ผลของเอ็น-(2-โพรพิลเพนทาโนอิล) ยูเรีย ต่อความบกพร่องของการเรียนรู้ ความจำ และการตายของเซลล์ประสาท หลังการอุดกั้นหลอดเลือดแดงคอมมอนแคโรติดทั้งสองข้างในหนูถีบจักร

นางสาว อรพรรณ วนขจรไกร

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EFEECTS OF N-(2-PROPYLPENTANOYL) UREA ON IMPAIRMENT OF LEARNING MEMORY AND NEURONAL CELL DEATH AFTER BILATERAL COMMON CAROTID ARTERIES OCCLUSION IN MICE

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Thesis Title	EFEECTS OF N-(2-PROPYLPENTANOYL) UREA ON IMPAIRMENT OF LEARNING MEMORY AND NEURONAL CELL DEATH AFTER BILATERAL COMMON CAROTID ARTERIES OCCLUSION IN MICE
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การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของเอ็น-(2-โพรพิลเพนทาโนอิล) ยูเรีย (วีพียู) ต่อความบกพร่องของการเรียนรู้ ความจำ และการตายของเซลล์ประสาท หลังการทำให้เกิดภาวะสมองขาดเลือดโดยการอุดกั้นหลอดเลือดแดงคอมมอนแคโรติดทั้งสองข้าง พบว่าหนูที่อยู่ในภาวะสมองขาดเลือดมีความบกพร่องของการเรียนรู้และความจำเมื่อทดสอบด้วยวิธีมอริสวอเตอร์เมสโดยหนูใช้เวลาในการหาแท่นพักนานขึ้น เช่นเดียวกับการทดสอบด้วยวิธีสะเต็บดาวพบอีกว่า หนูใช้เวลาอยู่บนแท่นพักลดลงและจำนวนครั้งที่ก้าวลงจากแท่นพักเพิ่มขึ้น นอกจากนี้ยังพบว่ามีการเพิ่มขึ้นของเอ็มดีเอในสมอง และพบการตายของเซลล์ประสาทในบริเวณซีเอวัน(CA1) และซีเอทรี(CA3) ของสมองส่วนฮิปโปแคมปัส เมื่อให้วีพียูหรือไพราซีแตม โดยวิธีการฉีดเข้าช่องท้องพบว่ามีผลทำให้หนูใช้เวลาในการหาแท่นพักลดลงในวันที่ 5 ของการทดสอบสารทดสอบ เมื่อทดสอบด้วยวิธีมอริสวอเตอร์เมส เช่นเดียวกันกับการทดสอบด้วยวิธีสะเต็บดาวพบว่าหนูที่ได้รับวีพียูหรือไพราซีแตมมีการลดลงของความบกพร่องทางการเรียนรู้และความจำ โดยที่ใช้เวลาอยู่บนแท่นพักนานขึ้นและจำนวนครั้งที่ก้าวลงจากแท่นพักลดลง เมื่อเทียบกับหนูในกลุ่มควบคุมพบว่าหนูกลุ่มที่ได้รับวีพียูหรือไพราซีแตมจะมีจำนวนเซลล์ประสาทที่มีชีวิตในบริเวณซีเอวันและซีเอทรีของฮิปโปแคมปัสมากกว่า นอกจากนี้หนูกลุ่มที่ได้รับวีพียูหรือไพราซีแตมมีการลดลงของเอ็มดีเอที่เพิ่มขึ้นจากภาวะสมองขาดเลือด ในการทดลองนี้ไม่พบความแตกต่างของอัตราการเคลื่อนไหวเมื่อทดสอบโดยวิธีโลโคมอเตอร์แอคติวิตี้ ไม่ว่าจะเป็นหนูกลุ่มควบคุมหรือหนูกลุ่มที่ได้รับสารทดสอบทั้งสองชนิด

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KEY WORD: N-(2-PROPYLPENTANOYL) UREA, VALPROATE ANALOG, LEARNING, MEMORY, CEREBRAL ISCHEMIA, MORRIS WATER MAZE, STEP DOWN TEST, LOCOMOTOR ACTIVITY TEST, OXIDATIVE STRESS

ORAPHAN WANAKHACHORNKRAI : EFFECTS OF N-(2-PROPYLPENTANOYL) UREA ON IMPAIRMENT OF LEARNING MEMORY AND NEURONAL CELL DEATH AFTER BILATERAL COMMON CAROTID ARTERIES OCCLUSION IN MICE. THESIS ADVISOR: ASSOC.PROF. BOONYONG TANTISIRA, Ph.D., THESIS CO-ADVISOR: ASSOC.PROF. MAYUREE TANTISIRA, Ph.D., 65 pages.

The present study aimed to investigate the effects of N-(2-propylpentanoyl) urea (VPU) on impairment of learning and memory and neuronal cell death after transient cerebral ischemia (bilateral occlusion of common carotid arteries, 2VO) in mice. The 2VO caused an impairment of learning and memory seen as an increase time to find platform in Morris Water Maze (MWM) test as well as a reduction in latency and an increase in number of errors in passive avoidance (step-down) test. In addition, an increases in brain malondialdehyde (MDA) as well as neuronal cell death in hippocampus CA1 and CA3 regions were also observed. Intraperitoneal administration (i.p.) of VPU or piracetam significantly improved performance in MWM test in 2VO mice on day 5 by decreasing the time to find platform. Similar results were observed in step-down test whereby administration of VPU or piracetam significantly increased the step-down latency and a decreased the number of errors in this task. In comparison to 2VO group, administration of VPU and piracetam was found to increase number of survival neurons in CA1 and CA3 regions of hippocampus. Additionally, treatment by VPU or piracetam significantly abolished an increase in MDA induced by 2VO. However, locomotor activity was not altered by neither piracetam nor VPU. Previous report of potentiation of GABA, inhibition of NMDA receptor and reduction of cortical glutamate elicited by VPU might underlie the neuroprotective effect observed in the present studies.

Based on the results obtained, it is suggestive that by means of multi-mechanisms previously reported of VPU, neuroprotective effect was then accomplished resulting in more survival neuronal cells of CA1 and CA3 of the hippocampus. Therefore, impairment of learning and memory induced by 2VO was subsequently improved. Similar to piracetam, VPU might be further developed for post stroke amnesia or anticonvulsant with positive effect on learning and memory.

Field of study	Physiology	Student's signature	Orapan Wanakhachornkrai
Academic year	2006	Advisor's signature	Boonyong Tantisira
		Co-advisor's signature	Mayuree Tantisira

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LIST OF ABBREVIATIONS

%	=	Percent
α	=	Alpha
β	=	Beta
γ	=	Gamma
μm	=	Micrometer
^{o}C	=	Degree Celsius
AEDs	=	Antiepileptic drugs
AMPA	=	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	=	Analysis of variance
B.W.	=	Body weight
bcl	=	B-cell lymphoma
CA1	=	Area 1 of Ammon's horn
Ca^{2+}	=	Calcium ion
CA3	=	Area 3 of Ammon's horn
CBF	=	Cerebral blood flow
Cl^{-}	=	Chloride ion
cm	=	Centrimeter
CMC	=	Carboxy methyl cellulose
CNS	=	Central Nervous System
CR	=	Condition response
CS	=	Condition stimulus
CSF	=	Cerebrospinal fluid
CT scan	=	Computed tomography
DC	=	Direct current
DG	=	Dentate gyrus
DNA	=	Deoxyribonucleic acid
e.g.	=	Exampli gratia (for example)
ED_{50}	=	Median effective dose

et al.	=	et alli (and other)
g	=	Gram
GABA	=	Gamma aminobutyric acid
GFs	=	Growth factors
HPLC	=	High performance liquid chromatography
hr	=	Hour
HSPs	=	Heat shock proteins
Hz	=	Hertz
i.p.	=	Intraperitoneal
IL	=	Interleukin
IP_3	=	Inositol triphosphate
K^+	=	Potassium ion
kg	=	Kilogram
M	=	Molar
MDA	=	Malondialdehyde
MES	=	Maximal electroshock seizure test
mg	=	Milligram
mg/kg B.W.	=	Milligram per kilogram body weight
min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
mm^2	=	Square millimeter
MRI	=	Magnetic resonance imaging
ms	=	Millisecond
mV	=	Millivolt
MWM	=	Morris Water Maze
Na^+	=	Sodium ion
NFkB	=	Nuclear factor kappa B
nm	=	Nanometer
NMDA	=	N-methyl-D-aspatate
nmol/g	=	Nanomolar per gram

NO	=	Nitric oxide
NSS	=	Normal saline solution
p53	=	Tumor protein p53
PARP	=	Poly ADP ribose polymerase
PTZ	=	Pentylenetetrazole
ROS	=	Reactive oxygen species
rpm	=	Round per minute
S.E.M.	=	Standard error of the mean
sec	=	second
TBA	=	Thiobarbituric acid
TBARS	=	Thiobarbituric acid-reacting substances
US	=	Unconditioned stimulus
V	=	Volt
VPA	=	N-dipropylacetic acid, Valproic acid
VPU	=	N-(2-propylpentanoyl) urea
2VO	=	Two vessel occlusion
w/v	=	Weight per volume

CHAPTER I

INTRODUCTION

Ischemic stroke may results from a variety of causes that reduced cerebral blood flow (CBF) and led to deprivation of both oxygen and glucose. Brief periods of ischemia result in severe damage in selectively vulnerable regions of the brain (Leker and Neufeld, 2003). A sudden cessation of blood flow resulting in a loss of high energy compounds lead to depolarization of neuronal membranes and increase in extracellular glutamate and other amino acid (Hara, Sukamoto, and Kogure, 1993). Increased extracellular levels of glutamate, commonly seen following cerebral ischemia, over activates post-synaptic receptors resulting in an increase in intracellular calcium and generation of oxygen free radicals. The combination of several cascades rapidly leads to neuronal cell death.

Hippocampal CA1 and CA3 neuron are instrumental for learning and memory in rodents, nonhuman primates, and human (Mohajari *et al*., 2003). In rats, hippocampal lesion leads to an impairment of spatial learning (Morris *et al*., 1982). Furthermore, pyramidal neurons of the CA1 regions of the hippocampus display a selective vulnerability to transient global cerebral ischemia can be observed. Regarding to duration of ischemia, duration of 20 min has been reported to provide neuronal damage in the CA1 region which is developed within 48-72 hr following ischemia (Kirino, 1982). In addition, cell death also occurred in the striatum and cortex due to long duration of ischemia.

N-(2-propylpentanoyl) urea (VPU) is an analog of valproate (VPA) which has been reported to possess anticonvulsant activity higher than that of VPA in both the Maximal electroshock seizure (MES) and the pentylenetetrazole (PTZ) tests. In a study using brain microdialysis in anesthetize rats, it was found that VPU decreased level of glutamate, aspartate, GABA and glycine (Sooksawate, 1995). Further study by microiontophoretic technique demonstrated that VPU was able to depress spontaneous

firing of both neurons of cerebral cortex and cerebellum (Khongsombat, 1997). Recently, it was reported that VPU reduced hippocampal level of glutamate, decreased lipid peroxidation and restored mitochondria dysfunction in pilocarpine-induced seizure model (Khongsombat, 2004). Accordingly, neuroprotective effect of VPU against β amyloid in vitro study has been demonstrated (Chantong, 2001).

On the basis of similarity of the cascade seen in cerebral ischemia and epilepsy, several new antiepileptic drugs (AEDs) have been tested as possible neuroprotective agents in animal models of stroke. Interestingly, some new AEDs were shown to be promising in counteracting experimentally induced brain ischemia (Calabresi *et al*., 2003). Therefore, the present studies were aimed to investigate neuroprotective effect of VPU against impairment of learning and memory induced by common carotid artery occlusion. Furthermore, effects of VPU on lipid peroxidation and neuronal cell loss in hippocampus (CA1 and CA3 regions) were also undertaken.

RESEARCH QUESTIONS

1. Can VPU improve learning and memory deficits induced by transient cerebral ischemia?
2. Can VPU protect neuronal cell death induced by transient cerebral ischemia?
3. Can VPU reduce oxidative stress induced by transient cerebral ischemia?

HYPOTHESIS

1. VPU can improve learning and memory deficits-induced by transient cerebral ischemia.
2. VPU can protect hippocampal neurons from transient cerebral.
3. VPU can reduce oxidative stress induced by transient cerebral ischemia.

OBJECTIVE

1. To investigate the effect of VPU on learning and memory impairment induced by transient cerebral ischemia.
2. To investigate the effect of VPU on neuronal cell death induced by transient cerebral ischemia in CA1 and CA3 regions of hippocampus.
3. To investigate the effect of VPU on level of oxidative stress induced by transient cerebral ischemia.

CHAPTER II

REVIEW LITERATURE

2.1 Cerebral ischemia

2.1.1. Cerebral ischemia

Cerebral ischemia resulted from a transient or permanent reduction in cerebral blood flow that is restricted to major brain artery. The reduction in flow is, in most cases, caused by the occlusion of cerebral artery either by an embolus or by local thrombosis. The major pathogenic mechanisms of this cascade include excitotoxicity, peri-infarct depolarization, inflammation and programmed cell death (Dirnagl, Iadecola, and Moskowitz, 1999).

Brain tissue has a relatively high consumption of oxygen and glucose, almost exclusively used in an oxidative phosphorylation for energy production. Impairment of blood flow restricts the delivery of substrates, oxygen and glucose, and impairs the energy required to maintain ionic gradients. With energy depletion, membrane potential is lost and depolarization of neuron and glia occur. Consequently, somatodendritic as well as presynaptic voltage-dependent Ca^{2+} channels become activated and excitatory amino acids are released into the extracellular space (White *et al*., 2000). At the same time, the energy-dependent processes, such as presynaptice reuptake of excitatory amino acids, are impeded, which further increases the accumulation of glutamate in the extracellular space. Activation of NMDA receptors and metabotropic glutamate receptors, via phospholipase C and IP_3 signaling, contribute to Ca^{2+} overload. As a result of glutamate-mediated over activation, Na^+ and Cl^- enter the neuron via channels for monovalent ions such as AMPA. Water follow passively, as the influx of Na^+ and Cl^- is much larger than the efflux of K^+. The ensuring edema can affect the perfusion of regions surrounding the core of the perfusion deficit negatively, and also have remote effects that are produced via increased intracranial pressure. Brain edema, which gives

rise to the earliest markers for the ensuing pathophysiology, studied with MRI and CT scan, is the one of major determinants of whether the patient survives beyond the first few hours after stroke (Moro *et al*., 2004).

An increase in the universal second messenger, Ca^{2+}, is thought to initiate a series of cytoplasmic and nuclear events that impact the development of tissue damage profoundly, such as activation of proteolytic enzymes that degrade cytoskeletal proteins, extracellular matrix proteins. Activation of phospholipase A_2 and cyclooxygenase generates free radical species that overwhelm endogenous scavenging mechanisms, producing lipid peroxidation and membrane damage (Adibhatla and Hatcher, 2006). The important role of oxygen free-radicals in cell damage associated with stroke is underscored by the fact that even delayed treatment with free radical scavengers can be effective in experimental focal cerebral ischemia. Oxygen free radicals also serve as important signaling molecules that trigger inflammation and apoptosis. Nitric oxide (NO) synthesized by the Ca^{2+}-dependent enzyme, neuronal nitric oxide synthase (NOS) reacts with a superoxide anion to from the highly reactive oxygen species, peroxynitrite, which promotes tissue damage (Iadecola, 1997).

In transient cerebral ischemia, when blood flow is restored (reperfusion), oxygen can enhance the biochemical reactions that generate free radicals in the cytosolic compartments or subcellular organelles and mitochondria. Moreover, during reperfusion, the endogenous antioxidative defense is likely to be perturbed as a result of overproduction of oxygen radicals (Chan, 2001). Another component that contributes to cell damage is inflammation. In inflammatory phase, endothelial adhesion receptors are upregulated and white cells adhere to the wall of blood vessels, invade the parenchyma and release cytotoxic cytokines, such as tumor necrocis factor, interleukin(IL) 1, and IL6 (De Keyser, Sulter, and Luiten, 1999).

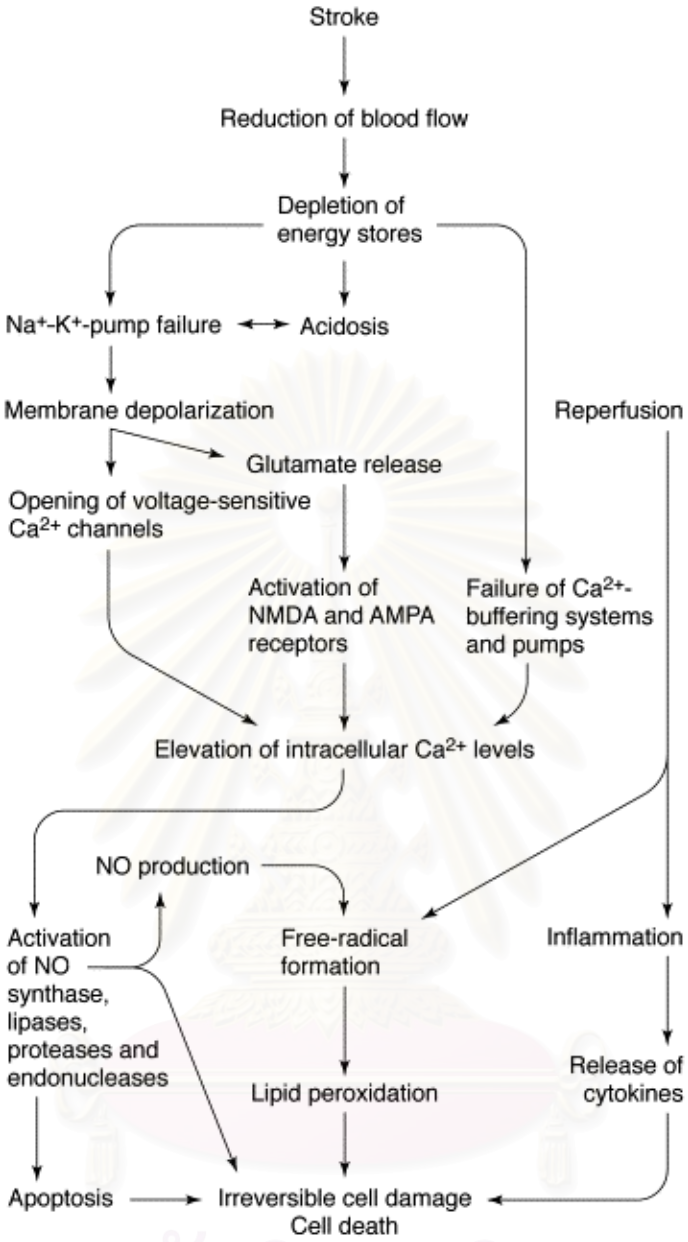

Figure 2.1 Neurotoxic cascades in ischemic brain injury (De keyser, Sulter, and Luiten, 1999).

2.1.1.1 Excitotoxic

Glutamate has an important physiological role as a neurotransmitter in the brain. It acts postsynaptically at several receptor types that are named for their prototypic pharmacological agonists. One major subtype of glutamate receptor involved in fast

synaptic excitation is the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid) receptor, a ligand-gated cation channel. Another glutamate receptor is the *N*-metyl-*D*-aspatate (NMDA). This receptor is crucial to many forms of a process known as excitotoxicity, during which the inability to respond properly to elevations in synaptic concentrations of glutamate overexcites neurons, It can cause acute cell death (necrosis) but can also initiate molecular events that lead to a delayed type of cell death, apoptosis. Glutamate is capable of activating multiple receptor systems. A crucial component of excitotoxicity is mediated through the NMDA receptor, which allows high levels of calcium entry. Such calcium selectively activates a variety of downstream events, many of which are toxic to the cell when excessive levels of activation are reached as show in Figure 2.2 (Lynch and Guttmann, 2002).

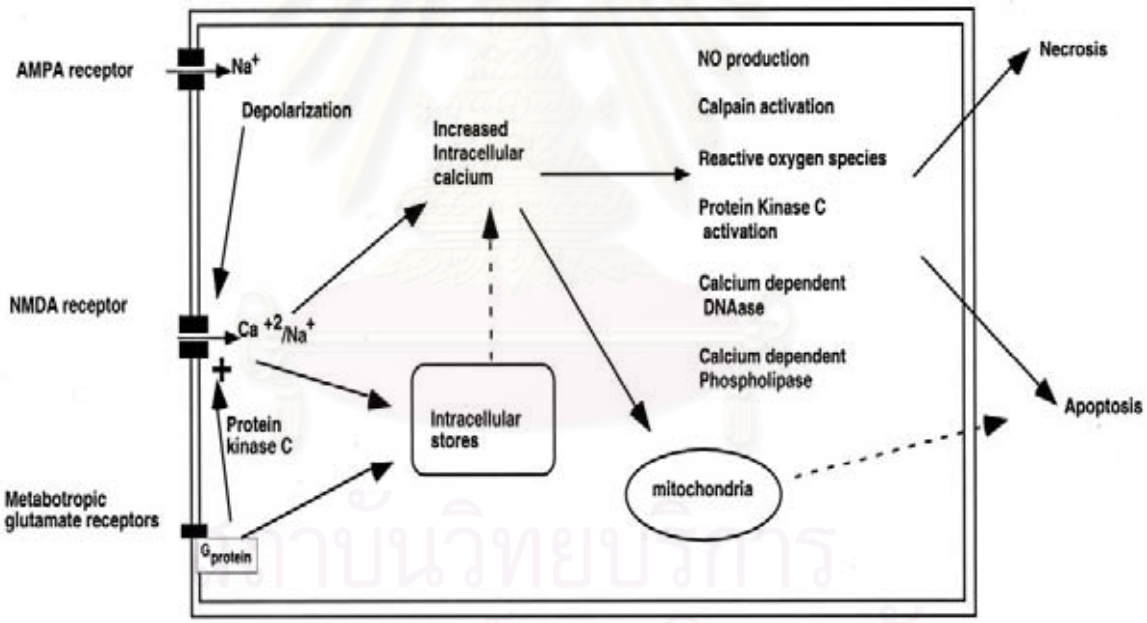

Figure 2.2 Excitotoxicity, glutamate receptors, and multiple messenger systems (Lynch and Guttmann, 2002)

In addition, the intracellular signaling pathways activated during excitotoxicity trigger the expression of genes that initiate post-ischemic inflammation, another pathogenic process that contributes to ischemic injury. Thus, excitotoxicity is a prime

target for stroke therapy. The therapeutic efficacy of strategies that inhibit excitotoxicity *in vivo* and *in vitro*, which, at present, focus on the inhibition of specific glutamate receptors, provides evidence for the pathophysiological role of excitotoxicity (Dirnagl, Iadecola, and Moskowitz, 1999).

2.1.1.2 Peri-infarct depolarization

Ischemic neurons and glia depolarize owing to the shortage of energy supply, and the release of K^+ and glutamate. In the core region of the affected brain tissue, cells can undergo an anoxic depolarization and never repolarize. In penumbra regions cell can repolarize, but at the expense of further energy consumption. The same cell can depolarize again in response to increasing glutamate or K^+ levels, or both, which accumulate in the extracellular space. Repetitive depolarization so call peri-infarct depolarizations occurs. When the number of depolarization increases, the infarcts grow larger (Dirnagl, Iadecola, and Moskowitz, 1999).

2.1.1.3 Inflammation

A phase of inflammation, which is characterized by migration of neutrophill leukocytes into the tissue, activation of glia cells secretion of potentially cytotoxic substance by these inflammatory cells, is also involved in the pathophysiology of cell death following global ischemia. In response to the regions of selective neuronal damage. Activation of microglia and astrocytes precedes neuronal damage, it synthesize and secrete the proinflammatory cytokines interleukin and tumor necrosis factor and activate the isoform of inducible nitric oxide synthase which all can be neurotoxic (Clark *et al*., 1995).

2.1.1.4 Apoptosis

Brain cells that are compromised by excessive glutamate-receptor activation, Ca^{2+} overload, oxygen radicals or by mitochondrial and DNA damage can die by

necrosis or apoptosis. This depends on the nature and intensity of the stimulus and type of cell (Leist and Nicotera, 1998). Necrosis is the predominant mechanism that follows acute, permanent vascular occlusion, whereas in milder injury, cell suicide becomes unmarked, particularly within ischemic penumbra. In fact, apoptosis may be responsible for up to 50% of cellular death in ischemia (Choi, 1996). Both extra- and intracellular signals that have been reported to initiate this process after brain injury were identified. The mechanisms leading to apoptotic death in brain injury are intricate and involve several possible pathways including a nuclear factor kB (NFkB) dependent pathway, a p53 dependent pathway and activation of inducible proapoptotic members of the bcl family (Bonfoco *et al.,* 1995, Clemens *et al.,* 1997, Matsushita *et al.,* 1998). Induction of these factors leads to the formation of the caspases, which are present in cells as proenzymes and are cleaved to their active from by other caspases (Thornberry and Lazebnik, 1998). The caspase 3 in turn activates DNA breaking enzymes and energy consuming DNA repair enzyme such as PARP, leading eventually to breakdown of DNA and cell death (Namura *et al*., 1998).

2.1.2 Common pathologic mechanisms in cerebral ischemia and epileptic seizures

Synaptic and cellular events initiated by acute energy deprivation caused by brain ischemia have been shown to be similar to those triggered by abnormal neuronal discharge induce by epilepsy. In both these pathological conditions, an acute membrane deporalization is caused by postsynaptic Na^{+} and Ca^{2+} influx via voltage-operated channels as well as via ligand-gated channels. A compromised energy supply occurs not only during brain ischemia but also when the hyperactive neuron is maintained in a sustained depolarized state by the epileptic discharge. The result of this altered metabolic state is the inhibition of the Na^{+}-K^{+} pump and rundown in transmembrane ion gradients. After the failure of the pump, the slow increase of the extracellular potassium concentration cause a further depolarization of the neuron. Not only does the process involve postsynaptic neuronal element, but also affects presynaptic nerve terminals and glia cells. Consequently, excitatory amino acids are

massively released into extracelluler space after either ischemia or sustained paroxymal neuronal activity. At the same time, the energy dependent processes are impeded, which further increases the accumulation of glutamate in the extracellular space. Activation of NMDA receptors and metabotropic glutamate receptors contributes to the calcium overload. This latter event may initiate a cascade of cytoplasmatic and nuclear events that generate tissue damage such as cell swelling, activation of proteolytic enzymes that degrade cytoskeletal proteins, generation of free radicals, increased nitric oxide production, impaired mitochondrial activity and possibly apoptosis (Calabresi *et al*., 2003, Leker and Neufeld, 2003).

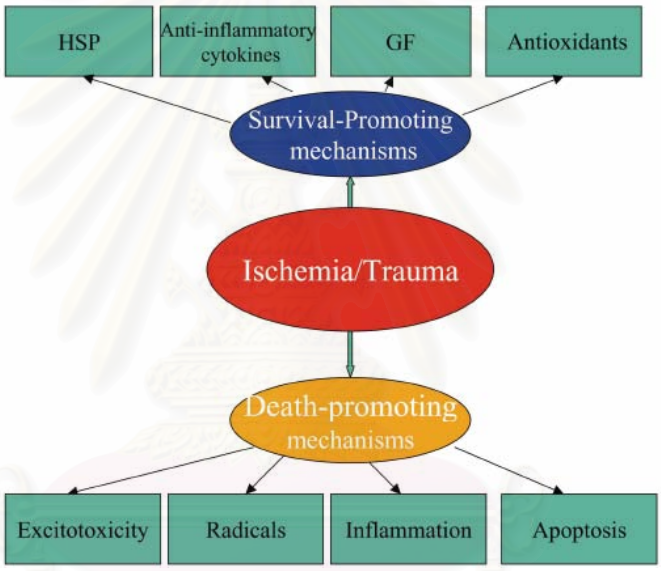

Figure 2.3 Endogenous protective and damaging mechanism triggered after ischemic and epileptic brain injury. Brain injury triggers pathological pathways that may potentially harm brain cells. These mechanisms include excitotoxicity, formation of free radicals, inflammation and apoptosis among others. Auto-protective mechanisms are also induced by brain injury including formation of heat shock proteins (HSPs), anti-inflammatory cytokines, growth factors (GFs) and endogenous antioxidants. The balance between harming and protective mechanisms employed will ultimately determine the fate of the injured brain. (Leker and Neufeld,2003).

Interestingly an excessive release of excitatory amino acids and a reduced neuronal inhibition occurs in both of epilepsy and brain ischemia. Thus, recently the use of antiepileptic drugs (AEDs) as a possible neuroprotective strategy in brain ischemia is receiving attention, and many AEDs have been tested in animal models of stroke. Experimental studies utilizing global or focal ischemia in rodents have provided insights into the possible neuroprotactive action of the various AEDs. Studies of some antiepileptic drugs such as felbamate that has both glutamate antagonist and GABA agonist properties (Shuaib *et al.,*1996), Fosphenytoin (Chan *et al.,*1998), topiramate which has a broad spectrum antiepileptic activities (Yang *et al.,* 1998), levetiracetam that has anticonvulsant and antiepileptogenic effects (Hanon and Klitgaard, 2001) provide encouraging results.

2.2 Learning and memory

2.2.1 Learning

Learning refers to the process by which experiences change our nervous system and hence our behavior. We refer to these changes as memories. There are two types of learning: (1) nonassociative learning and (2) associative learning.

2.2.1.1 Nonassociative learning

Nonassociative learning describes the change in the behavioral response that occurs over time in response to a single type of stimulus. There are two types: habituation and sensitization. Habituation is a decrease in response to a benign stimulus when that stimulus is presented repeatedly. Sensitization is and enhanced response to a wide variety of stimuli after the presentation of an intense or noxious stimulus (Kandel, Schwaetz, and Jessell, 2000).

2.2.1.2 Associative learning

During associative learning, we form associations between events. Two types are usually distinguished: classical conditioning and instrumental conditioning.

Classical conditioning involves associating a stimulus that evokes a measurable response with a second stimulus that normally does not evoke this response. For example of classical conditioning is the salivation of a conditioned dog in response to a stimulus of sound. Normally, the dog salivates only in response to meat. After a sound of bell stimulus (the conditioned stimulus or CS) has been paired with the presence of a piece of meat (the unconditioned stimulus or US) in a sufficient number of conditioning trials, the sound alone is sufficient to cause the dog to salivate (salivation is the conditioned response or CR)(Bear, Connors and Paradiso, 2007).

In instrumental conditioning an individual learns to associate a response, a motor act, with a meaningful stimulus, typically a reward such as food. As an example, consider what happens when a hungry rat is placed in a box with a lever that dispenses food. In the course of exploring the box, the rat bumps the lever, and out pops a piece of food. After this happy accident occurs a few more times, the rat learns that pressing the lever lead to food reward.

As in classical conditioning, a predictive relationship is learned during instrumental conditioning. In classical conditioning, the subject learns that one stimulus (CS) predicts another stimulus (US). In instrumental conditioning, the subject learns that a particular behavior is associated with a particular consequence. Also like classical conditioning, timing is important. Successful instrumental conditioning requires the stimulus to occur shortly after the response (Bear, Connors and Paradiso, 2007).

2.2.2 Memory

The brain has numerous systems for performing functions related to sensation, action, and emotion, and each system contains billions of neurons with enormous numbers of interconnection. Memories are caused by changes in the sensitivity of synaptic transmission between neurons as a result of previous neural activity. This change in turn causes new pathways or facilitated pathways to develop for transmission of signals through the neural circuits of brain. The new or facilitated pathways are call memory traces. Memories are frequently classified according to the

type of information that is stored. One of these classifications divides memory into declarative memory and non declarative memory, as follows (Lynch, 2004).

2.2.2.1 Declarative (explicit memory)

Refers to the acquisition of facts, experiences, and information about event. It is a memory that is directly accessible to conscious awareness and can be declared. Declarative memory is relatively fast and flexible. Fact-based information, for example, can usually be expressed quickly by a number of different response systems. Declarative memory however, is not always reliable, as is evident in everyday problems with retrieval of information and forgetting. Declarative memory can be further sub-divided into semantic memory and episodic memory (Widmaier, Raff, and Strang, 2004).

Semantic memory refers to general knowledge of the world that is not linked to a particular temporal or spatial context. It includes our memory of the meanings of words–the kind of memory that lets us recalls. Its content is thus abstract and relational and is associated with the meaning of verbal symbols. Since it is a form of reference memory that contains information accumulated repeatedly throughout our lifetimes, semantic memory is usually spared when people suffer from amnesia, but it can be affected by some forms of dementia.

Episodic memory refers to information learned at a particular place and time in one's life. Asking an individual to recall what he or she ate for breakfast that morning, or what words were presented on a list he or she heard earlier all tap into episodic memory. To recall the target information correctly, the individual must be able to access information regarding the time place of the original event (Delis, Lucas, and Kopelman, 1999).

2.2.2.2 Non declarative (implicit memory /procedural)

Refers to various form of memory that is not directly accessible to consciousness. These include skill and habit learning, classic conditioning, the

phenomenon of priming, and other situations in which memory is expressed through performance rather than recollection. Non declarative memory is considered quite reliable but is often slow and inflexible. The information present in a learn skill, for example, can often be expressed most readily only by the response systems that were involved in the original learning of that skill (Milner, Squire, and Kandel, 1998).

Procedural memory refers to the process of retrieving information that underlies these skilled performances. The procedure is automatic and is performed without conscious attention to the mechanics involved. In fact, conscious attention to procedural information can often disrupt performance of skill. Furthermore, Priming is a phenomenon in which prior experience with perceptual stimuli temporarily and unconsciously facilitates the subject's ability to later detect or identify those stimuli (Widmaier, Raff, and Strang, 2004).

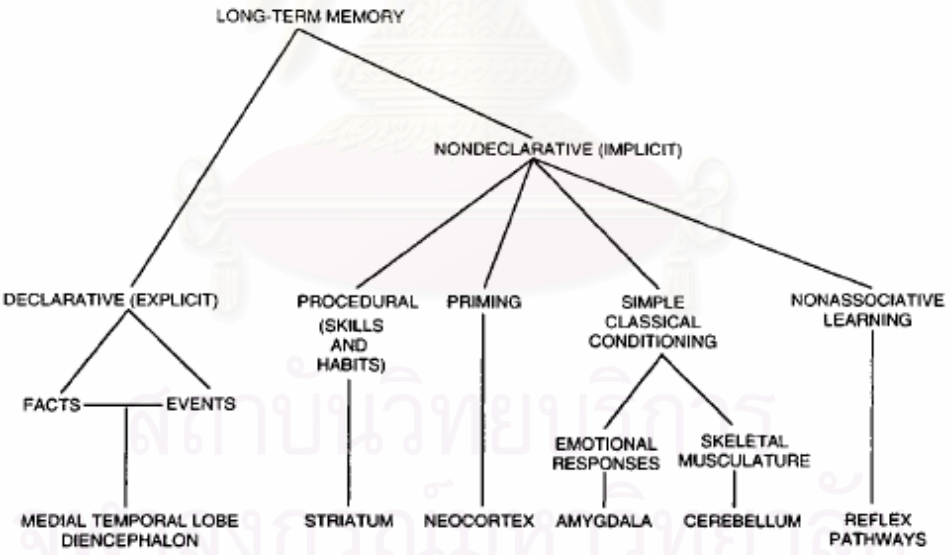

Figure 2.4 The taxonomy of mammalian memory system (Milner, Squire, and Kandel, 1998)

2.2.3 Process of memory

According to Kandel, Schwaetz, and Jessell (2000), memory is the process by which that knowledge of the world is encoded, consolidation, stored, and retrieved.

Encoding refers to the process by which newly learned information is attended to and processed when first encountered. The extent and nature of this encoding are critically important for determining how well the learn material will be remembered at later time. For a memory to persist and be well remembered, the incoming information must be encoded thoroughly and deeply. This is accomplished by attending to the information and associating it meaningfully and systematically with knowledge that is already well established in memory so as to allow one to integrate the new information with that one already knows. Memory storage is stronger when one is well motivated (Delis, Lucas, and Kopelman, 1999).

Consolidation refers to those processes that alter the newly stored and still labile information so as to make it more stable for long-term storage. Consolidation involves the expression of gene and synthesis of new proteins, giving rise to structural changes that store memory stably over time (Kandel, Schwaetz, and Jessell, 2000).

Storage refers to the mechanism and site by which memory is retained over time. One of the remarkable features about long-term storage is that it seems to have and almost unlimited capacity.

Retrieval refers to those processes that permit the recall and use of the stored information. Retrieval involves bringing together different kind of information that is stored separately in different storage sites. Retrieval of memory is much like perception; it is a constructive process and therefore subject to distortion, much as perception is subject to illusion. Retrieval of information is most effective when it occurs in the same context in which the information was acquired and in the presence of the same cues that were available to the subject during learning (Anderson, 1995).

Additionally, the term spatial memory is the part of memory responsible for recording information about one's environment and its spatial orientation. For example, a person's spatial memory is required in order to navigate around a familiar city, just as a rat's spatial memory is needed to learn the location of food at the end of a maze. It is often argued that a person's or an animal's spatial memories are summarized in a cognitive map. In general, mammals require a functioning hippocampus (particularly area CA1) in order to form and process memories about space. There is some evidence that human spatial memory is strongly tied to the right hemisphere of the brain (Tucker *et al*., 1999; Nunn *et* al., 1999). Spatial learning requires both NMDA and AMPA receptor, consolidation requires NMDA receptors, and the retrieval of spatial memories requires AMPA receptors (Liang *et al*., 1994). Because many people process spatial information using the right side of their brain, damage to the right side of the brain can often cause impairments in spatial memory and in the ability to learning and process spatial information. Usually, damage limited to the left side of the brain cause little disruption in spatial (Tucker *et al*., 1999; Nunn *et* al., 1999).

2.2.4 Amnesia

Amnesia is a severe disruption of memory without deficits in intelligence, attention, perception or judgment. It may occur following damage, such as chronic alcoholism, encephalitis, brain tumor, and stroke, to any of several brain structures which are critical for mamory. Amnesia can take 2 forms that are retrograde and anterograde (Joseph, 1996).

2.2.4.1 Anterograde amnesia

Anterograde amnesia is a selective memory deficit, resulting from brain injury, in which the individual is severely impaired in learning new information. Memories for events that occurred before the injury may be largely spared, but events that occurred since the injury may be lost. In practice, this means that an individual with amnesia may have good memory for childhood and for the years before the injury, but may remember little or nothing from the years since. Anterograde amnesia can occur

following damage to at least three distinct brain areas. The hippocampus seems to act as a gateway through which new fact information must pass before being permanently stored in memory. If it is damaged, no new information can enter memory - although older information which has already passed through the gateway may be safe (Joseph, 1996).

2.2.4.2 Retrograde amnesia

Retrograde amnesia is a form of amnesia resulting from brain injury in which the individual looses memories for the time period just prior to the injury. This time period may stretch from a few minutes to several years, and typically it is worst for event which occurred just before the injury (Bear, Connors and Paradiso, 2007).

2.3 Cerebral ischemia model

The goal of cerebral ischemia model is to reduce oxygen and glucose supply to brain tissue. This process produces brain injury via variety of cellular and molecular mechanisms that impair the energy required to maintain ionic gradients. The mechanisms involve a complex series of pathophysiology events that are dependent on the severity, duration, and location of ischemia within the brain. Cerebral ischemia experimental models are characterized as global, focal, and multifocal ischemia. Global ischemia occurs when cerebral blood flow (CBF) is reduced throughout most or all of brain, whereas focal ischemia is represented by a reduction in blood flow to a very distinct, specific brain region. In multifocal ischemia, there are is patchy pattern of reduced CBF (Traystman, 2003).

Global cerebral ischemia in rodent is an established model in experimental research on cerebral ischemia which is characterized morphologically by a selective neuronal damage in the hippocampus, striatum and cortex. At present, there are three experimental models which are commonly used: (1) the four-vessel occlusion (4VO) where the vertebral arteries are permanently occluded and the carotid arteries are clamped transiently for 10-30 min, (2) the two vessel occlusion plus hypotension (2VO+hypo) which is produced by transient occlusion of both common carotid arteries

and withdrawal of blood, (3) In gerbils a transient occlusion of both common carotid arteries (2VO) because these animals display an incomplete circle of Willis (Block, 1999).

With respect to histopathology, blood flow and metabolism of the three main models have been thoroughly characterized. Selective neuronal damage can be observed in the CA1 sector of the hippocampus lead to an impairment of spatial learning and memory. It develops within 48-72 hr following ischemia and is thus termed delayed neuronal death, in rats ischemia for 20 min has result to increase neuronal damage in CA1 sector. Furthermore, following ischemia of 20 or 30 min duration neuronal damage can also be observed in the striatum and cortex (Block, 1999).

The two-vessel (2VO) model was initially used to characterize cerebral energy state following incomplete ischemia. In this model, bilateral common carotid artery occlusion is coupled with systemic hypotension to produce a reversible ischemia. Furthermore, in this model, both of the ischemia and reperfusion which has ischemic/reperfusion injury to neurons are almost immediate. Moreover, following reperfusion hypoperfusion has been demonstrated. With this model, injury occurs within selectively vulnerable areas such as the CA1 of hippocampus, caudoputamen, and neocortex (Traystman, 2003).

2.4 The Morris Water Maze (MWM)

The Morris water maze, a commonly used test of spatial memory, was devised by Richard Morris about 20 years ago. It developed to test abilities to learn, remember and go to the place in space defined only by its position relative to cues (Block and Schwarz, 1998). This spatial version of the task is dependent on the hippocampus; it hippocampal biochemical mechanisms have been partly identified. It must be borne in mind that even in the spatial version of this task there is a procedural learning embedded. The learning of where to escape to is declarative information that hinges on

or is superposed upon the swimming-to-escape knowledge (Rossato *et al*., 2006). In this test, animal is placed in pool filled with water. Submerged just below the surface in one location is a small platform that allows animal to escape. Typically, an animal learns to escape from the water by locating the hidden platform with the help of visual cues around the pool. The relative simplicity of the MWM task is undoubtedly one of the reasons for its continuing success. The MWM task has often been used in the validation of rodent models. Through its much application, MWM testing gained a position at the very core of contemporary neuroscience research (D'Hooge and De Deyn, 2001).

2.5 Passive avoidance

One of the most common animal tests in memory research is the inhibition to imitate activities or learn habits. This task relies heavily on the dorsal hippocampus, where it uses a sequence of molecular events very remindful of those of long-term potentiation; but it also depends on the entorhinal and parietal cortex and is intensely modulated by the basolateral nucleus of the amygdala, in all of which it uses other sequences of molecular events (Rossato *et al*., 2006). The term passive avoidance is usually employed to describe experiments in which animal learns to avoid a noxious event by suppressing a particular behavior (Vohora, Pal, and Pillai, 2000). Step-down test is one of the most frequently used tests for passive avoidance. The test is usually based on electric current as source of punishment. In many tests, the floor of the apparatus is made up by a grid that can be electrified. After habituation to this apparatus the animal were placed into the platform for an acquisition trial and a foot shock is delivered as soon as the animal enters the grid, and 24 hr later the retention trial were performed. The latency to refrain from performing the punished act expresses the ability to avoid (Block, 1999).

2.6 Brain oxidative stress and lipid peroxidation

The role of oxygen free radicals and antioxidants in central nervous system (CNS) pathology is of major interest for many reasons. While accounting for only 2% of

the body weight, the brain uses 20% of the oxygen consumed by the resting body. Molecular oxygen is chemically a biradical with two unpaired electrons with equal spins in two antibonding orbitals. This has the consequence that oxygen primarily is reduced one step at a time, since reactions with paired electrons are forbidden. The results are the intermediates: superoxide anion radical, hydrogen peroxide and hydroxyl radical. Hydrogen peroxide is not a free radical but is still reactive, and intermediates are together often termed reactive oxygen species (ROS) (Marklund, 2005).

Oxidative stress has been defined as a disturbance in the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage. The brain consumes a large quantity of oxygen, making it particularly susceptible to oxidative stress. Natural formation of oxidant during mitochondrial electron transport, auto-oxidation of some neurotransmitter and initiation of events during hypoxia or ischemia, can result in oxidant formation and subsequent tissue damage (Warner, Sheng, and Batinic-Haberte, 2004).

There are numerous relatively stable small molecules and protein and DNA modifications formed in reactions with oxygen free radicals, which can be used as markers for oxidant injury. Peroxidative attack on polyunsaturated fatty acids results in the various classes of isoprostane, which are regarded as the most reliable markers of lipid peroxidation.

Malondialdehyde (MDA) is a late breakdown produce from peroxidized lipids and is generally detected as a reaction product with thiobarbituric acid (TBARS). The TBAR can be analyzed by spectrophotometry, fluorimetry and by HPLC with increasing specificity (Moore and Robert, 1998). A variety of other aldehydes and molecules can react with TBA, however, and MDA is also formed in prostanoid synthesis. The TBARS reaction therefore, has a limited specificity and utility in complex matrices such as tissue extracts, plasma and CSF.

2.7 Piracetam

Piracetam (2-oxo-1-pyrrolidine acetamide) is the most well-known nootropic agent. Nootropic refers to a class of drugs which have common properties including enhancement of learning and memory, enhancement of the resistance of the brain towards chemical and physical injuries, and a general lack of stimulant or sedative properties or other side effects. In clinical practice, nootropics are commonly used to treat brain disturbances caused by various chemical and physical agents, for the treatment of dementia and mild cognitive impairment, and as cerebroprotective agents in stroke (McDaniel, Maier, and Einstein, 2003).

Although much progress has been made in understanding the mechanism of action of piracetam since the early research, a unifying hypothesis has yet to receive widespread acceptance. Piracetam has no specific action at GABA receptor sites, on dopaminergic, serotenergic, adrenergic transmission, or any other known receptor, enzyme, or transporter system, with the exception of weak interaction at L-glutamate binding sites (Shorvon, 2001; Wischer *et al*., 2001). A multitude of biochemical effects have been observed in many systems after piracetam administration, but these are rarely consistent between experiments. Many different mechanisms have been suggested, but this is only further evidence that an exact mechanism is still not well established in the scientific community (Galeotti, Ghelardini, and Bartolini, 1996). There is also a large amount of experimental evidence which is in good agreement with the hypothesis that the effects of piracetam are due to increased membrane fluidity (Muller *et al*., 1997; Muller, Eckert, and Eckert 1999). Increased membrane fluidity would explain the improvement in membrane-bound functions after piracetam administration, including secondary messenger activity, ATP production and neurotransmission. In aged rodents, it has been observed that piracetam increases muscarinic, NMDA, and AMPA receptor density (Scheuer *et al*., 1999; Vaglenova and Vesselinov Petkov, 2001). Using animal model of cerebral ischemia it has been reported that pirecetam exert neuroprotective effects and improve Morris water maze performance (Tortiglione *et al*., 2002; Shang *et al*., 2006). Furthermore, piracetam has demonstrated the improvement

in passive avoidance task in both of pentylenetetrazole (PTZ) induced kindling and scopolamine induced amnesia rats (Pohle *et al*., 1997; Kumar *et al*., 2000).

2.8 N-(2-propylpentanoyl) urea (VPU)

VPU is a valproic acid analog which was synthesized by Boonard Saisorn and co-worker (1992). The structure of VPU modeled partially on barbiturate ring and VPA in the same molecule (Figure 2.5)

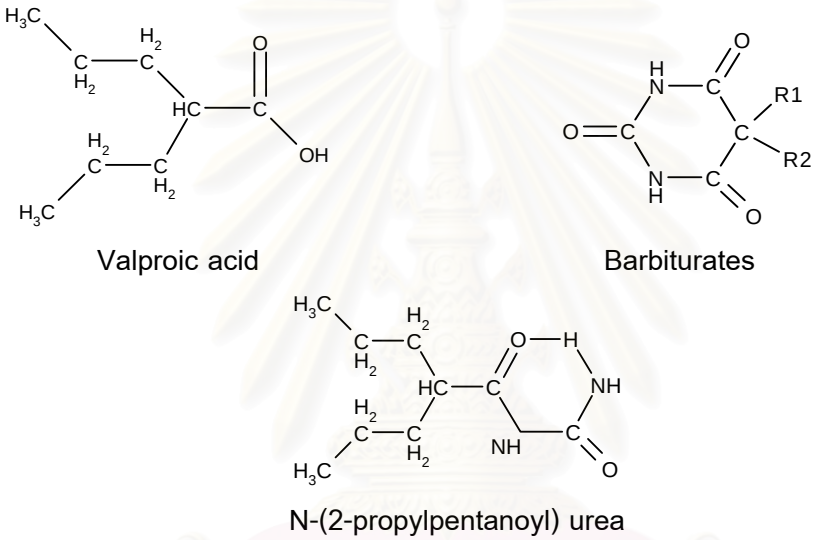

Figure 2.5 The structures of valproic acid, barbiturates, and VPU (modified from Saisorn, Patarapanich, and Janwitayanuchit, 1992)

VPU has been identified to possess an anticonvulsant activity. It demonstrated a higher protection than VPA in both of the MES and PTZ test, exhibiting an ED_{50} of 66 and 57 mg/kg respectively. Furthermore, VPU was found to produce less neurological side effects and higher protective index (9.5) than that of VPA (1.1) (Tantisira *et al*., 1997). Developmental toxicity with regards to effects on axial rotation and embryonic growth was lower in VPU-treated animals when compared with those of VPA-treated group. Furthermore, hepatotoxic effects of VPU were observed *in vivo* and *in vitro* only at large dose administration (Patchamart, 1996).

Study using microdialysis technique in anesthetized rats demonstrated that VPU decreased level of both cortical excitatory (aspatate and glutamate) and inhibitory (glycine and GABA) amino acid neurotransmitter. However, the depression was greatest on glutamate and least on glycine (Sooksawate, 1995). By microiontophoretic studies in anesthetized rats, it was found that VPU depressed spontaneous firing of both neurons of cerebral cortex and cerebellum (Khongsombat, 1997). *In vitro* study using beta amyloid protein-induced cytotoxicity in P19 embryonal carcinoma cells revealed neuroprotective effect of VPU as it was found to decrease percent cell death (Chantong, 2001).

Additionally, VPU at doses of 1-300 μM did not directly induce inward currents in acutely dissociated rat hippocampal pyramidal neuron. It was suggested that the potentiation of the $GABA_A$ currents by VPU may, at least in part, contributes to its mechanism of anticonvulsant action (Janthet, 2002). In 2003, Ponsup, by using the two-electrode voltage-clamp technique in *Xenopus laevis* oocytes, reported inhibitory effect, though rather weak, of VPU on NR1A/NR2B NMDA receptors subtype. Recently, Khongsombat (2004) reported the protection of VPU against pilocarpine-induced status epilepticus. VPU was found to reduce pilocarpine-induced increments of extracellular level of excitatory amino acid neurotransmitter, hippocampal neuronal damage and malondialdehyde (MDA) and restore mitochondria dysfunction by pilocarpine.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental animals

All experiments were performed on male ICR mice, weighing 25 - 30 g. All animal were purchased from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand.

Prior to testing, they were housed 10 mice per cage for one week in the Animal House of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. They were maintained under 12:12 light-dark cycle at a control temperature ($25 \pm 2^{0}C$). Standard food (C.P. Mice Food) and tap water were provided *ad libitum.*

All behavioral experiments were carried out in a room adjacent to that in which the mice were housed under the same conditions of temperature and humidity.

The experimental protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

3.2 Experimental instruments

1. Rotary Evaporater (Rotavapor R-114, Buchi)
2. pH meter (Sevenmulti, Switzerland)
3. Stop watches (Seiko)
4. Morris water maze set
5. Step down set
6. Locomotor activity set (UGO Basile, Comerico, Italy)

7. Automatic micropipette (Pipet-Lite™, U.S.A.)
8. Automatic mixer (Vertex, U.S.A.)
9. Homogenizer (Glas-Col, Terre Haute, U.S.A.)
10. Centrifugeter (Sorvoll, GLC-2B, U.S.A.)
11. Spectrophotometer (Shimadzu, UV1201, Japan)
12. Conical centrifuge tube (Nunc, Denmark)
13. Cryostat (Leica, Germany)
14. Slide and cover glass (China)

3.3 Drugs and chemicals

1. N-(2-propylpentanoyl) urea (VPU)

   VPU was kindly supplied by Assistance Professor Chamnan Pattarapanich (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand).
2. Piracetam (Sigma, USA)
3. Carboxy methyl cellulose sodium salt (Fluka, Finland)
4. Ethanol 95% (GPO, Thailand)
5. Chloroform (Lab-scan LTD, Ireland)
6. Methanol (Lab-scan LTD, Ireland)
7. Normal saline solution (Thai Nakorn Patana Co., Ltd., Thailand)
8. Nembutal (Sanifi-Synthelabo, Thailand)
9. Sodium hydrogen phosphate-2-hydrate (Ajex Finecham, Australia)
10. Sodium dihydrogen phosphate-2-hydrate (Ajex Finecham, Australia)
11. Acetic acid (Sigma, USA)
12. Sodium dodycyl sulfate (Sigma, USA)
13. Thiobarbitulic acid (Sigma, USA)
14. N-Butanol (Sigma, USA)
15. Pyridine (Sigma, USA)
16. 1,1,3,3-Tetraethoxy-propane (Malondialdehyde) (Sigma, USA)
17. Cresyl violet (Sigma, USA)

18. Xylene (TJ Baker, USA)

19. Permount (Sigma, USA)

20. Ethanol absolute (Merck, Germany)

3.4 Experimantal methods

3.4.1 Drug preparation and administration

0.5 % w/v Carboxy methyl cellulose (CMC) was used to suspend VPU and to dissolve piracetam. It was prepared by dissolving CMC in 40°c distilled water with the aid of magnetic stirrer. The doses of substances were expressed in milligram of substances per kilogram body weight (mg/kg B.W.). All tested substances were administered intraperitoneally (i.p.). The volumes of injection were 0.1 ml/25 g B.W.

3.4.2 Cerebral ischemia-induced learning and memory impairment

Mice were subjected to cerebral ischemia induced by 2VO plus hypotension (Figure 3.1). In brief, the mice were anesthetized with sodium pentobarbital (Nembutal sodium solution, 60 mg/kg B.W., intraperitoneal injection). Under deep anesthesia, the neck skin of mice was midline vertically incised. The common carotid arteries were exposed, carefully separated from the adjacent veins and vagus nerves, and then occluded by arterial clips. While the arteries were clamped, the tip of tail was cut and blood (0.3 ml) was withdrawn. After 20 min, the arterial clips were removed. The skin incision was closed and the mice were kept in an air-conditioned room at 25 °C. Sham-operated mice were subjected to the same procedure without arterial clamping and bleeding. Twenty four hour after the operation MWM were carried out (Xu *et al*., 2000; Watanabe *et al*., 2003).

Animal were divided into seven groups of at lease eight mice each as followed

1) Sham operated group administered with NSS
2) Sham operated group administered with 0.5% CMC
3) 2VO group administered with NSS
4) 2VO group administered with 0.5% CMC
5) 2VO group administered with VPU 50mg/kg B.W.
6) 2VO group administered with VPU 100mg/kg B.W.
7) 2VO group administered with piracetam 100mg/kg B.W.

To study the effect of VPU on impairment of learning and memory induced by transient cerebral ischemia all mice received tested substance by intraperitoneal injection for 8 consecutive days. Three behavioral tests, MWM test. Step-down test and test for spontaneous locomotor activity were performed. MWM was tested for 5 consecutive days. The step-down test was performed 6 days after 2VO. Spontaneous locomotor activity test 8 days after 2VO. After the behavioral tests the animal were sacrificed for estimation of lipid peroxidation and morphology study as shown in Figure 3.2.

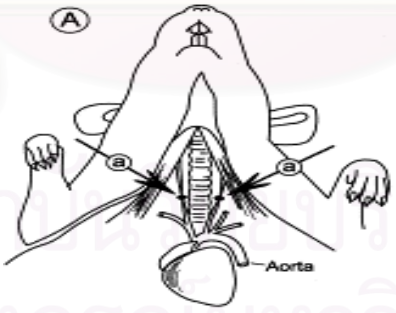

Figure 3.1 Experimental mice model of cerebral ischemia (bilateral common carotid artery occlusion, two vessel occlusion: 2VO)

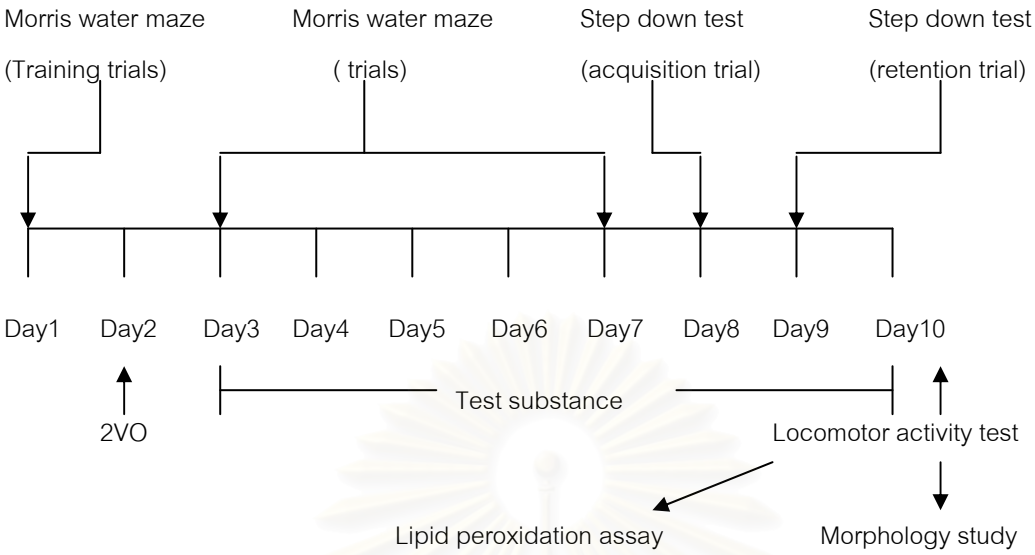

Figure 3.2 Diagram of experimental schedule

3.4.3 Behavior tests

3.4.3.1 Morris water maze (MWM)

After 24 hr of cerebral ischemia, the MWM was performed. The procedure used was modified from those described by Morris (1984). The MWM consisted of a black circular pool of water, 70 cm in diameter and depth 13 cm. Water in the pool was maintained at 25$\pm$ 1^{0}C. A platform (6 cm diameter) was situated 1 cm below the surface of the water. The pool was divided into four quadrants with platform in a fix position in one quadrant all the time. It was placed in a large room and surrounded by various visual cues in fix position as seen in Figure 3.3. Daily swimming consisted of four trials in which the mouse was placed into the water from four different starting points and the time to find platform (escape latency) were recorded and average 4 trials for each animal per day. In training day, if animal could not find platform the examiner would guide animal onto platform for 10 sec. However if the animal could not find the platform in all 4 trials point the animal were then excluded from experiments. After operation animals were tested again with an administration of tested substance 30 min before daily testing for 5 consecutive days. A maximum time point of

60 sec was allowed during which the mice had to find the platform and climb onto it for 15 sec (Watanabe *et al*., 2003).

Figure 3.3 Equipment for Morris water maze test

3.4.3.2 Step-down test

Step-down test is a test for passive avoidance. It was conducted using an apparatus of a chamber (Figure 3.4). The inside dimensions of the chamber are, length 35 cm; width 23 cm; and height 20 cm. The cage floor is made of evenly spaced stainless steel bars (3 mm diameter) that are space 11 mm apart, and a wooden platform (5cm diameter, 4 cm height) set on grid in one corner. Electric stimulation was given though the grid connected with scrambled shock generator (1 Hz, 1 ms, 36 V DC). Step-down experiment was started after 24 hr of the last MWM testing; Mice were placed in a box to get adapted to environment for 3 min without electric shock. Administration of tested substance was given 30 min before testing in which mice were placed in the box and electric shock was delivered. Upon electric shock mice escaped from the grid floor back onto the platform. The time that elapsed

until the mice stepped down from the platform was record as initial latency and error was counted as initial error whenever the mice stepped down from the platform. The duration of training test was 5 min and the shock was maintained during this period. Twenty-four hours after training, mice were placed on the platform for retention test. The electric shocks were again delivered for 5 min. The time that elapsed until the mice stepped down from the platform was record as retention latency and error was counted as retention error whenever the mice stepped down from the platform (Luo, Yin and Wei, 2003).

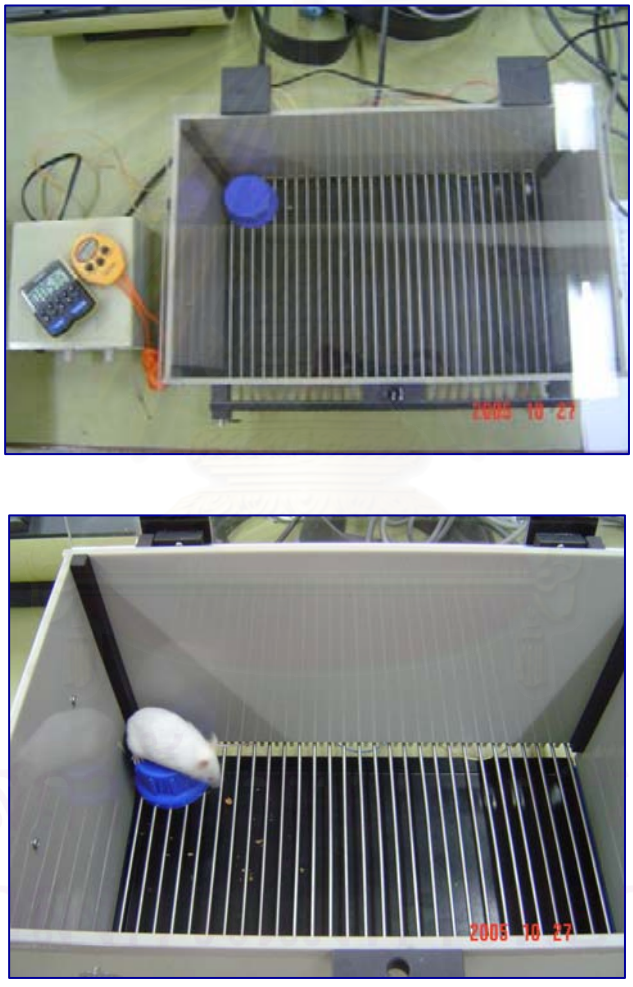

Figure 3.4 Equipment for step down test

3.4.3.3 Spontaneous locomotor activity test

Each animal was placed in an activity cage consisting of a chamber and counting device (Figure 3.5). The inside dimensions of the activity cage are, length 35 cm; width 23 cm; and height 20 cm. The cage floor is made of evenly spaced stainless steel bars (3 mm diameter) that are spaced 11 mm apart, connected to the circuit counting unit. The registered numbers or counts of movements were recorded at 5 min intervals. The apparatus was placed in light and sound attenuated, and ventilated testing room with other behavioral testing apparatus (Jain *et al.*, 2002; Gupta, Veerenda, and Srivastava, 2003).

Figure 3.5 Activity meter

3.4.4 Estimation of marker of oxidative stress

After the behavioral testing, the animals were decapitated and the brains were quickly removed, cleaned with ice-cold saline and store at -80^{o}C. There were at least eight mice per group.

3.4.4.1 Tissue preparation

Brain tissue samples were thawed and homogenized with 10 times (w/v) ice-cold 0.1 M phosphate buffer (pH 7.4). Aliquots of homogenates from mice brain were separated and used to determine lipid peroxidation.

3.4.4.2 Measurement of lipid peroxidation

MDA, a measure of lipid peroxidation, was measured. The reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulphate (8.1%) were added to 0.1 ml of processed tissue samples and then heated at 100^{0}C for 60 minutes. The mixture was cooled under tap water and 5 ml of *n*-butanol:pyridine (15:1) and 1 ml of distilled water were added and then vortexed vigorously. After centrifugation at 2500 rpm for 20 minutes, the organic layer was separated. Supernatant were collected and absorbance was measured at 532 nm using a spectrophotometer (Gupta, Veerenda, and Srivastava, 2003). The concentration of MDA was expressed as nmol/g tissue.

3.4.4 Morphology study

After the behavioral testing the animals, containing at least four mice per group, were subjected to morphological evaluation for the neuronal damage in hippocampal formation (CA1 and CA3). It was evaluation by a Cresyl violet staining technique. The mice were decapitated the whole brains were removed and quickly frozen in dry ice. Subsequently, coronal sections (10μm thick) were cut at the level of the hippocampus (1.5-mm. caudal to the bregma) by using a cryostat and stained with 1% Cresyl violet for a microscopic observation. The CA1 and CA3 subfields of hippocampus were photographed (X40), and then the number of pyramidal cell per 0.068 mm^2 in CA1 and CA3 subfields (Figure 3.6) was counted. Only the neuron with a distinct nucleus was counted as an undamaged cell. Average surviving cell numbers were counted over consistent fields, over both hemisphere, and three sections in each

brain. The degree of neuronal cell damage at the selective hippocampal CA1 and CA3 area (Figure 3.7) was expressed as the density of surviving CA1 and CA3 pyramidal cells according to the following equation: Density = the number of surviving CA1 or CA3 pyramidal cells / the area of CA1 or CA3 region (0.068mm^2) (Ni *et al.*,1995; Nanri *et al.*,1998).

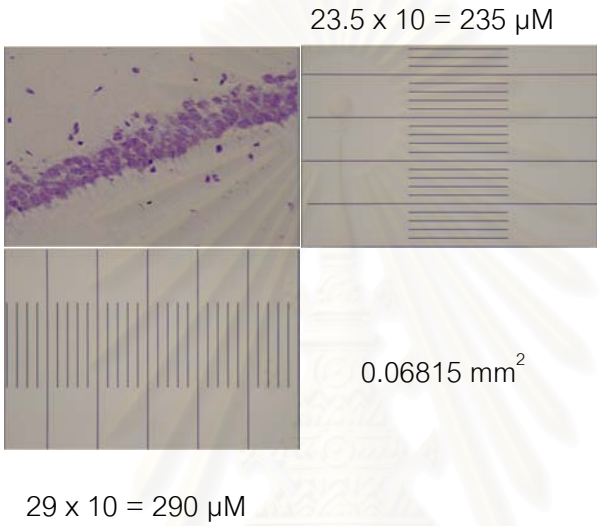

Figure 3.6 The photographed (X40) of CA1 regions of hippocampus measure with micrometer (X40).

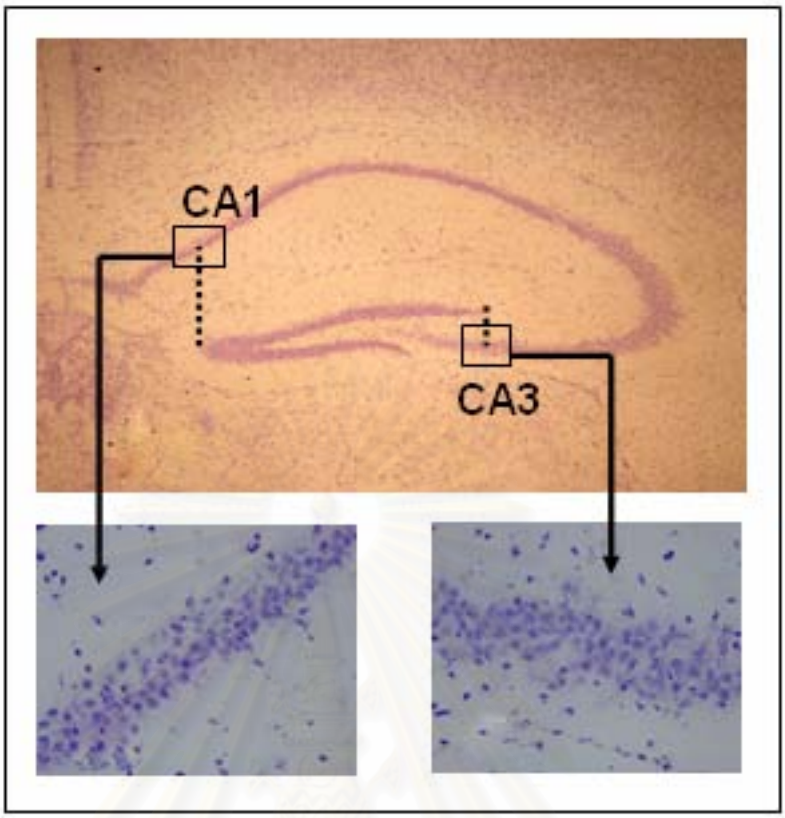

Figure 3.7 Illustration of area selected for the evaluation of survival neurons in CA1 and CA3 regions of hippocampus. Tip and tail of dentate gyrus (DG) was used to allocate the CA1 and CA3 respectively

3.5 Data analysis

The data of behavioral, biochemical test and morphology study are represented as mean value for the group ± standard error of the mean (S.E.M). The differences between various groups were assessed by one-way ANOVA followed by Duncan post-hoc test. A difference with $P < 0.05$ was considered to be statistical significance.

CHAPTER IV

RESULTS

4 Effects of VPU on impairment of learning and memory induced by cerebral ischemia.

4.1 Effects of 2 VO on spatial learning and memory performance.

The MWM performance in 2VO and sham-operated mice as measured by latency to reach the hidden platform during 5 consecutive days was summarized in Figure 4.1. The mean search times to find the platform on the training day between those of 2VO were 39.89 $\pm$ 2.10 and 38.00 $\pm$ 2.71 sec (treated with NSS and CMC respectively) and sham-operated mice were 33.28 $\pm$ 3.71 and 33.27 $\pm$ 3.45 sec (treated with NSS and CMC respectively) did not differ. During days 1-5, mice subjected to 2VO required longer time to locate the hidden platform than sham-operated mice. The escape latencies of 2VO on day 5 were 33.39 $\pm$ 4.07 and 28.00 $\pm$ 3.55 sec (treated with NSS and CMC respectively) and sham-operated mice 11.72 $\pm$ 3.10 and 6.73 $\pm$ 0.71 sec (treated with NSS and CMC respectively).

4.2 Effects of VPU on spatial learning and memory performance in 2VO mice.

Twenty four hours after cerebral ischemia the MWM test was performed. VPU (either 50 or 100 mg/kg B.W.) was given intraperitoneal injection to animal 30 minutes before testing. The administration of VPU 50 mg/kg B.W. or piracetam 100 mg/kg B.W. significantly improved learning and memory performance on day 5. On day 5 the escape latencies of VPU treated mice at doses of 50 and 100 mg/kg B.W. were 16.53 $\pm$ 3.61 and 23.50 $\pm$ 1.44 sec, whereas it was 12.93 $\pm$ 1.44 sec in piracetam treated group (Figure 4.2)

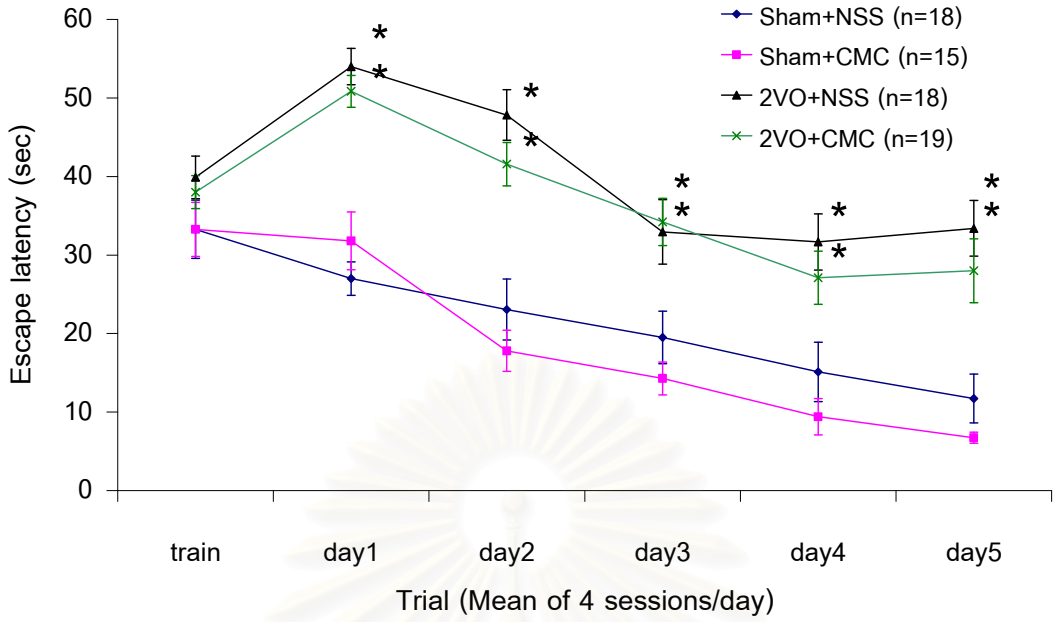

Figure 4.1 The MWM performance of 2VO - and sham - operated mice. The escaping latency onto the platform was measured on 5 consecutive days. Each data point represents the mean ± S.E.M. of four trials. Significant difference was assessed with one-way ANOVA followed by Duncan post-hoc test

* *P*< 0.05 denotes statistically significant difference from sham-operated mice treated with CMC

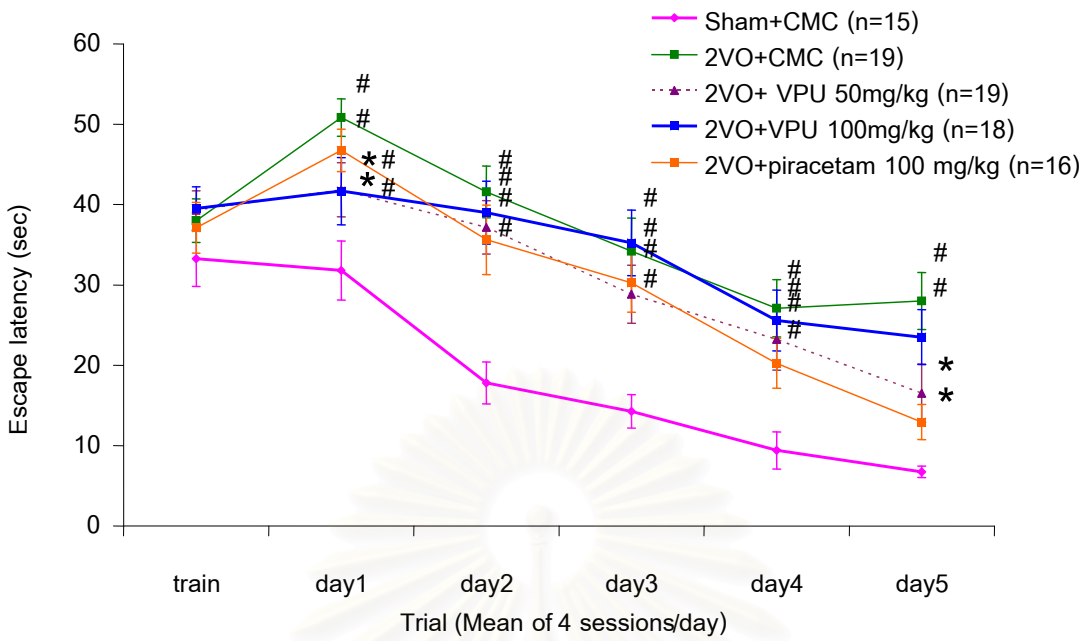

Figure 4.2 Effects of VPU on 2VO-induced disruption of memory in the MWM performance. Mice received vehicle and VPU at doses of 50, 100 mg/kg B.W. and piracetam 100 mg/kg B.W. once daily. The escaping latency onto the platform was measured in 5 consecutive days. Each data point represents the mean ± S.E.M. of four trials. Significant difference was assessed with one-way ANOVA followed by Duncan post-hoc test

\# $P < 0.05$ denotes statistically significant difference from sham-operated mice

* $P < 0.05$ denotes statistically significant difference from 2VO-operated mice

4.3 Effects of 2VO on step-down passive avoidance in mice.

Impairment of learning and memory in step-down test was observed after cerebral ischemia. 2VO caused a significant reduction in both step-down latency and increase in the number of errors on retention trial. Step-down latencies were 147.33 $\pm$ 126.00 and 187.53 $\pm$ 28.65 sec in sham-operated mice (treated with NSS and CMC respectively). In 2VO mice, they were 34.83 $\pm$ 18.57 and 26.47 $\pm$ 15.59 sec (treated with NSS and CMC respectively). Number of errors were 2.17 $\pm$ 0.50 and 1.67 $\pm$ 0.53 in sham-operated mice (treated with NSS and CMC respectively) and they were 5.00 $\pm$ 1.14 and 5.26 $\pm$ 0.78 (treated with NSS and CMC respectively) as shown in Figure4.3.

4.4 Effects of VPU on step-down passive avoidance in 2VO mice.

Similar to piracetam (100mg/kg B.W.), treatment with VPU at 50 and 100 mg/kg B.W., i.p., significantly reversed the reduction in step-down latency and number of errors of 2VO mice in retention trial as shown in Figure 4.4. In VPU treated mice step-down latency was 127.58 $\pm$ 26.00 and 197.00 $\pm$ 28.09 sec and numbers of errors were 2.53 $\pm$ 0.47 and 1.72 $\pm$ 0.61 for the doses of 50 and 100 mg/kg B.W., respectively. In piracetam treated group, step-down latency and number of errors were 105.86 $\pm$ 21.32 sec and 2.36 $\pm$ 0.45 respectively.

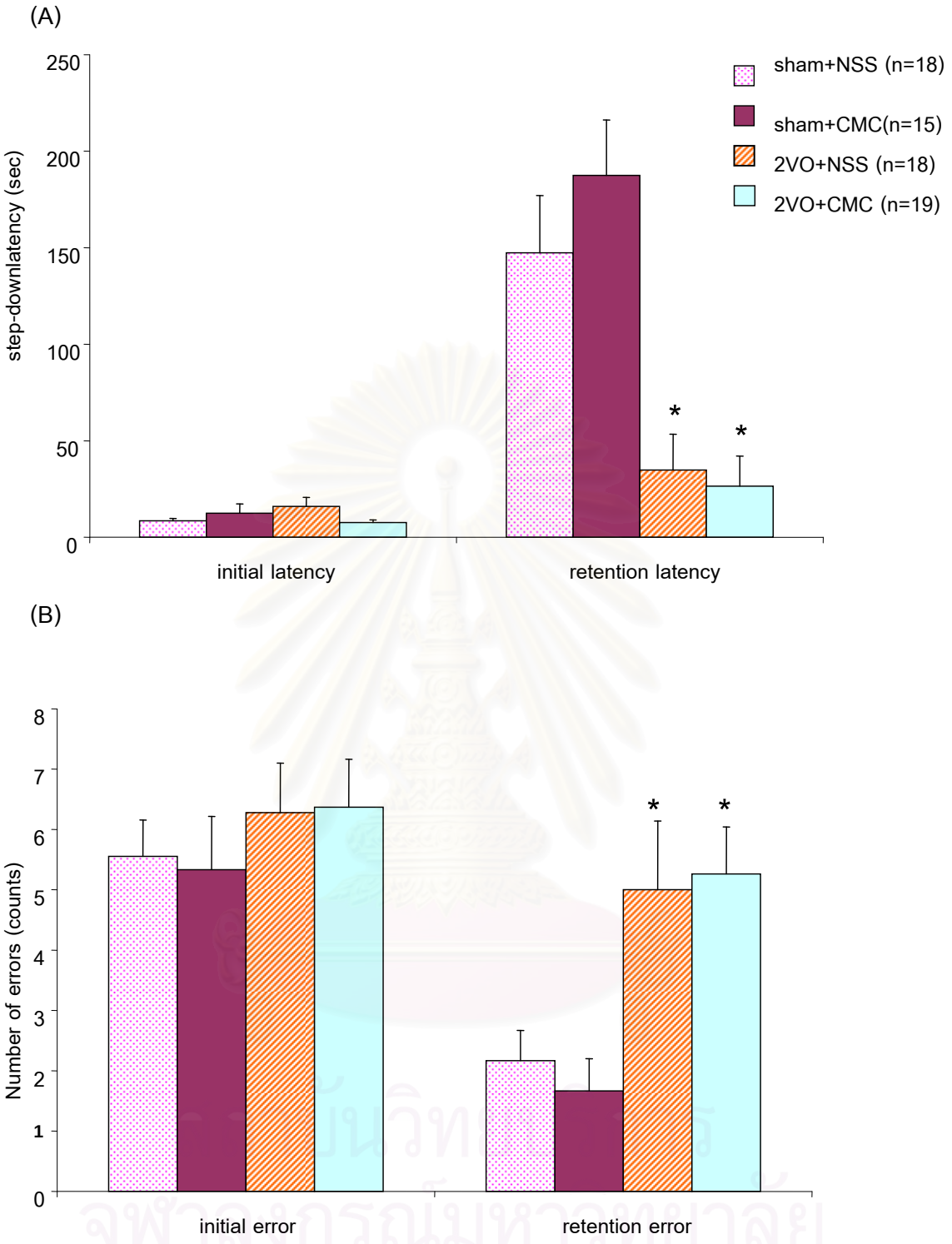

Figure 4.3 Step-down latency (A) and number of errors (B) of 2VO- and sham–operated mice. Data were expressed as mean ± S.E.M. of four trials. Significant difference was assessed with one-way ANOVA followed by Duncan post-hoc test

*$P < 0.05$ denotes statistically significant difference from sham-operated mice treated with CMC

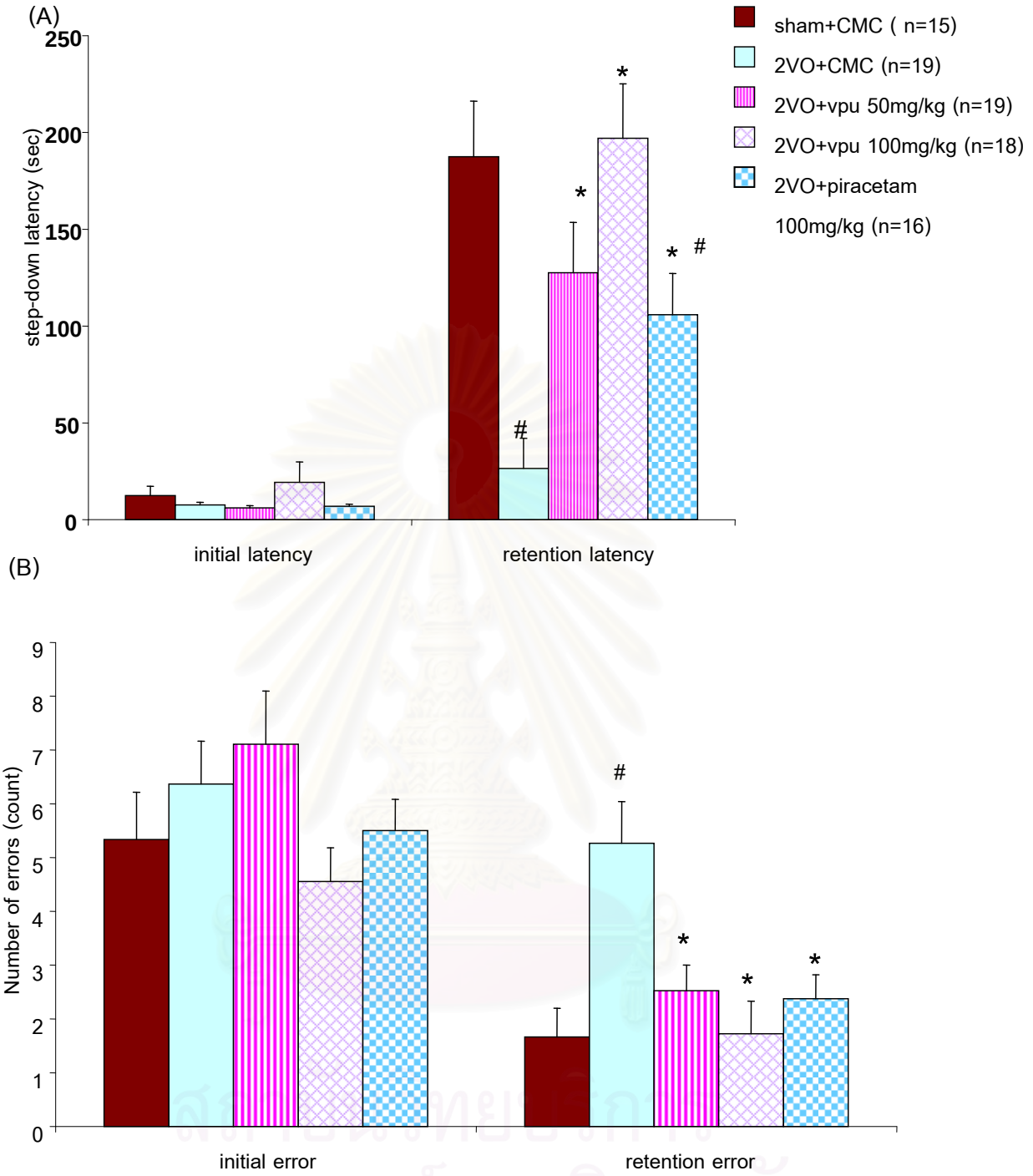

Figure 4.4 Effects of VPU on 2VO-induced disruption of memory in step-down test. Mice were treated by vehicle, VPU (50,100 mg/kg B.W.) and piracetam (100 mg/kg B.W.) once daily. Step-down latency (A) and number of errors (B) were expressed as mean ± S.E.M. of four trials. Significant difference was assessed with one-way ANOVA followed by Duncan post-hoc test

\# $P < 0.05$ denotes statistically significant difference from sham-operated mice

* $P < 0.05$ denotes statistically significant difference from 2VO-operated mice

The spontaneous locomotor activity did not differ between 2VO and sham-operated mice as shown in Figure 4.5.

4.6 Effects of VPU on spontaneous locomotor activity in mice.

As shown in Figure 4.5, administration of VPU at doses 50 and 100 mg/kg B.W. as well as administration of piracetam 100 mg/kg B.W. had no effect on spontaneous locomotor activity in 2VO mice.

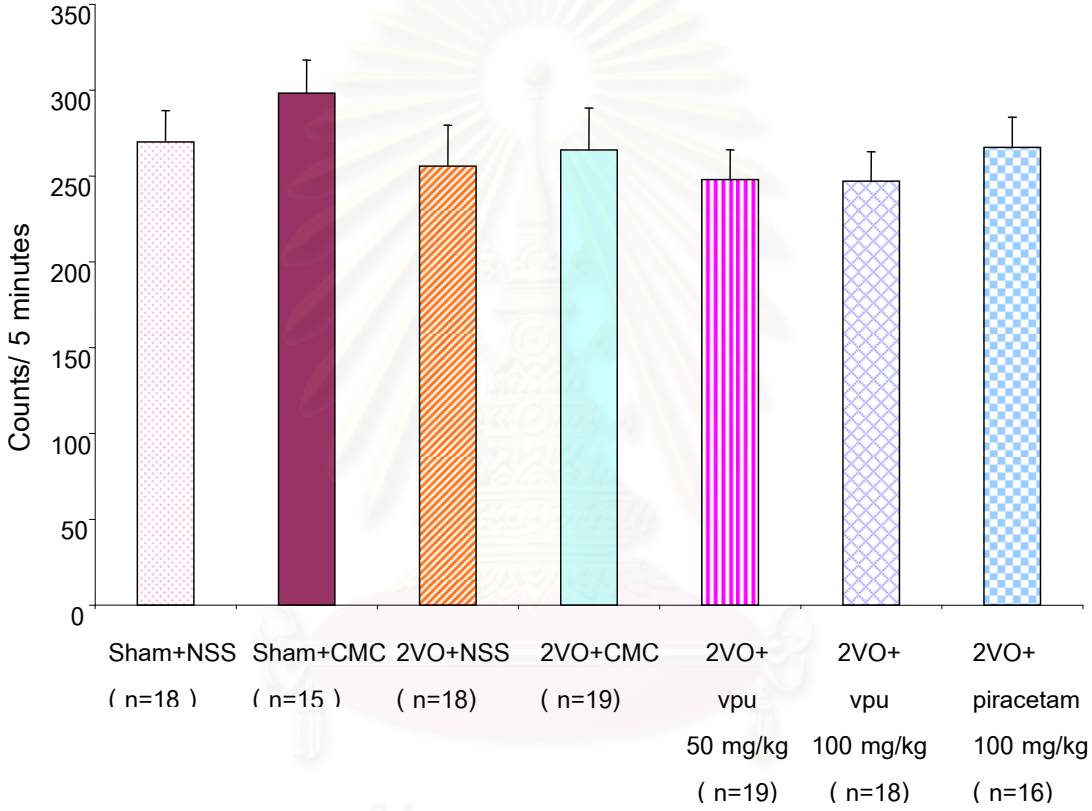

Figure 4.5 Spontaneous locomotor activity of mice receiving vehicle, VPU at doses of 50,100 mg/kg B.W. and piracetam 100 mg/kg B.W. On the ordinate: count/5 min, the values are expressed as the mean $\pm$ S.E.M. Significant difference was assessed with one-way ANOVA followed by Duncan post-hoc test

4.7 Effects of 2VO on brain lipid peroxidation in mice.

In comparison to sham-operated mice, significant increase in MDA levels was observed in 2VO mice. MDA levels of 2VO and sham-operated mice treated with NSS were 22.49 $\pm$ 1.90 and 11.75 $\pm$ 1.41 nmol/g tissue, whereas MDA levels of 2VO and sham-operated mice treated with CMC were 21.14 $\pm$ 1.50 and 11.81 $\pm$ 1.78 nmol/g tissue respectively (Figure 4.6).

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4.8 Effects of VPU on brain lipid peroxidation in 2VO mice.

The effect of VPU on lipid peroxidation was significantly decreased in 2VO mice in both VPU and piracetam-treated group. Administration of VPU at doses 50 and 100 mg/kg B.W. reduced MDA levels to 15.02 $\pm$ 1.57 and 10.16 $\pm$ 1.23 nmol/g tissue respectively. In piracetam-treated group MDA was reduced to 9.34 $\pm$ 1.28 nmol/g tissue (Figure 4.7).

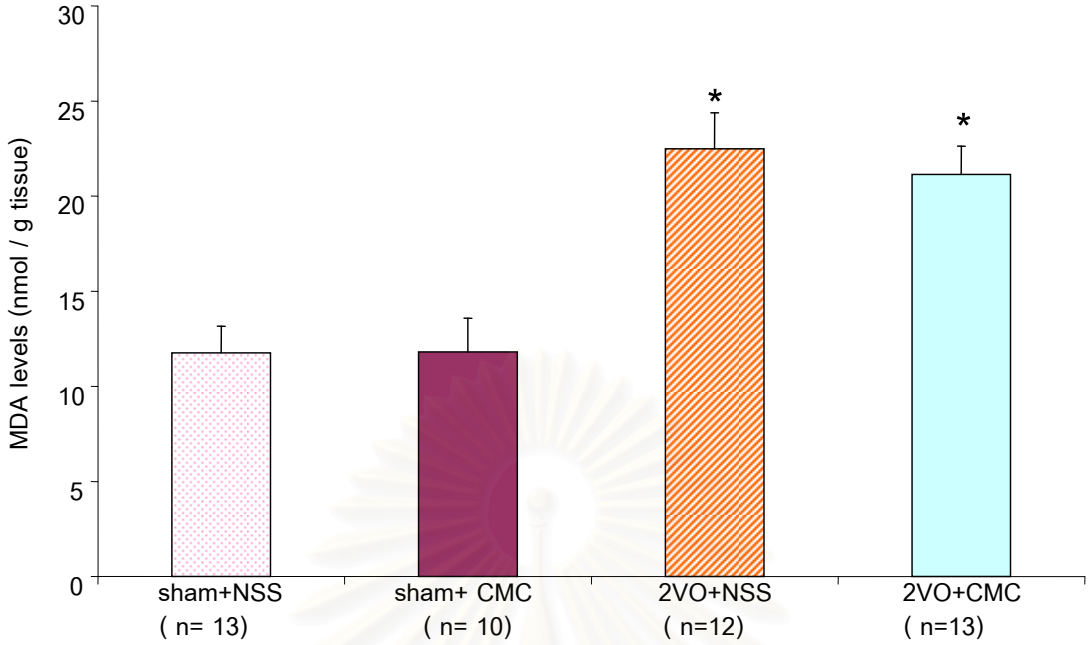

Figure 4.6 Brain levels of MDA in 2VO- and sham-operated mice after 8 days of 2VO. On the ordinate: MDA was expressed as nmol/g tissue (mean ± S.E.M.). Significant difference was assessed with one-way ANOVA followed by Duncan post-hoc test

*$P<$ 0.05 denotes statistically significant difference from sham-operated mice treated with CMC

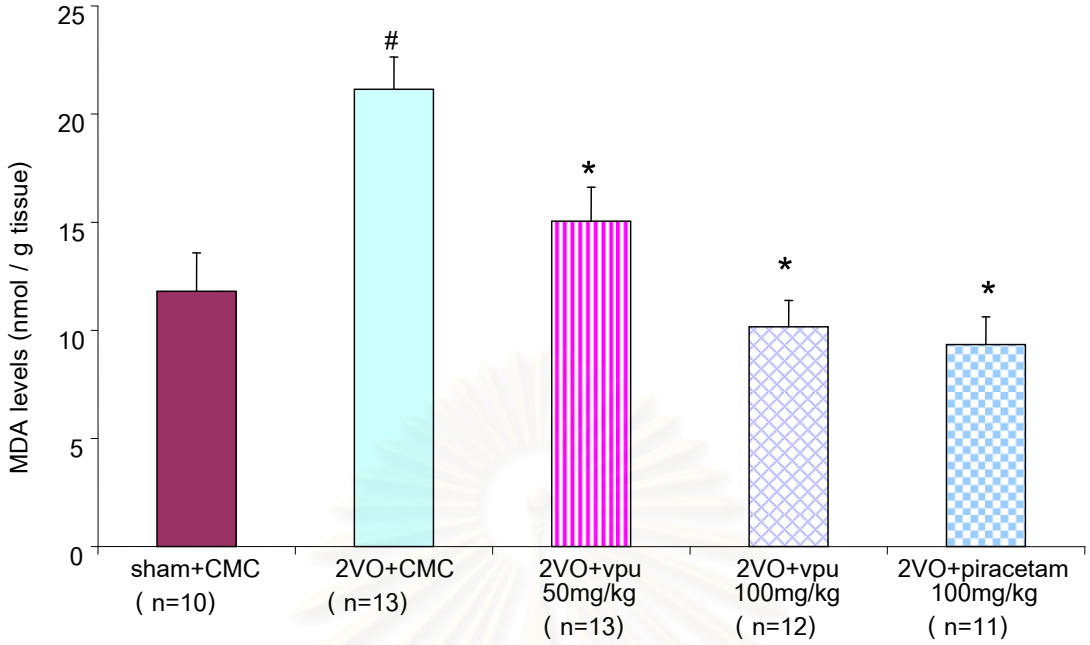

Figure 4.7 Effects of VPU on brain levels of MDA in 2VO-operated mice. Mice received vehicle, VPU at doses of 50 or100 mg/kg B.W. and piracetam 100 mg/kg B.W. On the ordinate: MDA was determined after 8 days of 2VO and expressed as nmol/g tissue (mean $\pm$ S.E.M.). Significant difference was assessed with one-way ANOVA followed by Duncan post-hoc test.

\# $P< 0.05$ denotes statistically significant difference from sham-operated mice

* $P< 0.05$ denotes statistically significant difference from 2VO-operated mice

4.9 Effects of 2VO on CA1 and CA3 pyramidal neurons in mice.

To determine the relative survival of pyramidal neurons in the CA1 and CA3 regions after cerebral ischemia, an average surviving cell numbers were counted over consistent fields, over both hemispheres, and over tree sections in each brain.

Figure 4.8 Illustrated histological changes observed in CA1 and CA3 regions of the hippocampus. Neuronal loss, shrinkage and dark staining of neurons were observed in both CA1 and CA3 regions of the hippocampus in 2VO mice. As shown in Figure 4.9, the number of pyramidal neurons in both regions of both hemispheres was decreased in 2VO mice.

4.10 Effects of VPU on CA1 and CA3 pyramidal neurons in 2VO mice.

As seen in Figure 4.10, administration of VPU50 and 100 mg/kg B.W. and piracetam 100 mg/kg B.W. attenuated neuronal damage caused by cerebral ischemia. Administration of VPU at doses 50 and 100 mg/kg B.W. have number of survival cells were 68.86 $\pm$ 2.17 and 59.94 $\pm$ 5.00 in CA1, 65.93 $\pm$ 3.85 and 59.97 $\pm$ 3.16 in CA3 respectively. In piracetam-treated group number of survival cells were 66.46 $\pm$ 2.27 and 66.92 $\pm$ 2.39 in CA1 and CA3 respectively.

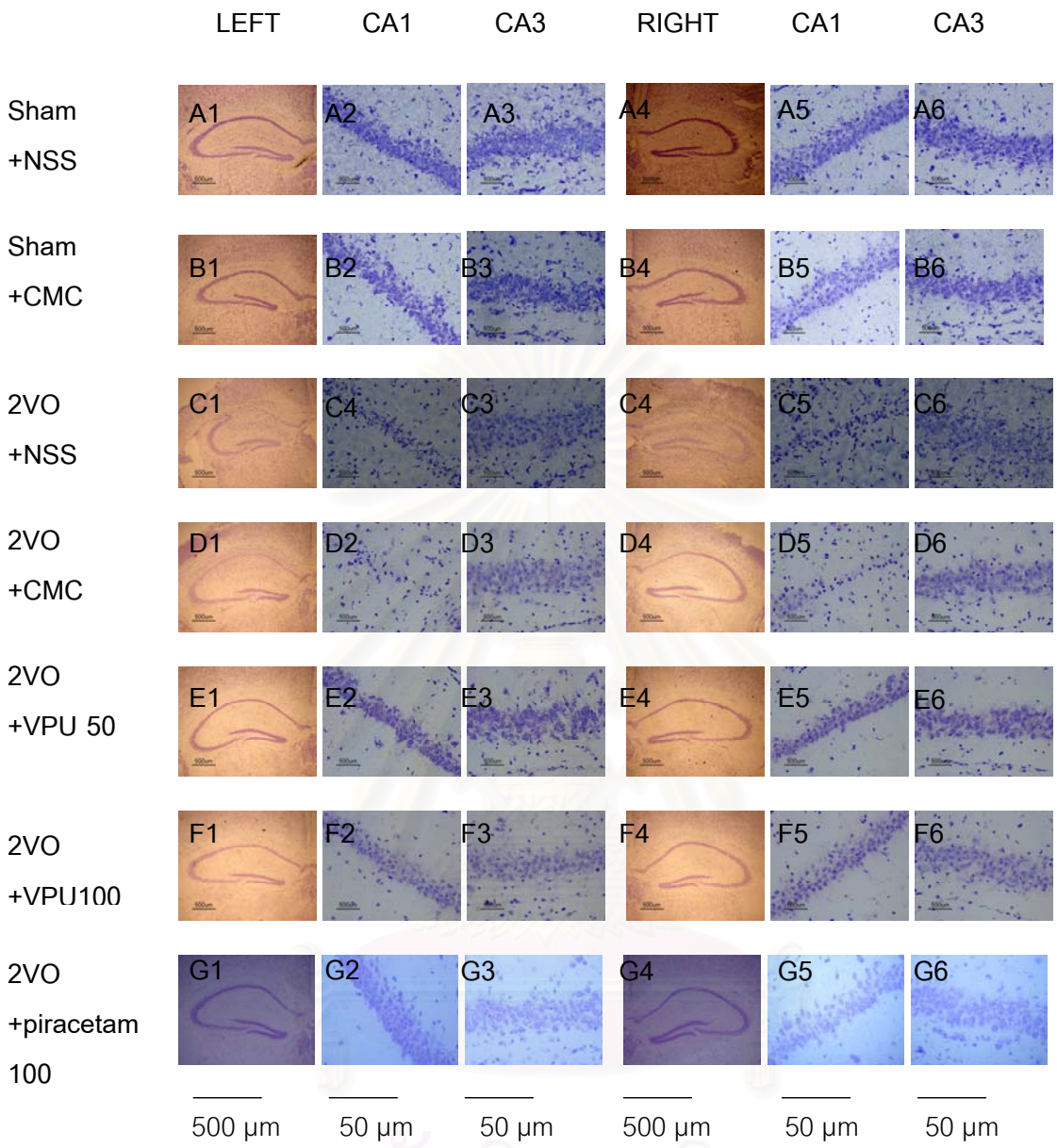

Figure 4.8 Nissl staining with cresyl violet of CA1 and CA3 neurons in transverse left and right hippocampal slices. A_1-A_6 in sham+NSS, B_1-B_6 in sham+CMC, C_1-C_6 in 2VO+ NSS, D_1-D_6 in 2VO+ CMC, E_1-E_6 in 2VO+VPU 50mg/kg B.W., F_1-F_6 in 2VO+VPU 100mg/kg B.W. and G_1-G_6 in piracetam-treated group.

Scale bar are 500 µm for column A_1-G_1 and A_4-G_4.

Scale bar are 50 µm for column A_{2-3}-G_{2-3} and column A_{5-6}-G_{5-6}.

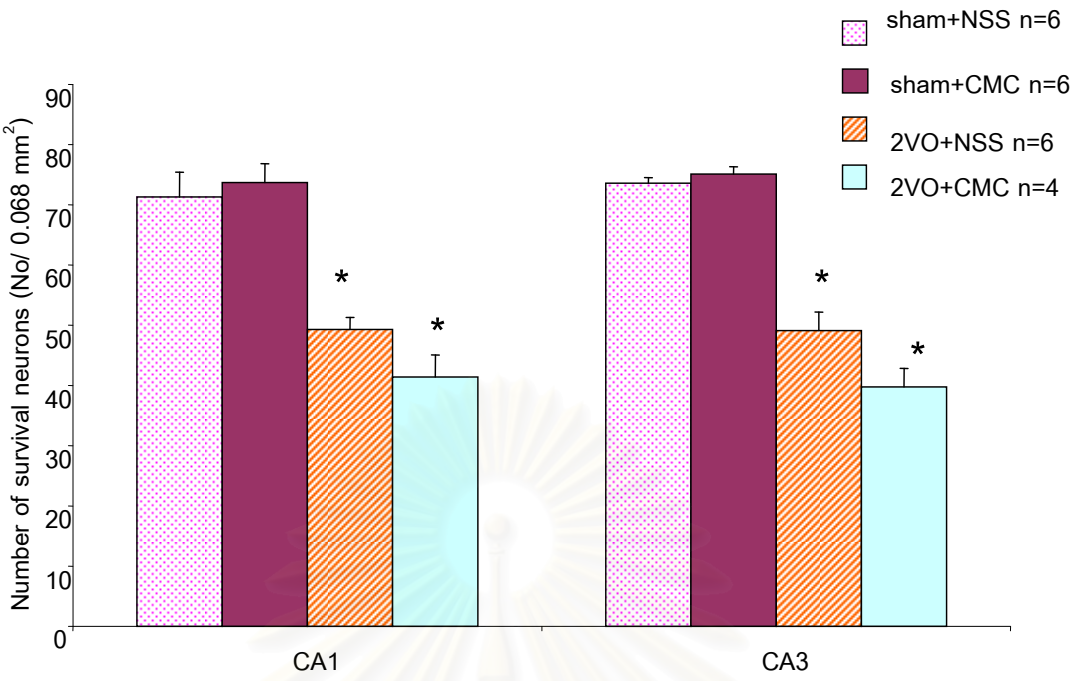

Figure 4.9 Number of survival CA1 and CA3 hippocampal pyramidal neurons in 2VO and sham-operated mice (mean ± S.E.M.). Significant difference was assessed with one-way ANOVA followed by Duncan post-hoc test

* $P < 0.05$ denotes statistically significant difference from sham-operated mice treated with CMC

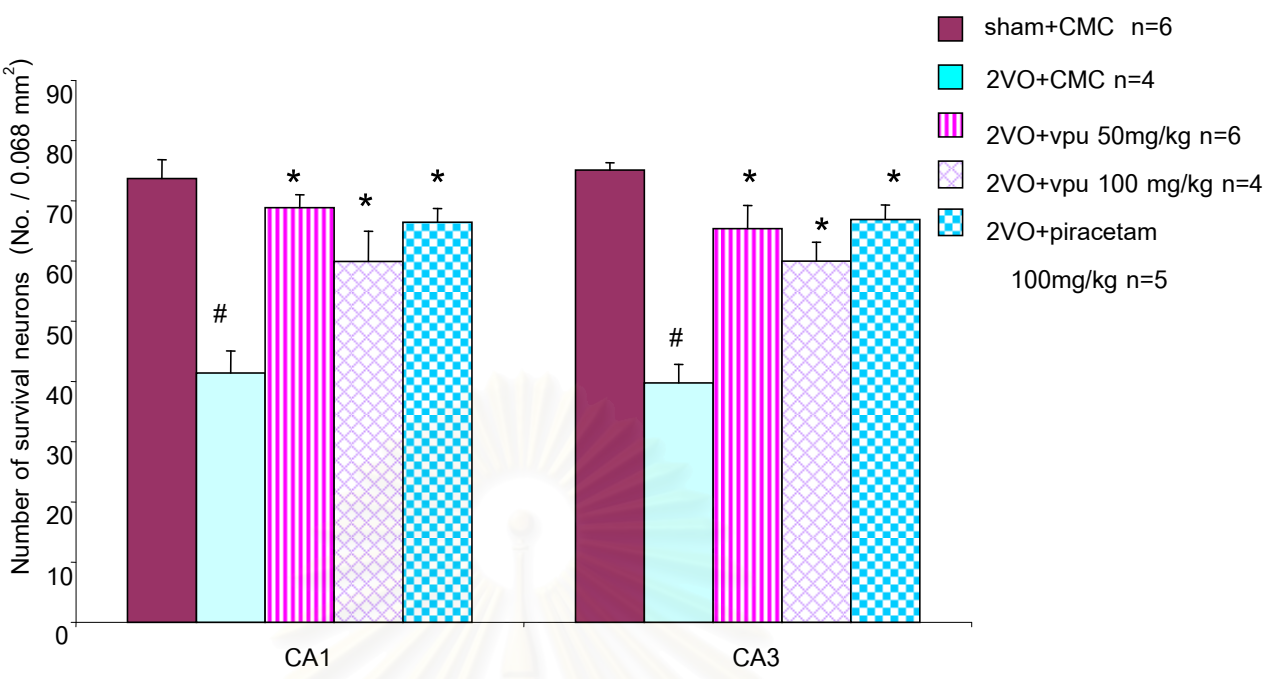

Figure 4.10 Effects of VPU on CA1 and CA3 pyramidal neurons in 2VO-operated mice. Mice received vehicle, VPU at doses of 50 or100 mg/kg B.W. and piracetam 100 mg/kg B.W. (mean ± S.E.M.). Significant difference was assessed with one-way ANOVA followed by Duncan post-hoc test

# *P*< 0.05 denotes statistically significant difference from sham-operated mice

* *P*< 0.05 denotes statistically significant difference from 2VO-operated mice

CHAPTER V

DISCUSSION AND CONCLUSION

Synaptic and cellular events initiated by acute energy deprivation caused by brain ischemia have been shown to be similar to those triggered by abnormal neuronal discharge induced by epilepsy (Calabresi *et al.*, 2003). In brief, sudden cessations of blood flow lead to depolarization of membrane result in massive release of glutamate. Glutamate activation of NMDA receptors and metabotropic glutamate receptors contribute to Ca^{2+} overload. As a result of glutamate-mediated over activation, Na^{+} and Cl^{-} enter the neuron via channels. Water follow passively, as the influx of Na^{+} and Cl^{-} is much larger than the efflux of K^{+}. The ensuring edema can affect the perfusion resulting to cell necrocis. Moreover, an increase in intracellular calcium can generate oxygen free radicals. Massive generation of free radical when reperfusion lead to cell apoptosis. The combinations of several cascades rapidly lead to neuronal cell death especially in hippocampus which is known for its importance in learning and memory process (Shuaib *et al.*, 1996). Therefore, recently some AEDs have been tested in animal models of focal or global ischemia for possible neuroprotective effect against brain ischemia. Though the existing data are rather scant and insufficient but it appears that only AEDs with multiple mechanisms of action seem to have some potential in conferring a degree of neuroprotective (Stepien, Tomaszewski, and Gzvezwar, 2005). For example, Topiramate which exhibited several pharmacological properties such as enhancement of GABAergic, attenuated of voltage-gated sodium current, and block AMPA/kainate receptors has shown to be a promising neuroprotectant in model of cerebral ischemia in many studies (Yang *et al.*, 1998; Lee, Kim, and Kim, 2000).

Previously we found that VPU which was the valproic analogue possessed anticonvulsant activity with various possible mechanisms. By patch clamp technique it was found that VPU could potentiate GABA current (Janthet, 2002). Cortical glutamate of normal and pilocarpine-treated rats was reduced by the administration of VPU (Sooksawate, 1995; Chunngam, 1996; Khongsombat, 2004). Furthermore NMDA

receptors expressed on *Xenopus* oocytes was slightly blocked by VPU (Ponsup, 2003). In addition, neuroprotective effects of VPU against β amyloid were demonstrated in *in vitro* study (Chantong, 2001). Thus we consider investigating the protective effects of VPU against cerebral ischemia using MWM to assess learning and memory ability in conjunction with an evaluation of neuronal cell loss in hippocampus and lipid peroxidation.

The development of animal models of ischemia induced amnesia is vital to the analysis of the functional consequences of ischemic damage and to testing the behavioral efficacy of potential therapeutic drugs (Xu *et al*., 2000). Transient cerebral ischemia produced by two vessel occlusion (2VO) is the one of the model of global ischemia which mainly in use today (Block, 1999). Transient global ischemia leads to damage in selective neuronal in the CA1 sector in hippocampus which resulting in long term learning deficits. In accordance with previous studies (Olsen *et al*., 1994, Block and Schwarz, 1998; Li *et al*., 1999; Hartman *et al*., 2005) we found that mice with transient cerebral ischemia increased escape latency in MWM but decreased step-down latency while the number of errors was increased indicating that transient cerebral ischemia impaired spatial memory (MWM task) and passive avoidance task. Based on the finding that 2VO had no effects on locomotor activity test it is apparent that the memory deficit induced by 2VO in MWM and step-down tasks was not resulted from motor system deficit.

Piracetam, a nootropic agent has been shown to improve cognitive performance in a number of animal model systems (Moran *et al*., 2002). Furthermore, it has been reported that piracetam reduced infarct brain volume induced by permanent middle cerebral artery occlusion and subsequently improved passive avoidance task (Kumar *et al*., 2000; Tortiglione *et al*., 2002; Mcdaniel, Maier, and Einstein, 2003). In the present study piracetam was used as a positive control demonstrating an improvement of memory deficit induced by transient cerebral ischemia. Similarly, intraperitoneal injection of VPU significantly improved 2VO-induced deficit in learning and memory observed in MWM and step-down tests while no alteration in motor activity was

observed. However the effects of VPU seemed to be comparatively delayed in onset. Similar results of other AED, felbamate, have been reported in model of cerebral ischemia (Shuaib *et al*., 1996).

It is generally known that neurons in CA1 regions of hippocampus are the most vulnerable cells to damage induced by transient cerebral ischemia resulting in cognitive deficits (Kim *et al*., 2006). Impairment of performances in MWM and passive avoidance tests in parallel with neuronal loss in hippocampus has been reported in 2VO models (Hartman *et al*, 2005). In line with their finding, we found that number of survival cells in CA1 and CA3 of 2VO mice is significantly lower than those of sham group and deficit in MWM and step-down tests was accordingly observed. Administration of VPU and piracetam which demonstrated alleviating effect on learning and memory deficit was found to increase number of survival cells in CA1 and CA3 regions. These results suggest that protective effects against cell death of VPU on neurons in hippocampus may underlie its positive effect on learning and memory deficit of 2VO mice.

Free radical formation has been demonstrated during cerebral ischemia. Moreover, reperfusion after cerebral ischemia could generate additional burst of free radicals. Reoxygenation during reperfusion provides oxygen as a substrate for numerous enzymatic oxidation reactions in the cytosolic compartment or subcellular organelles and mitochondria leads to cell injury and death (Chan, 2001).

Several synthetic compounds and natural products that could reduce the oxidative stress and improve the memory have been evaluated in animal models of cerebral ischemia (Gupta, Singh and Sharma, 2003). Therefore, the effect of VPU on the oxidative stress parameters (levels of MDA) in brain of mice was further investigated. We found that the levels of MDA in 2VO mice were significantly increased when compared to those of sham-operated mice. This finding agrees well with previous report that bilateral common carotid artery occlusion followed by reperfusion generated reactive oxygen species (ROS) (Nakashima *et al*., 1999). Excessive generation of ROS results in the lipid peroxidation of the cell membrane and subsequent damage is

reflected by accumulation of MDA, a late breakdown product produced from peroxidized lipids (Halliwell, 1991). Similar to piracetam, intraperitoneal injection of VPU significantly abolished an increase in MDA level in 2VO group. Considering that glutamate plays a major role in the pathology observed in ischemia, numerous studies have tried to counteract excitotoxicity by blocking NMDA or AMPA receptors, and preventing excitatory amino acid release (Leker and Neufled, 2003). Moreover, brain excitability is finely tuned by the opposite influence of excitatory glutamatergic activity and the inhibitory influence mainly exerted by GABAergic systems (Green, Hainsworth, and Jackson, 2000). It is likely that VPU which possesses multiple mechanisms leading to inhibition of excitatory transmission and potentiation of inhibitory transmission might protect neurons against damage by ROS by inhibiting cascades proximal to lipid peroxidation. Thereby a reduction of MDA was observed.

In conclusion, the present study has demonstrated positive effect of VPU on learning and memory deficit in brain ischemia-reperfusion models. In addition, neuroprotective effects of VPU on CA1 and CA3 regions of hippocampus which might underlie the positive effect on learning and memory deficit was also demonstrated. Our findings suggest not only the advantage of VPU as anticonvulsant with positive effect on learning and memory but also the possibility of VPU to be further developed for treatment of post stroke amnesia as piracetam.

REFERENCES

Adibhatla, R.M., Hatcher, J.F. Phospholipase A_2, reactive oxygen species, and lipid peroxidation in cerebral ischemia. Free radical Biology and Medicine. 40(2006): 376-387.

Anderson, J.R. Retrieval of memories. In, Learning and memory, an integrated approach. 1st edition. pp.273-313. Canada: John wiley and sons, Inc., 1995.

Bear, M.F., Connors, B.W., and Paradiso, M.A. Memory system. In, Neuroscience Exploring the brain. 3rd edition. pp. 725-760. USA: Lippincott Williams and Wilkins, 2007.

Block, F. Global ischemia and behavioral deficits. Progress in neurobiology. 58(1999): 279-295.

Block, F., and Schwarz, M. Global ischemic neuronal damage relates to behavioral deficits: A pharmacological approach. Neuroscience. 82(3) (1998): 781-803.

Bonfoco, E. Krainic, D., Ankarcrona, M., Nocetera, P., and Lipton, A.S. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proceeding of the National Academic of Sciences of the United States of America. 92(1995): 7162-7166.

Calabresi, P., Cupini, L.M., Centonze, D., Pisani, F., and Bernardi, G. Antiepileptic drugs As a possible neuroprotective strategy in brain ischemia. Annual Neurology. 53(2003): 693-702.

Chan, P.H. Reactive oxygen radicals in signaling and damage in the ischemia brain. Journal of cerebral blood flow and metabolism. 21(2001): 2-14.

Chan, S.A., Reid, K.H., Schurr, A.,Miller, J.J., Iyer, V. and Tseng, M. Fosphenytoin reducee hippocampal neuronal damage in rat following transient global ischemia. Acta Neurochirurgica. 140(1998): 175-180.

Chantong, B. Effect of valproic acid and valproyl urea on betaanyloid protein-induced cytotoxicity in P19 embryonal carcinoma cells. Master's Thesis, Chulalongkorn University, 2001.

Choi, D.W. Ischemia-induced neuronal apoptosis. Current Opinion in neurobiology.6 (1996): 667-672.

Chunngam T. Preliminary studies of Anticonvulsion mechanism of N-(2-propylpentanoyl) urea. Master's Thesis, Department of Pharmacology, Graduate School, Chulalongkorn University, 1996.

Clark, W.M., Leuten, J.D., Lessor, N., Woodward, W. and Coull, B.M. Time course of ICAM-1 expression and leukocyte subset infiltration in rat forebrain ischemia. Molecular Chemistry Neuropathology. 26(1995): 213-230.

Clemens, J.A., Stephens, D.T., Dixon, E.P., Smalstig, E.B., Mincy, R.E., Rash, K.S., and Little, S.P. Global cerebral ischemia activates nuclear factor-kappa B prior to evidence of DNA fragmentation. Molecular Brain Research. 48(1997): 187-196.

D'Hooge, R., and De Deyn, P.P. Applications of the morris water maze in the study of Learning and memory. Brain Research Review. 36(2001): 60-90.

De Keyser, J., Sulter, G., and Luiten, P.G. Clinical trials with neuroprotective drugs in acute ischaemic stroke: are we doing the right thing? Trends in Neuroscience. 22(1999): 535-540.

Delis, D.C., Lucas, J.A., and Kopelman, M.D. Memory. In, Fogel, B.S., Schiffer, R.B., and Rao, S.M. (eds.), Synopsis of Neuropsychiatry, pp. 169-191. USA: Lippincott Williams and Wilkins., 1999.

Dirnagl, U., Iadecola, C., and Moskowitz, M.A. Pathobiology of ischeamic stroke: an integrated view. Trands Neuroscience. 22(1999): 391-397.

Galeotti, N., Ghelardini, C., and Bartolini, A. Piracetam and aniracetam antagonism of centrally active drug-induced antinociception. Pharmacology Biochemistry and Behavior. 53(4) (1996):943-50.

Green, A.P., Hainsworth, A.H., and Jackson, D.M. GABA potentiation: a logical pharmacological approach for the treatment of acute ischaemic stroke. Neuropharmacology. 39(2000): 1483-1494.

Gupta, R., Singh, M., and Sharma, A. Neuroprotective effect of antioxidants on ischemia and reperfusion-induced cerebral injury. Pharmacological Research. 48(2003): 209-215.

Gupta, Y.K., Veerenda Kumar, M.H., Srivastava, A.K. Effect of Centella asiatica on pentylenetetrazole-induced kinding, cognition and oxidative stress in rats. Pharmacology Biochemistry and Behavior. 74(3) (2003): 579-585.

Halliwell, B. Reactive oxygen species in living system: source, biochemistry and role in human disease. The American Journal of Medicine. 99(1991): 145-225.

Hanon, E., and Klitgaard, H. Neuroprotective properties of the novel antiepileptic drugs levetiracetam in the rat middle cerebral artery occlusion model of focal cerebral ischemia. Seizure. 10(2001): 287-293.

Hara, H., Sukamoto, T., and Kogure, K. Mechanism and pathogenesis of ischemia-induced neuronal damage. Progress in Neurobiology. 40(6) (1993): 645-670.

Hartman, E., Lee, J.M., Zipfel, G.J., and Wozniak, D.F. Characterizing learning deficits and hipppocampal neuron loss following transient global cerebral ischemia in rats. Brain Research. 1043 (2005): 48-56.

Iadecola, C. Bright and Dark sides of nitric oxide in ischemic brain injury. Trands Neurosciences. 20(1997): 132-139.

Jain, N.K., Patil, C.S., Kulkarni, S.K. and Singh, A. Modulatory role of cyclooxygenase inhibitors in aging-and scopolamine or lipopolysaccharide-induced cognitive dysfunction in mice. Behavior Brain Research. 18(133) (2)(2002): 369-376.

Janthet, S. Effect of N-(2-propylpentanoyl) urea on $GABA_A$ receptor in acutely dissociated rat hippocampal neurons. Master's Thesis, Interdepartment Program of Physiology, Graduate School, Chulalongkorn University, 2002.

Joseph R. The hippocampus, amygdale, memory and amnesia. In, Neuropsychiatry, neuropsychology, and clinical neuroscience. 2nd edition, pp. 206-234. USA: Lippincott Williams and Wilkins., 1996.

Kandel, E.R., Schwaetz, J.H., and Jessell, T.M. Learning and memory, In, Principle of Neuroscience, 4th edition, pp. 1227-1246. Newyork: McGraw-Hill, 2000.

Khongsombat, O. Effects of N (2'-propylpentanoyl)-2-pyrolidinone and N-(2-propylpentanoyl) urea on neuron of cerebral cortex and cerebellar Purkinje cells in rats. Master's Thesis, Interdepartment Program of Physiology, Graduate School, Chulalongkorn University, 1997.

Khongsombat, O. Effect of N-(2-propylpentanoyl) urea on pilocarpine-induced seizure in rats. Doctoral's Thesis, Interdepartment Program of Physiology, Graduate School, Chulalongkorn University, 2004.

Kim, D.H., Jeon, S.J., Son, K.H., Jung, J.W., Lee, S., Yoon, B.H., Choi, J.W., Cheong, J.H., Ko, K.H., and Ryo, J.H. Effect of flavonoid, oroxylin A, on transient cerebral hypoperfusion-induced memory impairment in mice. Pharmacology Biochemistry and Behavior. 85(2006): 658-668.

Kirino, T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Research. 239(1982): 57-59.

Kumar, V., Singh, P.N., Muruganundam, A.V., and Bhattacharya, S.K. Effect of Indian *Hypericum perforatum* Linn on animal models of cognitive dysfunction. Journal of Ethnopharmacology. 72(2000): 119-128.

Lee, S-K, Kim, J-E., and Kim, J-E. Protective effect of topiramate against hippocampal neuronal damage after global ischemia in the gerbils. Neuroscience Letters. 281(2000): 183-186.

Leist, M. and Nicotera, P. Apoptosis, Excitotoxicity, and Neuropathology. Experimental Cell Research. 239(1998): 183-201.

Leker, R.R., and Neufeld, M.Y. Anti-epileptic drugs as possible neuroprotectants in cerebral ischemia. Brain Research Reviews. 42(2003): 187-203.

Li, A.J., Omura, Y., Sasaki, K., Suzuki, K., and Hori, T., Protective effect of acidic fibroblaset growth factor against ischemia-induced learning and memory deficits in two tasks in gerbils. Physiology and Behavior. 66(4) (1999): 577-583.

Liang, K.C., Hon, W., Tyan, Y.M., and Liao, W.L. Involvement of hippocampal NMDA and AMPA receptors in acquisition, formation and retrieval of spatial memory in the morris water maze. Chinese Journal of Physiology. 37(4) (1994): 201-212.

Luo, J., Yin, J.H., and Wei, Q. The effect of calcineurin activator, extracted from Chinese herbal medicine, on memory and immunity in mice. Pharmacology Biochemistry and Behavior. 75(4) (2003): 749-754.

Lynch, D.R., and Guttmann, R.P. Excitotoxicity: Perspectives Based on *N*-Methyl-D-Aspatate receptor subtypes. The Journal of Pharmacology and Experimental Therapeutics. 300(3) (2002): 717-723.

Lynch, M.A. Long-term potentiation and memory. Physiology review. 84(2004): 87-136.

Marklund, S.L. Endogenous free radicals and antioxidants in the brain. In, Beal, M.F., Lang, A.E., and Ludolph, A.C. (eds.), Neurodegenerative disease, Neurobiology, Pathogenesis and therapeutics. 1st edition. pp. 3-32. USA: Cambridge University Press., 2005.

Matsushita, K., Matsuyama, T., Kitagawa, K., Matsumoto, M.M Yanagihara, T., and Sugita, M. Alteration of Bcl-2 family proteins precede cytoskeletal proteolysis in the penumbra, but not in infarct canters following focal cerebral ischemia in mice. Neuroscience. 83(2) (1998): 43-48.

Mcdaniel, M.A., Maier, S.F., and Einstein, G.O. Brain-Specific Nutrients: A memory Cure? Nutrition. 19(2003): 957-975.

Milner, B., Squire, L.R., and Kandel, E.R. Cognitive neuroscience and the study of memory. Neuron. 20(1998): 446-468.

Mohajeri, M.H., Saini, K., Li, H., Crameri, A., Lipp, H.P., Wolfer, D.P., and Nitsch, R.M. Intact spatial memory in mice with seizure-induced partial loss of hippocampul Pyramidal neurons. Neurobiology of Disease. 12(2003): 174-181.

Moore, K., and Roberts, L.I. Measurement of lipid peroxidation. Free Radical Research. 28(1998): 659-671.

Moran, T.H., Capone, G.T., Knipp, S., Davisson, M.T., Reeves, R.H., and Gearhart, J.D. The effects of piracetam on cognitive performance in mouse model of Down's Syndrome. Physiology and Behavior. 77(2002): 403-409.

Moro, M.A., Cardenas, A., Hurtado, O., Leza, J.C., and Lizasoain, I. Role of nitric oxide after brain ischemia. Cell Calcium. 36(2004): 265-275.

Morris, R.G.M. Development of a water-maze procedures for studying spatial learning in the rat. Journal of Neuroscience Methodology. 11(1984): 47-60.

Morris, R.S.M., Garrud, P., Rawlins, J.N.P. and O'Keefe, J. Place navigation impaired in rat with hippocampal lesions. Nature. 297(1982): 681-683.

Muller, W.E., Eckert, G.P., and Eckert, A. Piracetam: novelty in a unique mode of action. Pharmacopsychiatry. 32 Suppl (1999)1: 2-9.

Muller, W.E., Koch, S., Scheuer, K., Rostock, A., and Bartsch, R. Effects of piracetam on membrane fluidity in the aged mouse, rat, and human brain. Biochemecal Pharmacology. 24; 53(2) (1997):135-140.

Nakashima, M., Niwa, M., Iwai, T., and Uematsu, T. Involvement of free radicals in cerebral vascular reperfusion injury evaluated in a transient focal cerebral ischemia model of rat. The Journal of Biological Chemistry. 7(274) (19)(1999): 13541-13546.

Namura, S.,Zhu, J., Fink, K., Endres, M., Srinivasan, A., Tomoselli, K.J., Yuan, Y., and Moskowitz, M.A. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. The Journal of Neurosciences. 18(1998): 3659-3668.

Nanri, M., Yamamoto, J., Miyake, H., and Watanabe, H. Protective effect of GTS-21, a novel nicotinic receptor agonist, on delayed neuronal death induced by ischemia in gerbils. Japanese Journal of Pharmacology. 76(1998): 23-29.

Ni, J-W., Matsumoto, K., Li, H.B., Murakami, Y., and Watanabe, H. Neuronal damage and decrease of central acetylcholine level following permanent occlusion of bilateral common carotid arteries in rats. Brain Research. 673(1995): 290-296.

Nunn, J.A., Graydon, F.J.X., Polkey, C.E., and Morris, R.G. Differential spatial memory impairment after right temporal lobectomy demonstrated using temporal titration. Brain. 122(1999): 45-59.

Olsen, G.M., Scheel-Kruger, J., Malter, A., and Jensen, L.H. Relation of spatial learning of rats in the morris water maze task to the number of viable CA1 neurons following four-vessel occlusion. Behavioral Neuroscience. 108(1994): 681-690.

Patchamart, W. Hepatotoxicity of N-(2-propylpentanoyl) urea in rats. Master's Thesis, Interdepartment Program of Pharmacology, Graduate School, Chulalongkorn University, 1996.

Pohle, W., Becker, A., Grecksch, G., Juhre, A., and Willenberg, A. Piracetam prevents pentylenetetrazol kindling-induced neuronal loss and learning deficits. Seizure. 6(1997): 467-474.

Ponsup, P. Effectof N-(2-propylpentanoyl) urea on NMDA receptors suptype NR1A/NR2B expressed in *Xenopus laevis* oocytes. Master's Thesis, Chulalongkorn University, 2003.

Rossato, J.I., Zinn, C.G., Furini, C., Bevilaqua, L.R.M., Medina, J.H., Cammarota, M., and Izquierdo, I. A link between the hippocampal and the striatal memory systems of the brain. Annals of the Brazillian Academy of Sciences. 78(2006): 515-523.

Saisorn, B., Patarapanich, C., and Janwitayanuchit, W. Synthesis of monouric analogues of valproic acid. Thai Journal Pharmaceutical Sciences. 16(1992): 145-150.

Scheuer, K., Rostock, A., Bartsch, R., and Muller, W.E. Piracetam improves cognitive performance by restoring neurochemical deficits of the aged rat brain. Pharmacopsychiatry. 2 Suppl (1999)1:10-6.

Shang, Y-Z, Miao, H., Cheng, J-J., and Qi, J-M. Effect of amelioration of flavonoids from stem and leaves of *Scutellaria baicalensisGeorgi* on cognitive deficits, neuronal damage and free eadicals disorder induced by cerebral ischemia in rats. Biological and Pharmaceutical Bulletin. 29(4)(2006): 805-810.

Shorvon, S. Pyrrolidone derivatives. Lancet. 358(2001):1885-92.

Shuaib, A., Wagaar, T., Ijaz, M.S., Wishart, T., and Howlett, W. Neuruprotection with felbamate: a 7- and 28-day study in transient forebrain ischemia in gerbils. Brain Research. 727(1996): 65-70.

Sooksawate, T. Anticonvulsant effects of N-(2-propylpentanoyl) urea. Master's Thesis, Interdepartment Program of Pharmacology, Graduate School, Chulalongkorn University, 1995.

Stepien, N., Tomaszewski, M., and Czuczwar, S.J. Profile of anticonvulsant activity and neuroprotective effects of novel and potential antiepileptic drugs-an update. Pharmacological Reports. 57(2005): 719-733.

Tantisira, B., Tantisira, M.H., Patarapanich, C., Sooksawate, T., and Chunngam, T. Preliminary evaluation of anticonvulsant activity of valproic analog: N-(2-propylpentanoyl) urea. Research Communication Molecular Pathological Pharmacology. 97(2) (1997): 151-164.

Thornberry, N.A., and Lazebnik, Y. Caspase: Enemies within. Science. 281(5381) (1998): 1312-1316.

Tortiglione, A., Minale, M., Pignataro, G., Amorozo, S., DiRenzo, G., and Annunziato, L. The 2-oxopyrrodinaceramide piracetam reduces in farct brain volume induced by permanent middle cerebral artery occlusion in male rats. Neuropharmacology Reports. 57(2002): 719-733.

Traystman, R.J. Animal models of focal and global cerebral ischemia. National Research Council, Institute of Laboratory Animal Resorces Journal. 44(2) (2003): 85-95.

Tucker, D.M., Hartry-Speiser, A., McDougal, L., Luu, P., and deGrandpre, D. Mood and spatial memory: emotion and right hemisphere contribution to spatial cognition. Biological Psychology. 50(1999): 103-125.

Vaglenova, J., Vesselinov Petkov, V. Can nootropic drugs be effective against the impact of ethanol teratogenicity on cognitive performance? European Neuropsychopharmacology. 11(1) (2001):33-40.

Vohora, D., Pal, S.N., and Pillai, K.K. Effect of locomotor activity on the passive avoidance test for the evaluation of cognitive function. Indian Journal of Pharmacology. 32(2000): 242-245.

Warner, D.S., Sheng, H., and Batinic-Haberte, I. Oxidants, antioxidant and ischemic brain. The Journal of Experimental Biology. 207(2004): 3221-3231.

Watanabe, H., Zhao, Q., Matsumoto, K., Tohda, M., Murakami, Y., Zhag, S.H., Kang, T.H., Mahakunakorn, P., Murayama, Y., Sakakibara, I.,Aimi, N., and Takayama, H. Pharmacological evidence for antidementia effect of Choto-san (Gouteng-san) a traditional Kampo medicine. Pharmacology Biochemistry and Behavior. 75(3) (2003): 635-643.

White, B.C, Sullivan, J.M., DeGracia, D.J., O'Neil, B.J., Neumar, R.W., Grossman, L.I., Rafols, J.A., and Krause, G.S. Brain ischemia and reperfusion: molecular mechenisms of neuronal injury. Journal of the Neurological Sciences. 179(2000): 1-33.

Widmaier, E.P., Raff, H. and Strang, K.T. Conciousness, the brain, and behavior. In, Vander, Sherman, Lucino's (eds.). Human physiology: The mechanism of body function. 9th edition. pp. 259-261. New Yoke: McGraw-Hill, 2004.

Wischer, S., Paulus, W., Sommer, M., and Tergau, F. Piracetam affects facilitatory I-wave interaction in the human motor cortex. Clinical Neurophysiology. 112(2) (2001):275-9.

Xu, J., Murakami, Y., Matsumoto, K., Tohda, M., Watanabe, H., Zhong, S., Yu, Q., and Shen, J. Protective effect of Oren-geoku-to (Huang-Lian-Jie-Du-Tang) against impairment of learning and memory induced by transient cerebral ischemia in mice. Journal of Ethnopharmacology. 73(2000): 405-413.

Yang, Y., Shuaib, A., Li, Q., and Siddigui, M.M. Neuroprotection by delayed administration of topitamate in rat model of middle cerebral artery embolization. Brain Research. 804(1998): 169-176.

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