistochemical evidence of gliosis, suggesting that the PTBR ligand was a good indirect marker of neuronal loss in experimental thiamine deficiency. Such a finding could have implications for the use of non-invasive approaches in assessment of neuronal loss in thiamine deficiency disorders in humans.

Claims to originality

Chapter 2 showed for the first time that ^{3}H-PK11195, a specific ligand for the "peripheral-type" benzodiazepine receptor, could be used as an indirect marker of neuronal loss in thiamine deficiency. Increased densities of ^{3}H-PK11195 binding sites were seen in selective brain regions. Such increases in ^{3}H-PK1195 binding were seen in areas which showed histological evidence of marked gliosis.

Chapter 3 is the first report of increased ED1-immunostaining (a marker for macrophages), as well as increased immunostaining for serum albumin in selective brain regions of thiamine deficient animals. These findings were suggestive of increased permeability of the blood-brain barrier and a macrophage response in the early stage of thiamine deficiency, preceding major neuropathological lesions and neurological symptoms of thiamine deficiency.

Chapter 4 contains a novel study of the putative neuroprotective effect of MK801 in non-convulsing thiamine deficient animals. This study was designed to ascertain neuronal loss due to thiamine deficiency *per se* and thiamine deficiency associated convulsions. Results of the study suggested that at the doses and times tested MK801 offered no neuroprotection in any of the brain regions examined.

Chapter 5 contains the first reported study comparing ligands for the central benzodiazepine receptor (CBR) and the "peripheral-type" benzodiazepine receptor (PTBR) as markers of neuronal loss in experimental thiamine deficiency. Results of the study suggested that the PTBR ligand, rather than the CBR ligand, offers a potential ligand for use in Positron Emission Tomography assessment of neuronal loss in thiamine deficiency disorders in humans.

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