ฤทธิ์ของสารสกัดเอธิลอะซิเตทของบัวบก (*Centella asiatica*) ในการป้องกันการชัก และภาวะบกพร่องของการเรียนรู้ที่ถูกเหนี่ยวนำโดยสารเพนธีลีนเตตระโซล

พันตรีหญิงอนุสรา วัฒนจันทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PROTECTIVE EFFECTS OF *Centella asiatica'* S ETHYL ACETATE FRACTION AGAINST PENTYLENETETRAZOLE-INDUCED SEIZURE AND LEARNING IMPAIRMENT

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อนุสรา วัฒนจันทร์ : ฤทธิ์ของสารสกัดเอธิลอะซิเตทของบัวบก (*Centella asiatica*) ในการป้องกันการชัก และภาวะบกพร่องของการเรียนรู้ที่ถูกเหนี่ยวนำโดยสารเพนธีลีนเตตระโซล (PROTECTIVE EFFECTS OF *Centella asiatica*' S ETHYL ACETATE FRACTION AGAINST PENTYLENETETRAZOLE-INDUCED SEIZURE AND LEARNING IMPAIRMENT อ.ที่ปรึกษา: ร.ศ.ดร.บุญยงค์ ตันติสิระ อ.ที่ปรึกษาร่วม: PROF. HIROSHI WATANABE, ร.ศ. ดร. มยุรี ตันติสิระ, 115 หน้า ISBN 974-53-1503-6

การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลและกลไกการออกฤทธิ์ของสารสกัดเอธิลอะซิเตทของบัวบกโดยใช้โมเดลต่างๆ ในสัตว์ทดลอง ศึกษาฤทธิ์ในการกันชักในสัตว์ทดลองที่ถูกเหนี่ยวนำด้วยสารเพนธีลีนเตตระโซล (พีทีแซท) พิษ ปฏิกิริยาระหว่างสารสกัดบัวบกกับยากันชัก ผลต่อการเปลี่ยนแปลงของระดับสารสื่อประสาทบริเวณฮิปโปแคมปัส การเปลี่ยนแปลงทางสรีรวิทยาไฟฟ้าของตัวรับกาบา ตลอดจนฤทธิ์ในการป้องกันกระบวนการเกิดการชัก และภาวะบกพร่องของการเรียนรู้

ขนาดสารสกัดเอธิลอะซิเตทของบัวบกที่สามารถกันชักในหนูถีบจักรที่ถูกเหนี่ยวนำด้วยพีทีแซทจำนวนครึ่งหนึ่ง เมื่อให้โดยการป้อน คือ 673 มก/กก น้ำหนักตัว และขนาดที่มีความเป็นพิษต่อระบบประสาทส่วนกลางในหนูถีบจักรจำนวนครึ่งหนึ่งเมื่อศึกษาโดยวิธี rotarod test คือ 415 มก/กก น้ำหนักตัว แต่อย่างไรก็ตามพบว่าสารสกัดเอธิลอะซิเตทของบัวบกมีความปลอดภัยมาก เนื่องจากขนาดของสารสกัดที่ทำให้หนูถีบจักรตายจำนวนครึ่งหนึ่งมีค่าสูงกว่า 5,000 มก/กก น้ำหนักตัว จากการศึกษาปฏิกิริยาที่มีต่อยากันชักที่ใช้อยู่ในปัจจุบัน พบว่ามีการเสริมฤทธิ์ในการกันชักระหว่างสารสกัดเอธิลอะซิเตทของบัวบกกับยากันชักฟีนีไทอิน วาลโปรเอท และกาบาเพนติน และเมื่อพิจารณาประกอบกับความเป็นพิษต่อระบบประสาทส่วนกลาง พบว่า เมื่อให้ร่วมกับกาบาเพนตินจะทำให้ค่าดรรชนีปกป้อง (protective index, TD_{50}/ED_{50}) กว้างขึ้น โดยไม่พบผลดังกล่าวในการให้ร่วมกับฟีนีไทอิน และวาลโปรเอท เมื่อทำการศึกษาโดยวิธีไมโครไดอะลัยซีส พบว่าในหนูขาวที่ได้รับสารสกัดเอธิลอะซิเตทของบัวบกขนาด 700 มก/กก น้ำหนักตัว ซึ่งสามารถกันชักจากการถูกเหนี่ยวนำด้วยพีทีแซทได้นั้น ระดับของแอสพาเตทและกลูตาเมทลดลงเล็กน้อย ในขณะที่ระดับของกลัยซีนและกาบามีแนวโน้มเพิ่มขึ้น ในทางตรงข้ามหนูขาวที่ได้รับสารสกัดเอธิลอะซิเตทของบัวบกในขนาดที่เท่ากันแต่ไม่สามารถกันชักได้ ยังคงพบระดับแอสพาเตทที่สูงขึ้นจากการเหนี่ยวนำด้วยพีทีแซท นอกจากนี้การศึกษาทางสรีรวิทยาไฟฟ้าพบว่าสารสกัดเอธิลอะซิเตทของบัวบกในขนาดต่ำ 0.1 ถึง 3 ไมโครกรัม/มล เมื่อให้ร่วมกับกาบา จะสามารถเพิ่มการทำงานของตัวรับกาบาได้เล็กน้อย แต่ในขนาดสูงขึ้น (50 ไมโครกรัม/มล) จะยับยั้งการทำงานของตัวรับกาบาได้บางส่วน เมื่อพิจารณาผลการทดลองทั้งหมดประกอบกันทำให้เชื่อว่าน่าจะมีสารที่ทำหน้าที่ด้านฤทธิ์กันเป็นองค์ประกอบอยู่ในสารสกัดเอธิลอะซิเตทของบัวบก นอกจากนี้ยังพบว่าสารสกัดเอธิลอะซิเตทของบัวบกไม่มีฤทธิ์ในการป้องกันกระบวนการเกิดการชักและไม่มีผลต่อระดับไลปิดเปอร์ออกซิเดชั่น แม้ว่าดูเหมือนจะมีฤทธิ์ในการป้องกันความบกพร่องของการเรียนรู้และความจำ ตลอดจนการอยู่รอดของเซลล์ประสาทพีรามิดอลในโมเดลของพีทีแซท-คินลิ่ง แต่ยังไม่สามารถสรุปได้ชัดเจนจากการศึกษาในครั้งนี้

การศึกษานี้แสดงให้เห็นว่า ฤทธิ์ในการกันชักของสารสกัดเอธิลอะซิเตทของบัวบกน่าจะมีความสัมพันธ์กับการลดระดับสารสื่อประสาทอะมิโนชนิดกระตุ้น ร่วมกับการเพิ่มขึ้นของระดับสารสื่อประสาทอะมิโนชนิดยับยั้ง ประกอบกับการเพิ่มการทำงานของตัวรับกาบาของเซลล์สมองบริเวณฮิปโปแคมปัส นอกจากนี้ความสามารถในการเสริมฤทธิ์กับยากันชักที่ใช้อยู่ในปัจจุบันได้แสดงถึงศักยภาพของสารสกัดจากบัวบกที่จะสามารถพัฒนาต่อไปเพื่อใช้เสริมฤทธิ์กับยากันชักในผู้ป่วยโรคลมชักได้ แต่ทั้งนี้ต้องทำการแยกสารออกฤทธิ์ซึ่งคาดว่าจะมีหลายชนิดและอาจมีฤทธิ์ทางเภสัชวิทยาที่แตกต่างกันออกจากกันก่อนที่จะทำการทดสอบในขั้นตอนต่อไป

สาขาวิชา สรีรวิทยา (สหสาขาวิชา) ลายมือชื่อนิสิต .....

ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา .....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

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ANUSARA VATTANAJUN: PROTECTIVE EFFECTS OF *Centella asiatica*' S ETHYL ACETATE FRACTION AGAINST PENTYLENETETRAZOLE-INDUCED SEIZURE AND LEARNING IMPAIRMENT. THESIS ADVISOR: ASSOC.PROF. BOONYONG TANTISIRA, THESIS CO-ADVISOR: PROF. HIROSHI WATANABE, ASSOC.PROF. MAYUREE TANTISIRA, 115 pp. ISBN 974-53-1503-6

The purpose of this study was to investigate the effects and underlying mechanisms of *Centella asiatica*'s ethyl acetate fraction (EACA) in animal model of epilepsy. We investigated an anticonvulsant activity of EACA against pentylenetetrazole (PTZ)-induced seizure, toxicity, drug interaction, possible mechanisms on neurochemical (amino acids neurotransmitter level in the hippocampus) and electrophysiological changes (GABA receptor current) as well as its effects on epileptogenesis and learning impairment in different animal models.

Orally given EACA, produced anticonvulsant activity against PTZ test in mice exhibiting the median effective dose (ED_{50}) of 673(299-1575) mg/kg BW, whereas the median neurotoxic dose (TD_{50}) as assessed by rotarod test was 415(147-1169) mg/kg BW. EACA seems to be very safe as the LD_{50} was found to be higher than 5,000 mg/kg BW. In isobolographic analysis for drug interaction, its additivity on some currently available antiepileptic drug namely phenytoin, valproate and gabapentin were observed. In relation to neurotoxicity, combination of gabapentin and EACA demonstrated a broader margin between the effective and the neurotoxic doses, while the other two combinations did not. Further study using microdialysis technique, demonstrated that in rats that EACA 700 mg/kg BW could protect the animals from PTZ-induced seizure, both aspartate and glutamate were gradually decreased while the glycine and GABA tended to increase. On the contrary, an increment of aspartate induced by PTZ was noted in animals receiving the same dose of EACA but demonstrated convulsion. By electrophysiological study, a slight potentiation of the GABA-induced current was observed when EACA at low concentration of 0.1 – 3 μg/ml were co-applied with GABA. However, the GABA-induced current was partially blocked at higher concentration of EACA (50 μg/ml). Taken all together the results obtained suggest the existence of several active constituents with antagonizing pharmacological profiles in EACA. In addition, EACA produced no effect on epileptogenesis and had no effect on lipid peroxidation. Though EACA seemed to have positive effect on learning and memory deficit and survival pyramidal neurons in PTZ-kindled mice, the results obtained was not yet conclusive.

The present studies suggested that the anticonvulsant activity of EACA might be, at least, related to a slight decrease of the level of excitatory amino acid neurotransmitter in conjunction with a small increase of inhibitory amino acid neurotransmitter of hippocampus including a slight potentiation of GABA-induced current. In addition, the additivity on some AEDs suggested the potential of EACA to be further developed as adjunctive medication for epileptic patients after identification and separation of the active substances which may be numerous and exhibiting different pharmacological profiles.

Field of study Physiology (Inter-depatrment) Student's signature Anusara Vattanajun

Academic year 2005 Advisor's signature Boonyong Tantisira

Co-advisor's signature H. Watanabe

Co-advisor's signature Mayuree Tantisira

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LIST OF ABBREVIATIONS

α	=	Alpha
β	=	Beta
γ	=	Gamma
µg	=	Microgram
µl	=	Microlitre
µm	=	Micrometre
µM	=	Micromolar
MΩ	=	Megaohm
ºC	=	Degree celsius
aCSF	=	Artificial cerebrospinal fluid
AD	=	Alzheimer's disease
AEDs	=	Antiepileptic drugs
AMPA	=	Alpha-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid
ANOVA	=	Analysis of variance
APV	=	2-amino-5-phosphonovaleric acid
BDZ	=	Benzodiazepine
BW	=	Body weight
CA	=	*Centella asiatica*
CA1	=	Area 1 of Ammon's horn
CA2	=	Area 2 of Ammon's horn
Ca^{2+}	=	Calcium ion
CA3	=	Area 3 of Ammon's horn
$CaCl_2$	=	Calcium chloride
Cl^-	=	Chloride ion
cm	=	Centimetre
CNS	=	Central nervous system
CR	=	Conditioned response
CS	=	Conditioned stimulus
CsCl	=	Cesium chloride
CSF	=	Cerebrospinal fluid

DC	=	Direct current
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DZP	=	Diazepam
EACA	=	Ethyl acetate fraction of *Centella asiatica*
ED_{50}	=	Median effective dose
EEG	=	Electroencephalogram
e.g.	=	Exampli gratia (for example)
EGTA	=	O,O′- Bis(2-aminoethyl)ethylene-glycol-N,N,N′,N′-tetraacetic acid
EPSP	=	Excitatory postsynaptic potential
et al.	=	et alii (and other)
etc.	=	et cetera
g	=	Gram
GABA	=	Gamma aminobutyric acid
GAD	=	Glutamic acid decarboxylase
GBP	=	Gabapentin
GC	=	Generalized clonic-tonic convulsion
Glu	=	Glutamate
^3H	=	Tritium (mass 3 isotope of hydrogen)
h	=	Hour
H_2O	=	Water
H_2O_2	=	Hydrogen peroxide
HEPES	=	N-[2-hydroxyethyl]piperazine-N′ [2-ethanesulfonic acid]
HPLC	=	High performance liquid chromatography
Hz	=	Hertz
I.D.	=	Inner diameter
i.p.	=	Intraperitoneal
K^+	=	Potassium ion
KCl	=	Potassium chloride
Kg	=	Kilogram

LD_{50}	=	Median lethal dose
LTP	=	Long-term potentiation
M	=	Molar
mA	=	Milliampere
MDA	=	Malondialdehyde
MES	=	Maximal electroshock seizure test
mg	=	Milligram
Mg^{2+}	=	Magnesium ion
$MgCl_2$	=	Magnesium chloride
mGluR	=	Metabotropic glutamate receptor
$MgSO_4$	=	Magnesium sulfate
min	=	Minutes
ml	=	Millilitre
mm	=	Millimetre
mM	=	Millimolar
mm^2	=	Square millimetre
ms	=	Millisecond
mV	=	Millivolt
Na^+	=	Sodium ion
Na_2-ATP	=	Disodium-adenosine triphosphate
NaCl	=	Sodium chloride
$NaHCO_3$	=	Sodium bicarbonate
nm	=	Nanometre
NMDA	=	*N*-methyl-D-aspartate
nmol	=	Nanomolar
NMR	=	Nuclear magnetic resonance
NSS	=	Normal saline solution
O.D.	=	Outer diameter
OPA	=	O-phthaldialdehyde
PHT	=	Phenytoin
PI	=	Protective index
p.o.	=	Per os
PSS	=	Physiological salt solution

PTZ	=	Pentylenetetrazole
rpm	=	Round per minutes
s.c.	=	Subcutaneous
SE	=	Status epilepticus
sec	=	Second
S.E.M.	=	Standard error of the mean
TBARS	=	Thiobarbituric acid-reacting substances
TD_{50}	=	Median neurotoxic dose
TLE	=	Temporal lobe epilepsy
UCS	=	Unconditioned stimulus
V	=	Volt
VPA	=	Valproate
v/v	=	Volume per volume
w/v	=	Weight per volume
w/w	=	Weight per Weight

CHAPTER I

INTRODUCTION

Worldwide, approximately 1-3% of the population suffers from epilepsy. For most of the patient population, pharmacotherapy represents the mainstay of treatment. However, advances in brain imaging and seizure mapping techniques have made surgery an option for patients in whom a defined resectable seizure focus can be identified. Vagal nerve stimulation represents a third possible option for patients whose seizures cannot be controlled by existing antiepileptic drugs (AEDs) or those who are not viable candidates for surgery (White, 2002). In addition, the ketogenic diet has resurfaced as a treatment option in certain types of epilepsy (Benbadis and Tatum, 2001). Unfortunately, despite these available therapeutic options, a significant number of patients with epilepsy continue to live with uncontrolled seizures, often at the expense of significant drug-induced adverse effects. More than half of them had some sort of cognitive problems with abnormal behavioral manifestations. These abnormalities are related to multiple factors including seizure type, age of onset, location of the focus, seizure frequency and type of electroencephalogram (EEG) pattern (Bornstein *et al*., 1988). Another factor that may affect cognition is AED medication. Although it is understood that the beneficial results of seizure suppression are of great clinical importance, there are indications of cognitive side effects of the drugs administered at the therapeutic doses especially with the polytherapy (Gillham *et al*., 1990; Nichols *et al*., 1993). Thus, there is a need for drugs, which can suppress epileptogenesis as well as prevent cognitive impairment.

It has been reported that the aqueous extract of *Centella asiatica* (CA), an indigenous herbaceous plant, has cognition-enhancing properties with an associated decrease in the brain oxidative stress parameters of the normal rats (Veerendra and Gupta, 2002). The aqueous extract of CA decreased the pentylenetetrazole (PTZ)-kindled seizures and showed improvement in the learning deficit induced by PTZ kindling as evident by decreased seizure score and increased latencies in passive avoidance behavior (Gupta *et al*., 2003). In addition, it was shown that the alcoholic extract of CA increased the gamma aminobutyric acid (GABA) level, which is a key

function of antiepileptic agent, in the central nervous system (CNS) in rats with dose-dependent manner (Brinkhaus *et al*., 2000). Therefore, it is possible that CA may be beneficial as adjuvant to AED in such a way of preventing cognitive impairment. However, a few data was available regarding the effect of CA in animal model of epilepsy.

In my preliminary study, it has been found that the ethyl acetate fraction of CA (EACA) showed anticonvulsant activity against PTZ-induced seizure. Therefore, this study aims to evaluate the mechanism(s) of anticonvulsant activity of an EACA in PTZ-induced convulsion with regards to its effect on the course of PTZ kindling-induced learning deficit in mice. Additionally, an adjuvant characteristic of the EACA to the currently existing AEDs was also examined.

CHAPTER II

REVIEW LITERATURES

2.1 *Centella asiatica* (CA)

CA is a plant in Family Umbelliferae, Subfamily Hydrocolyte. Its synonyms are Indian Pennywort (English), Hydrocolyte asiatique (French), Tsubo-kusa (Japanese), Luei Gong Gen (Chinese) etc. (Brinkhaus *et al*., 2000). This plant is known in Thai as Bua Bok (บัวบก).

2.1.1 Morphological description:

CA is a prostrate, perennial herb. The stem is glabrous, rooting at the nodes. The leaves are fleshy, orbicular-reniform, crenate, base cordate and often lobed and long-petioled (Fig. 1). The flowers are red, pink or white, in fascicled umbels. The fruits are oblong, dull brown, laterally compressed, 8 mm long, the pericarp hard, thickened, woody and white (Brinkhaus *et al*., 2000).

Figure 1 *Centella asiatica*

2.1.2 Major chemical constituents:

The major constituents in CA are the triterpenes asiatic acid and madecassic acid, and their derived triterpene ester glycosides, asiaticoside and madecassoside. Their structures are shown in Figure 2.

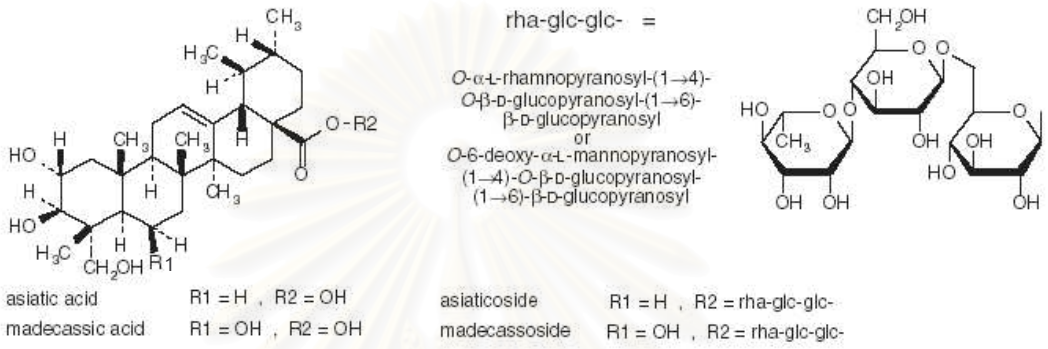

Figure 2 Structures of asiatic acid, madecassic acid, asiaticoside and madecassoside

2.1.3 Traditional applications:

The indications for the use of CA in the field of traditional medicine are extremely wide like gastrointestinal, dermatological, infectious, nephrological, neurological and other diseases (Brinkhaus *et al*., 2000).

- *Gastrointestinal disease:* it is used to treat dysentery, diarrhea, colicky abdominal pain, indigestion, gastric ulcers, gastritis and inflammatory diseases of the liver.

- *Dermatological diseases:* it was used as adjuvant therapy in the treatment of leprous lesion.

- *Infectious diseases:* it was used as adjuvant treatment in cholera, syphilis and also used to treat influenza as well.

- *Nephrological and urogenital diseases:* it is used to treat inflammatory diseases of the urogenital system and also used as a diuretic.

- *Neurological and psychiatric diseases:* it is employed to treat psychiatric problems, epilepsy and hysteria.

- *Other diseases:* in addition to the conditions already mentioned CA continues to be used to treat asthma, anemia, high blood pressure, diabetics and inflammatory diseases of the eye and is also useful to combat physical and mental exhaustion.

2.1.4 Medical research:

The pharmacological activity of CA is thought to be due to several saponin constituents, including asiaticoside, asiatic acid and madecassic acid.

- *Antiinflammatory:* Madecassol was reported to reduce acute skin reactions in radiotherapy-treated rats (Chen *et al*., 1999).

- *Antioxidant*: Asiaticoside enhanced induction of antioxidant levels at an initial stage of healing (Shukla *et al*., 1999).

- *Antitumor:* The methanolic extract of CA has shown significant cytotoxicity to Ehrlich ascites tumor cells and Dalton's lymphoma ascites tumor cells (Babu *et al*., 1995).

- *Antiulcer:* An extract of CA significantly inhibited gastric ulceration induced by cold and restraint stress in Charles-Foster rats (Chatterjee *et al*., 1992).

- *Wound-healing:* Titrated extract of CA stimulates collagen and glycosaminoglycan synthesis in rats surgically inserted with stainless steel wound chambers (Maquart *et al*., 1999).

- *Central Nervous system (CNS):* Asiaticoside derivatives were found to inhibit or reduce hydrogen peroxide (H_2O_2) induced cell death and lower intracellular free radical concentration, protecting against the effects of beta-amyloid neurotoxicity (Mook-Jung *et al*., 1999). An extract of CA was found to increase brain GABA levels in dose dependent manner (Chatterjee *et al*., 1992). The aqueous extract of CA has cognition-enhancing properties which were associated with a decrease in the brain oxidative stress parameters of the normal rats (Veerendra and Gupta, 2002). The aqueous extract of CA decreased the PTZ-kindled seizures and improved the learning deficit induced by PTZ kindling as evident by decreased

seizure score and increased latencies in passive avoidance behavior (Gupta *et al*., 2003).

2.2 Epilepsy

Epilepsy is one of the most common neurologic disturbance affecting humans. Up to almost 10% of the population will experience at least one seizure in their lifespan. Of these, nearly half will be subject to recurrent seizures. Seizures and epilepsy are particularly troublesome for patients because of their unpredictable occurrence and associated abrupt loss of competence. This often results in severe social and personal morbidity with attendant loss of self confidence, personal safety, financial and recreational independence (Filloux, 2005). In recent years, important advances have been made in the diagnosis and treatment of seizure disorders. However, understanding of the cellular and molecular mechanisms by which epilepsy develops, or epileptogenesis, is still incomplete (Chang and Lowenstein, 2003).

2.2.1 Terminology: seizure and epilepsy

The term “seizure” refers to a transient change of behavior due to the disordered, synchronous and rhythmic firing of populations of CNS neurons. The term “epilepsy” refers to a disorder of brain function characterized by the periodic and unpredictable occurrence of seizures. Seizures can be “nonepileptic” when evoked in a normal brain by treatments such as electroshock or chemical convulsions or “epileptic” when occurring without apparent provocation (McNamara, 1994).

2.2.2 Epileptic seizure classification

Epileptic seizures have been classified into partial seizures which begin focally at a cortical site and consciousness is not lost and generalized seizures, which entail widespread involvement of the cortex of both hemispheres from outset and the essential clinical feature is loss of consciousness (McNamara, 1994; Filloux, 2005). Both partial and generalized seizure types can each be divided into:

2.2.2.1 Partial seizures

- Simple partial seizure

: associated with preservation of consciousness

- Complex partial seizure

: associated with impairment of consciousness and majority originate in the temporal lobes.

2.2.2.2 Generalized seizures

- Absence seizures

: characterized by the abrupt cessation of ongoing activities associated with a blank stare lasting a few to 30 sec and followed by an abrupt return to normal behavior.

- Myoclonic seizures

: consists of a brief, shock-like contraction of muscles, which may be confined to part of one extremity or may be generalized.

- Tonic seizures

: consists of the sustained muscle contraction.

- Clonic seizures

: consists of muscle contraction alternating with periods of muscle relaxation.

- Tonic-clonic seizures

: involves muscle groups throughout the body, associated with loss of consciousness and typically lasts 30-60 sec.

2.2.3 Epileptogenesis

Epileptogenesis is thought of as a cascade of dynamic biological events altering the balance between excitation and inhibition in neural networks. The term applies to any of the progressive biochemical, anatomical, and physiological changes leading up to recurrent seizures. Understanding these changes and clarifying factors which are and are not beneficial is a key to prevent the onset of epilepsy (Jacobs *et al.*, 2001).

Complex partial epilepsy is the most common form of epileptic syndrome, accounting for approximately 40% of all cases of epilepsy. However, understanding of the cellular and molecular mechanisms by which complex partial epilepsy develops is still incomplete. The most prevalent of these syndromes features complex partial epilepsy arising from the mesial temporal lobe, because its surgical resection virtually eliminates epileptic seizures in 80-90% of patients who are refractory to conventional medical therapy (McNamara, 1994).

Histologic abnormalities of the resected tissues provided clues to the pathogenesis of the disorder. The most common abnormality observed is hippocampal sclerosis (Liu *et al*., 1995; Blümcke *et al*., 1999). In hippocampal sclerosis, there is selective loss of neurons in the dentate hilus and the hippocampal pyramidal-cell layer, with relative preservation of dentate granule cells and a small zone of pyramidal cells (CA2). The dense gliosis that accompanies the loss of neurons causes shrinkage and hardening of tissue. It is possible that hippocampal sclerosis represents a pathologic final common pathway to partial epilepsy from a number of different causes (McNamara, 1994; Chang and Lowenstein, 2003).

Detailed studies of the morphologic changes in hippocampal sclerosis have led to several hypotheses about the mechanism of epileptogenesis in this condition (Fig. 3). The best-described change is the sprouting of mossy-fiber axons from dentate granule cells (Parent and Lowenstein, 1997). Normally, excitatory input to the hippocampus comes directly to the hippocampal dentate granule cells from the neighboring entorhinal cortex, whereas inhibitory input arises locally from interneurons in the inner molecular layer. The dentate granule cells sprout mossy fiber axons, which extend to pyramidal neurons as part of the hippocampal output pathway. Normal dentate granule cells appear to be relatively resistant to hypersynchronous activation and may actually serve to inhibit the propagation of seizures from the entorhinal cortex (Engel, 2001). In hippocampal sclerosis, however, these cell sprout mossy-fiber axons that are directed back into the inner molecular layer (Tauck and Nadler, 1985; Sutula *et al*., 1989; Babb *et al*., 1991), possibly because the neurons to which they usually extend have been lost. There is some evidence that these aberrant mossy fibers instigate a recurrent excitatory circuit by

forming synapses on the dendrites of neighboring dentate granule cells (Okazaki *et al*., 1995; Wuarin and Dudek, 1996). Although such a circuit is a plausible explanation for hyperexcitability, the causative role of mossy-fiber sprouting in epileptogenesis is still largely speculative. There is, for example, strong evidence that newly sprouted axons also form synapses on inhibitory interneurons as part of a feedback mechanism, rather than simply increase excitation (Sloviter, 1992; Kneisler and Dingledine, 1995).

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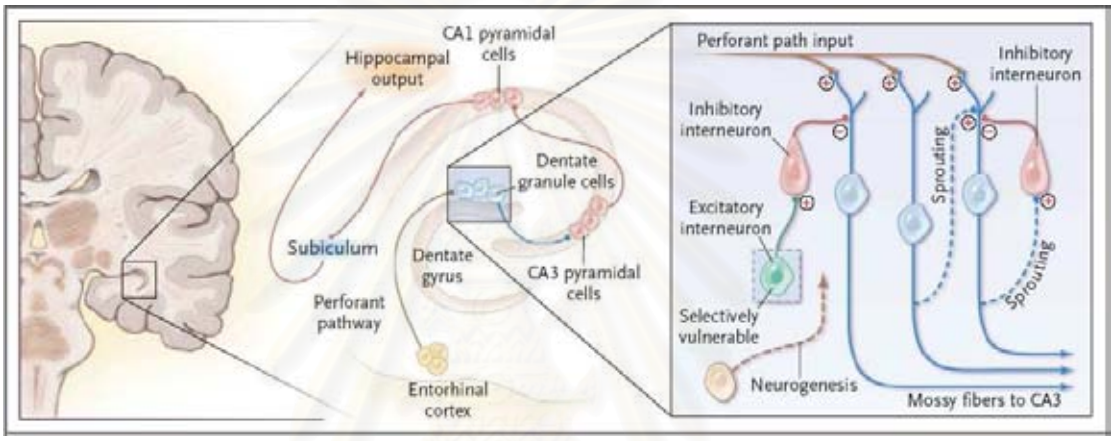


Figure 3 Hippocampal sclerosis is the most common identified pathological feature in cases of mesial temporal lobe epilepsy. Normally, input to the hippocampus comes from the entorhinal cortex to the dentate granule cells through the perforant path. Dentate granule cells project to the CA3 sector as the first step in the hippocampal output pathway. A close-up of the dentate granule cell layer reveals several morphologic changes characteristic of hippocampal sclerosis that may play a part in epileptogenesis. Newly sprouted mossy fibers from dentate granule cells can synapse on dendrites of neighboring dentate granule cells, resulting in a recurrent excitatory circuit. They can also sprout onto inhibitory interneurons. Excitation interneurons, which normally activate inhibitory interneurons, may be selectively vulnerable to brain insults. Finally, neurogenesis of new dentate granule cells continues into adult life, and these neurons may integrate themselves into abnormal circuits. (Chang and Lowenstein, 2003).

Some investigators have suggested that the selective vulnerability of certain neurons may be a mechanism of epileptogenesis in hippocampal sclerosis. In animal models, excitatory interneurons located within the dentate gyrus, which normally activate inhibitory interneurons, appear to be selectively lost. Loss of these excitatory cells would be expected to impair the inhibitory feedback and feed-forward mechanisms that act on dentate granule cells, resulting in hyperexcitability. This explanation is plausible, but the evidence that dentate granule cells can project directly onto inhibitory interneurons raises the possibility of a compensatory mechanism. For these reasons, the implications of selective cell loss in the hippocampus, although highly suggestive of a mechanism of epileptogenesis, are still not completely understood (Chang and Lowenstein, 2003).

An intriguing hypothesis lies in the phenomenon of neurogenesis. Almost all neurons in the brain are postmitotic and do not divide in adults, but progenitor cells in the dentate gyrus of the hippocampus are known to divide. Postnatal neurogenesis in the hippocampus can occur throughout life (Eriksson *et al*., 1998). In an animal model of temporal-lobe epilepsy induced with the use of pilocarpine, seizures can trigger increased mitotic activity in a proliferative area of dentate gyrus, resulting in the differentiation of new dentate granule cells (Parent *et al*., 1997). This process may be independent of mossy fiber sprouting, which appears to involve mature dentate granule cells rather than newly differentiated ones (Parent *et al*., 1999). The functional significance of neuronal generation in the hippocampus after a brain insult is uncertain, although some evidence suggests that new dentate granule cells become abnormally integrated into neuronal circuits. The potential clearly exists for an imbalance between excitation and inhibition as new neurons differentiate and form synaptic connections. Since the genesis of dentate granule cells recapitulates similar processes early in the development of the nervous system, this mechanism could be relevant to epileptogenesis both early and later in life (Scharfman *et al*., 2000).

In addition to these morphologic features, changes at the molecular level may also be important. The most prominent of these are alterations in the composition and expression of $GABA_A$ receptors on the surface of hippocampal

dentate granule cells. Normally, $GABA_A$ receptors in adults, which consist of five subunits, serve as inhibitors, hyperpolarizing the neuron by allowing passage of chloride ions when activated. In the pilocarpine model of temporal lobe epilepsy, however, the expression of various $GABA_A$ receptors subunits in dentate granule cells is altered, and these altered receptors have heightened sensitivity to zinc, which is abundant in mossy fiber terminals (Brooks-Kayal *et al*., 1998). Because this molecular change precedes the onset of spontaneous seizures, it is a plausible mechanism of epileptogenesis. Such findings have implication for treatment, since various anticonvulsant drugs act through the $GABA_A$ receptors (McNamara, 1994).

2.2.4 Animal model of epilepsy

Mechanisms related to partial seizures have been exploited experimentally in the development of animal models of temporal lobe epilepsy (TLE). Many (but certainly not all) models exhibit spontaneous seizures of limbic origin. Some exhibit primarily evoked seizures, like the kindling model. Other models have spontaneous seizures, like the kainate or pilocarpine models, but rarely have complex partial seizures as seen in many patients. No animal model completely mimics the features of human epilepsy. Clearly, there are experimental advantages to have models which exhibit varying features of the clinical spectrum of limbic epilepsy. This facilitates elucidation of critical pathophysiological mechanisms. Having a single model that exhibits all the features and variability evident in the clinical presentation of limbic epilepsy would be as difficult to study as the patients themselves (Jacobs *et al*., 2001; Coulter *et al*., 2002).

⊙ Pentylenetetrazole induced seizure test (PTZ test) and kindling

PTZ is a selective blocker of the chloride ionophore coupled to $GABA_A$ receptor and has convulsant effects on acute administration (Sejima *et al*., 1997). The behavioral seizure that results from the administration of subcutaneous PTZ is markedly different from the maximal electroshock seizure (MES) test. Depending on the dose administered, PTZ can produce myoclonic jerks; repeated clonic seizures of the vibrissae, forelimbs, and hindlimbs without loss of righting reflex; clonic seizures of the limbs with loss of righting reflex; and loss of righting

reflex followed by tonic extension of the forelimbs and hindlimbs. The importance of this point relies on the observation that the pharmacologic profile of the PTZ test differs depending on the endpoint selected (White *et al*., 1998).

The amino acidic pattern may play a role in PTZ-induced seizures (Sechi *et al*., 1997). Examination of PTZ-induced convulsions revealed that the levels of aspartate, glutamate, glycine and taurine were elevated in the cerebrospinal fluid (CSF) during myoclonic jerks and more distinctly immediately after generalized clonic-tonic convulsions (GC). During the recovery period of postictal depression seen in EEG (5 min after GC), the amino acids levels were lower than in the control group. PTZ-induced irritative activity in the EEG disappeared in 24 h but the levels of amino acids remained abnormal (Halonen *et al*., 1992).

Kindling, a model of epileptogenesis, refers to the phenomenon whereby repeated electrical or chemical of the brain with initially subconvulsive currents or doses results in progressive reduction of the threshold for induction the development of partial and secondarily generalized seizures, reflecting epileptogenesis (Potschka and Loscher, 1999; Kupferberg, 2001; Godukhin, 2005). The kindling-induced enhanced seizure activity is understood to indicate a long-lasting alteration of the neuronal excitability, which can be regarded as suggestive of neuronal plasticity (Cain, 1989). As an epilepsy experimental model associating neuronal plasticity and seizures, kindling is unique in providing opportunities to study the ability of drugs to modify these progressive changes (Silva *et al*., 1998). In addition, Pitkanen (2002) suggested that compounds that alleviate the initial brain injury and delay the development of kindling reduce the risk of epileptogenesis after brain lesions. Three parameters were used to measure the antiepileptogenic effects: (1) percentage of animals that develop epilepsy, (2) latency from the insult to the first and second spontaneous seizure (e.g. delay of epileptogenesis), and (3) efficacy to prevent or delay the development of lowered seizure threshold (susceptibility to kindling or to seizures induced by chemoconvulsants) after epileptogenic insults.

Chemical kindling is an experimental model of epilepsy consisting of progressive behavioral and electrographic changes induced by repetitive

administration of initially subconvulsive amounts of excitatory drugs such as PTZ. The mechanisms by which enhanced susceptibility to convulsions results from chronic PTZ administration involve alterations in inhibitory and excitatory systems (Rocha *et al*., 1996). It impaired GABA-coupled chloride channel activity (Corda *et al*., 1990) and was also associated with a cognitive deficit (Becker *et al*., 1992). Examination of PTZ-kindled rat brains revealed a significant neuronal cell loss priority in vulnerable hippocampal CA1 and CA3 structures and in the hilus which was possibly the cause of observed cognitive deficits (Pohle *et al*., 1997). Following PTZ-kindling, the glutamatergic transmitter system is modified (Rocha *et al*., 1996; Sejima *et al*., 1997). The enhanced activity of glutamatergic systems induces an increased formation of free oxygen radicals which may amplify again the basal release of excitatory amino acids, thus increasing intracellular calcium which finally results in neuronal cell death. It is reported that free radical generation due to the increased activity of the glutamatergic transmitter plays a crucial role in neuronal cell death of PTZ-kindling in rats (Rauca *et al*., 1999; Han *et al*., 2000).

There are a few studies using chemical kindling in mice. However, Silva *et al*., (1998) indicated that mice can be used in a reliable model of PTZ-induced kindling as in rats. They found that the development of PTZ-induced kindling in mice was prevented by the coadministration of phenobarbital or diazepam. They also found that PTZ-induced kindling in mice increased the specific [^{3}H] glutamate binding in the cerebral cortex, therefore allowing screening new drugs that can interfere in the plastic changes believed to underlie epileptic phenomena. In addition, the major advantage of using mice is that a large number of animals can be kindled simultaneously at minimal cost. An additional benefit to the use of mice is that the amount of compound required for testing is smaller than that for rats due to the size difference (Kupferberg, 2001).

2.2.5 Amino acid neurotransmitters and their receptors in epilepsy

2.2.5.1 Excitatory neurotransmission

In the adult CNS, L-glutamate and L-aspartate are the most likely candidates for neurotransmitter action at excitatory amino acid receptors. They are used by some of the most widely distributed neuronal types (Siegel, 1999). These excitatory amino acids are undoubtedly involved in the generation of seizure events (Meldrum, 1984).

Glutamate is synthesized from glutamine by the action of the enzyme glutaminase in glutamatergic neurons (Daikhin and Yudkoff, 2000). Following synaptic release, glutamate exerts its pharmacological effects on several receptors, classified into ionotropic and metabotropic families. Ionotropic glutamatergic receptor can be subdivided in those sensitive to *N*-methyl-D- aspartate (NMDA) and those sensitive to α-amino-3-hydroxy-5-methyl-isoxazole-4-proprionate / kainite which are regarded as non- NMDA receptors (Watkins *et al*., 1990). The NMDA receptor is distinguished by having glycine as a co-agonist. NMDA receptors increase predominantly sodium and calcium conductances and non-NMDA receptors mediated excitation results in elevated sodium permeability (Monaghan *et al*., 1989; Zorumski and Thio, 1992).

The NMDA-type channel opens and closes relatively slowly in response to glutamate and thus contributes to the late phase of EPSP while the non-NMDA ionotropic receptor generates the early component. This late phase of the EPSP is normally small after a single presynaptic action potential, because of Mg^{2+} blockade of the channel. However, when the presynaptic neuron fires repeatedly so that the EPSPs summate to depolarize the postsynaptic cell by 20 mV or more, the NMDA receptor gives rise to a much larger current. This current is carried by Ca^{2+}. Thus activation of the NMDA receptor leads to the activation of calcium-dependent enzymes and certain second messenger-dependent protein kinases in the postsynaptic cell (Kandel *et al*., 2000).

It has come out during the last decade that apart from ionotropic receptors for glutamate, there exists a population of metabotropic glutamate receptors

(mGluRs). The eight mGluRs known so far are divided into three groups, according to their sequence homology, signal transduction mechanisms and agonist selectivity (Pin and Duvoisin, 1995). Group I includes mGluR1 and mGluR5, which are linked to the activation of phospholipase C. Group II and III include all other types, negatively coupled to adenylyl cyclase (Bordi and Ugolini, 1999). Recent studies revealed separate functions for two group receptor subtypes. Glutamate stimulation produces a single peak of intracellular Ca^{2+} mobilization through mGluR1, but Ca^{2+} oscillations through mGluR5 receptors (Nakanishi *et al*., 1998).

An imbalance in excitatory transmitters such as glutamate may, under certain circumstances, contribute to disease. Most cells in the brain have receptors that respond to L-glutamate. Excessive amounts of glutamate are highly toxic to neurons, an action called glutamate excitotoxicity (Kandel *et al*., 2000). NMDA channel activation has implications on two levels (Wasterlain *et al*., 1993, Fountain and Lothman, 1995). First, it results in a prolonged cellular depolarization, which allows more NMDA channels to open in a self-reinforcing process that may sustain status epilepticus. Second, it allows intracellular accumulation of Ca^{2+}, which can activate many pathological intracellular processes. Accumulation of intracellular ions may mediate acute necrotic cell death via osmotic stress and other mechanisms (Jope *et al*., 1991). Intracellular Ca^{2+} can also activate a cascade of events that may lead to apoptotic programmed cell death, e.g., by activating immediate early genes (Pollard *et al*., 1994). Apoptosis may occur during a longer time course than necrotic cell death and may continue after cellular depolarization has stopped. This mechanism can only be applied to locations where NMDA receptors are especially concentrated, such as the hippocampus and other parts of the limbic system, which explains why neuronal loss is selective to the hippocampus (Monaghan and Cotman, 1985). The resulting injury and long-term changes in the limbic system lead to subsequent epilepsy.

2.2.5.2 Inhibitory neurotransmission

GABA is the chief inhibitory neurotransmitter in the mammalian brain. Along with glycine, that primarily has effects in the spine, brainstem and retina, it is responsible for the vast majority of all inhibitory

neurotransmission in the CNS. Between 20-50% of all central synapses use GABA as their transmitter. The enzyme responsible for the formation of GABA from the amino acid glutamate is glutamic acid decarboxylase (Dawson *et al*., 2005).

There was three types of receptors for GABA in the mammalian CNS, designated A, B and C. The $GABA_A$ and $GABA_C$ receptors are GABA-gated chloride ion-conducting channels while the $GABA_B$ receptor is a member of the seven transmembrane helix-containing, guanine nucleotide-binging receptor G-protein-couple receptors. The $GABA_A$ and $GABA_C$ receptors were initially distinguished by their sensitivity to the ligand bicuculline with the $GABA_A$ being antagonized by it while the $GABA_C$ were insensitive (Bernard *et al*., 1998). While varieties of the $GABA_A$ receptor are found all over the CNS, the $GABA_C$ receptors are primarily found in the retina in which their physiological significance is a matter of dispute (Enz and Cutting, 1998). $GABA_B$ receptors are found in central and peripheral autonomic nervous system (Chen *et al*., 2005).

$GABA_A$ receptor complex consists of a number of binding sites for GABA itself, benzodiazepine, barbiturates, ethanol and picrotoxin which is a chloride channel blocker. When GABA binds to its recognition site on the $GABA_A$ receptor complex, an opening of the chloride channel occurs with the subsequent influx of Cl^- into a neuron, resulting in its hyperpolarization (Olsen *et al*., 2004). Benzodiazepine derivatives (diazepam, clonazepam) increase the frequency of the channel openings whilst barbiturates (phenobarbital) prolong the opening time of the channel. Both, benzodiazepines and barbiturates also enhance the affinity of $GABA_A$ receptors for the neurotransmitter. In contrast, binding GABA to the $GABA_B$ receptors result in the activation of phospholipase A-2 and the following synthesis of arachidonic acid from phospholipids. Arachidonic acid via regulatory G_i proteins is likely to modulate the activity of adenyl cyclase and cyclic AMP levels affect the neurotransmitter release (Czapinski *et al*., 2005).

Occurrence of GABA in the central nervous system was demonstrated in 1950 and in the same decade GABA was shown to inhibit seizure activity after its direct cerebral application in dogs (Meldrum, 1978). This gave rise to the assumption that GABAergic inhibition may be an important factor in the

suppression of seizure activity in epileptic patients. GABA itself was not a good candidate for an antiepileptic drug since it very poorly entered the brain through the blood-brain barrier. Much attention was paid to a synthesis of GABAergic agonists, which would easily penetrate into the central nervous system (Czapinski *et al*., 2005).

2.2.6 Drugs for treatment of epilepsy

The ultimate determinant in the successful treatment of epilepsy is the patient's ability to take medications consistently over a long period. To achieve the best outcomes in the treatment of epilepsy, the following are required of a medication: (a) efficacy; (b) lack of neurotoxicity at effective doses; (c) lack of systemic toxicity (e.g. teratogenicity and idiosyncratic effects); and (d) ease of use (convenient dosing schedule and lack of drug interactions) (Jacobs *et al*., 2001; Leppik, 2001).

Although the mechanisms of action of the currently marketed AEDs are still not completely understood, they ultimately involve alteration of the balance between neuronal excitation and inhibition (White, 1999). At the cellular level, three basic mechanisms are recognized: modulation of voltage-dependent ion channels (Na^+, Ca^{2+}, K^+), enhancement of GABA-mediated inhibitory neurotransmission and attenuation of excitatory (particularly glutamate-mediated transmission (Meldrum, 1996). The currently used AEDs according to their principle mechanisms of action are showed in Table 1. It is, however, increasingly recognized that many AEDs possess multiple primary mechanisms. Although all of the available AEDs are effective when prescribed for the appropriate epilepsy syndrome, they differ in their pharmacokinetic and side-effect profiles (Leppik, 2001).

Table 1 Main mechanisms of actions of old- and new-generation AEDs (Perucca., 2005)

	Blockade of voltage-dependent sodium channels	Increase in brain or synaptic GABA levels	Selective potentiation of $GABA_A$-mediated responses	Direct facilitation of chloride ion influx	Blockade of calcium channels	Other actions
First-generation AEDs						
Benzodiazepines	−	−	++	−	−	−
Carbamazepine	++	?	−	−	+ (L-type)	+
Ethosuximide	−	−	−	−	++ (T-type)	−
Phenobarbital	−	+	+	++	?	+
Phenytoin	++	−	−	−	?	+
Valproic acid	?	+	?	−	+ (T-type)	++
Second-generation AEDs						
Felbamate	++	+	+	−	+ (L-type)	+
Gabapentin	?	?	−	−	++ (N-, P/Q-type)	?
Lamotrigine	++	+	−	−	++ (N-, P/Q-, R-, T-type)	+
Levetiracetam	−	?	+	−	+ (N-type)	++
Oxcarbazepine	++	?	−	−	+ (N- and P-type)	+
Pregabalin	−	−	−	−	++ (N-, P/Q-type)	−
Tiagabine	−	++	−	−	−	−
Topiramate	++	+	+	−	+ (L-type)	+
Vigabatrin	−	++	−	−	−	−
Zonisamide	++	?	−	−	++ (N-,P-,T-type)	+

++, Primary action; +, secondary action; -, no action described; ?, controversial evidence; GABA, γ-aminobutyric acid. Modified from Perucca (8).

2.2.6.1 Targets for antiepileptic drug action

2.2.6.1.1 Ion channels

⊙ Na^+ channels

Brain voltage-gated sodium channels are the molecular targets of a number of chemically diverse antiepileptic drugs. These entire drugs act by inhibiting ionic current through the channel, but the precise way in which this result in protection against seizures is incompletely understood (Rogawski, 2002).

Voltage-dependent Na^+ channels are responsible for the upstroke of the neuronal action potential, and ultimately control the intrinsic excitability of the nervous system (Porter and Rogawski, 1992). The neuronal Na^+ channel has a multi-subunit structure that forms a Na^+-selective, voltage-gated pore through the plasma membrane (Ragsdale and Avoli, 1998). The main structural component of the neuronal Na^+ channel is the α-subunit, which forms the ion conducting pore and confers voltage dependency (Catterall, 1992). In the mammalian brain, the α-subunit associates with two auxiliary subunits designed β_1 and β_2. The β-

subunits are not required for basic Na^+ channel activity, but they modulate the expression and function of individual channels (Ragsdale and Avoli, 1998).

At normal membrane potentials, most Na^+ channels exist in a closed, resting state. Upon depolarization, the channel activates, facilitating ion flux. Thereafter, the Na^+ channel enters an inactivated state, from which it is not readily re-activated. Repolarization of the neuronal membrane rapidly converts the channel back to a resting state, from which it can respond to subsequent depolarizations (Catterall, 1992; Ragsdale and Avoli, 1998). Neuronal Na^+ channels can cycle through these functional states within a few milliseconds. This characteristic is essential for sustaining the rapid bursts of action potentials necessary for some normal brain functions, and is implicated in the production of epileptic discharges. The neuronal Na^+ channel represents one of the most important targets for AED action (Upton, 1994; MacDonald and Kelly, 1995; Meldrum, 1996; White, 1999).

Antiepileptic agents that act on Na^+ channels characteristically exhibit protective activity in the MES test, a widely used animal model for the screening of AEDs, and they are effective in the treatment of partial and generalized tonic-clonic seizures in humans (Ragsdale and Avoli, 1998; Rogawski and Porter, 1990). These drugs have the unique property that they block high-frequency repetitive spike firing, as is believed to occur during the spread of seizure activity, without affecting ordinary ongoing neuronal activity. This accounts for their ability to protect against seizures without causing a generalized impairment of brain function (Rogawski, 2002).

⊙ Ca^{2+} channels

Voltage-gated Ca^{2+} channels, like Na^+ channels, are multisubunit protein complexes that permit ion flux when gated open by membrane depolarization (Catterall, 2000). However, there are a larger number of functional Ca^{2+} channel types with a correspondingly greater diversity of functional roles in neurons (Rogawski, 2002). Voltage-sensitive Ca^{2+} channels can be broadly classified into low or high threshold, according to the membrane potential at which

they are activated (Hofmann *et al*., 1994). The low-threshold T-type Ca^{2+} channel is expressed predominantly in thalamocortical relay neurons, where it is believed to be instrumental in the generation of the rhythmic 3-Hz spike-and-wave discharge that is characteristic of generalized absence seizures (Coulter *et al*., 1989a). High-threshold Ca^{2+} channels are subclassified by their pharmacological properties into L-, N-, P-, Q- and R-types (Hofmann *et al*., 1994). These channels are distributed throughout the nervous system on dendrites, cell bodies and nerve terminals. The N-, P- and Q-type channels, in particular, have been implicated in the control of neurotransmitter release from presynaptic nerve terminals (Stefani *et al*., 1997). They represent potential anticonvulsant targets because blockade of these channels inhibits neurotransmitter release (Turner, 1998). However, as yet, there are no practical anticonvulsants that specifically target these channels, with the possible exception of gabapentin, which binds with high affinity to certain calcium channel subunits (Rogawski, 2002).

⊙ K^+ channels

Neuronal K^+ channels are large protein complexes that form tetrameric structures, the monomers of which are structurally and genetically related to the α- and α_1-subunits of the Na^+ and Ca^{2+} channel, respectively. The association of four subunits (monomers) in the neuronal membrane is required for the formation of a K^+-sensitive pore and, therefore, channel function (Barchi, 1998).

At the neuronal level, K^+ channels are intimately involved in excitability. They are responsible for the action potential downstroke or, more specifically, repolarization of the plasma membrane in the aftermath of Na^+ channel activation. Direct activation of voltage-dependent K^+ channels hyperpolarizes the neuronal membrane and limits action potential firing (Porter and Rogawski, 1992). Accordingly, K^+ channel activators have anticonvulsant effects in some experimental seizure models (Gandolfo *et al*., 1989; Rostock *et al*., 1996), whereas K^+ channel blockers precipitate seizures (Yamaguchi and Rogawski, 1992).

2.2.6.1.2 γ-aminobutyric acid-mediated inhibition

GABA is the predominant inhibitory neurotransmitter in the mammalian CNS. Impairment of GABA function is widely recognized to provoke seizures, whereas facilitation has an anticonvulsant effect (Löscher, 1998).

Several AEDs exert their effects, at least in part, by actions on the GABAergic system. Increased GABA synthesis, increased release, allosteric receptor facilitation and reduced inactivation have all been implicated in the mechanism of action of commonly used agents (Sills *et al*., 1999). The GABA system also represents the most successful target for the rational design of novel antiepileptic compounds (Löscher, 1998).

2.2.6.1.3 Glutamate-mediated excitation

Glutamate is the principle excitatory neurotransmitter in the mammalian brain. Focal injection of glutamate induces seizures in animal and over-activation of glutamatergic transmission or abnormal glutamate receptor properties are observed in certain experimental seizure models and human epilepsy syndromes. Inhibition of the neuronal release of glutamate and blockade of its receptors has received considerable attention in the search for novel AEDs (Meldrum, 2000).

Although none of the commonly used AEDs exert their pharmacological effects solely by an action on the glutamate system, blockade of ionotropic glutamate receptors is believed to contribute to the antiepileptic activity of several compounds (Upton, 1994; White, 1999).

2.2.6.2 Mechanisms of some antiepileptic drugs

2.2.6.2.1 Phenytoin (PHT)

Phenytoin (Fig. 4) is known by the brand name dilantin. PHT has become a first-line treatment for partial and generalized tonic-clonic seizures (Brodie and Dichter, 1996).

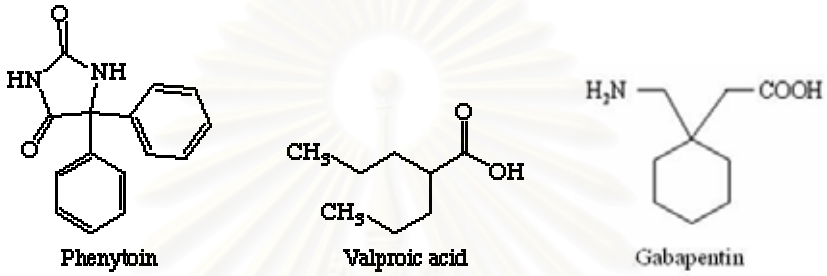


Figure 4 Molecular structure of phenytion, valproic acid and gabapentin (Kwan *et al*., 2001)

PHT is believed to exert its anticonvulsant effect primarily by an action on voltage-dependent Na^+ channels (Tunnicliff, 1996), binding to the fast inactivated state of the channel and reducing the frequency of sustained repetitive firing of action potentials, without affecting their amplitude or duration (McLean and Macdonald, 1983). PHT inhibits high-frequency repetitive firing in a voltage-dependent manner, with limitation of firing increased after depolarization and removed by hyperpolarization (Schwartz and Grigat, 1989). Na^+ channel blockade with PHT is also frequency-dependent, being more effective at higher frequencies of neuronal stimulation. An effect on persistent Na^+ currents has also been suggested (Lampl *et al*., 1998). The precise binding site for PHT on the Na^+ channel is unclear, and there may be a preferential effect on different subtypes of Na^+ channels (Song *et al*., 1996).

PHT is highly (average 90%) plasma protein bound, is extensively metabolized in the liver and consequently interacts with many drugs,

including other AEDs (Leppik, 2001). The half-life is about 22 hours, with a range of 7 to 42 hours. The half-life is shorter in children and longer in the elderly.

PHT has a relatively narrow therapeutic range, with the generally accepted values of 10-20 µg/ml being the usual effective range. It can cause a range of dose-related and idiosyncratic adverse effects. Neurotoxic symptoms (drowsiness, dysarthria, tremor, ataxia and cognitive difficulties) become increasingly likely when the plasma drug concentration exceeds 20 µg/ml. It may also be associated with mild-to-moderate cognitive side effects, especially in doses in the higher therapeutic range. The teratogenic effect of PHT has been the subject of much controversy (Brodie and Dichter, 1996; Leppik, 2001).

2.2.6.2.2 Valproic acid (VPA)

Valproic acid (Fig. 4) is known by the brand name depakene. VPA has proved to be an extremely useful AED, with a broad spectrum of activity and particular efficacy in the generalized epilepsies (Brodie and Dichter, 1996; Leppik, 2001).

VPA has been reported to block voltage-dependent Na^+ channels. It reduces sustained repetitive firing of mouse neurons in culture (McLean and MacDonald, 1986), inhibits Na^+ channels in *Xenopus leavis* myelinated neurons (van Dongen *et al*., 1986) and reduces Na^+ currents in neocortical neurons (Zona and Avoli, 1990). VPA may also block T-type Ca^{2+} channels like those implicated in the spike-wave activity of absence seizures. However, the reduction of T-type Ca^{2+} currents observed with VPA in rat primary afferent neurons in modest and requires relatively high drug concentrations (Kelly *et al*., 1990). In addition, VPA appears to have no effect on Ca^{2+} channel conductance in rat thalamic neurons (Coulter *et al*., 1989c).

There is evidence to suggest that VPA elevates whole brain GABA levels and potentiates GABA responses, possibly by enhancing glutamic acid decarboxylase(GAD) activity and inhibiting GABA degradation (Löscher, 1999). Anecdotal reports suggest that the drug also augments GABA release and blocks

GABA uptake (Sills *et al*., 1996). Single doses of VPA decrease brain levels of the excitatory amino acid aspartate, without influencing those of glutamate or GABA (Schechter *et al*., 1978). Decreases in aspartate concentration have been shown to correspond with the period of anticonvulsant activity in animal models (Chapman *et al*., 1983).

VPA is highly bound (90%) to proteins in the blood. Thus, only 10% is free or unbound and able to enter the brain. More than 95% of VPA is broken down in the liver by several different metabolic pathways. The half-life ranges from 8 to 16 hours, with shorter times in children and longer times in the elderly. Common side effects of VPA are dose-related tremor, weight gain due to appetite stimulation, some gastric irritation, thinning or loss of hair (usually temporary), and menstrual irregularities, including amenorrhea. Sedation is unusual, although stupor and encephalopathy occur in rare cases (Brodie and Dichter, 1996; Leppik, 2001).

2.2.6.2.3 Gabapentin (GBP)

Gabapentin (Fig. 4) is known by the brand name neurontin. It is a novel compound, structurally related to GABA, which is effective in the adjunctive treatment of partial seizures, with or without secondary generalization (Brodie and Dichter, 1996; Leppik, 2001). It was originally designed as a GABAmimetic that could freely cross the blood-brain barrier. However, subsequent studies have shown that GBP does not directly interact with GABA receptors or transporters (MacDonald and Greenfield, 1997).

There is evidence that GBP may increase the synthesis (Taylor *et al*., 1992) and nonvesicular release of GABA (Gotz *et al*., 1993), and may prevent its metabolism (Leach *et al*., 1997). Using ^{1}H NMR spectroscopy, GBP has been shown to elevate GABA concentrations in the occipital cortex of epileptic patients. Whether this observation is the result of enhanced synthesis, increased release or reduced metabolism of GABA remains to be determined (Petroff *et al*., 1996a).

The identification of a specific binding site for GBP in the mammalian brain, and its subsequent unveiling as the $\alpha_2\delta$-subunit of the L-type voltage-dependent Ca^{2+} channel, suggested another potential pharmacological mechanism (Gee *et al*., 1996).

The bioavailability of GBP is not dose-proportional; it decreases as the dose increases. It is not metabolized, and does not induce or inhibit hepatic metabolism. It is not bound to plasma proteins and displays linear pharmacokinetics at usual dosages. Consequently, drug-drug interactions are not an issue with GBP. The half-life of GBP in otherwise healthy patients with epilepsy is generally 4 to 9 hours. Because the elimination of GBP is entirely renal, patients with renal insufficiency usually need lower dosages and less frequent dosing (Benbadis and Tatum, 2001). Side effects of GBP are generally mild to moderate and transient. The most commonly reported dose-related side effects are drowsiness, ataxia, dizziness, nystagmus and fatigue. Weight gain occurs in up to 5% of patients and is often accompanied by ankle edema. Teratogenicity has not been a major issue in animals, although limited data are available from human experience (Leppik, 2001).

2.3 Learning and memory

2.3.1 Learning

Learning is the acquisition and storage of information as a consequence of experience. It is measured by an increase in the likelihood of a particular behavioral response to a stimulus. Generally, rewards or punishments are crucial ingredients of learning. There are two types of learning: (1) nonassociative learning and (2) associative learning (Widmaier, *et al*., 2004; Rhoades and Pflanzer, 2003).

2.3.1.1 Nonassociative learning

In nonassociative learning, we learn about a single type of stimulus and adapt to it according to its relevance to our desires or survival. This type of behavior, in which we learn to decrease our response to a repeated stimulus, is called habituation. In contrast, we can become more sensitized to certain other sounds. For example, we learn to recognize the sound of a fire alarm and respond

accordingly. Sensitization is thus an increase in response to a stimulus (Rhoades and Pflanzer, 2003).

2.3.1.2 Associative learning

In associative learning, an animal acquires an understanding about the relationship or association between one stimulus and another. This learning process is called classical conditioning. An example of classical conditioning is the salivation of a conditioned dog in response to a stimulus of light. Normally, the dog salivates only in response to a piece of food. After a light stimulus (the conditioned stimulus, or CS) has been paired with the presence of a piece of meat (the unconditioned stimulus, or UCS) in a sufficient number of conditioning trials, the light alone is sufficient to cause the dog to salivate (salivation is the conditioned response, or CR). The dog has been conditioned to salivate in response to light.

The other type of associative learning is called operant conditioning. In this type of learning, animals acquire an understanding of the relationship between their own behavior and subsequent reward (or punishment). With this type of learning, animals tend to repeat behaviors that are rewarded and avoid behaviors that are punished (Rhoades and Pflanzer, 2003).

2.3.2 Memory

Memory is the relatively permanent storage form of the learned information. The term memory encoding defines the neural processes that change an experience into the memory of that experience - in other words, the physiological events that lead to memory formation (Widmaier *et al*., 2004). Memories are frequently classified according to the type of information that is stored. One of these classifications divides memory into declarative memory and procedural (or skill) memory, as follows (Guyton and Hall, 2006)

Declarative memory is the retention and recall of conscious experiences that therefore can be put into words (declared). One example is the memory of having perceived an object or event and, therefore, recognizing it as familiar and maybe even knowing the specific time and place when the memory was

instigated. A second example would be one's general knowledge of the world such as names and facts. The hippocampus, amygdale and diencephalons – all parts of the limbic system – are required for the formation of declarative memories (Widmaier *et al*., 2004).

Procedural memory can be thought of as the memory of how to do things. In other words, it is the memory for skilled behaviors independent of any conscious understanding, as for example, riding a bicycle. Individuals can suffer severe deficits in declarative memory but have intact procedural memory. The category of procedural memory also includes learned emotional responses, such as fear of thunder, and the classic example of Pavlov's dog, which learned to salivate at the sound of a bell after the bell, had previously been associated with food. The primary areas of the brain involved in procedural memory are regions of sensorimotor cortex, the basal nuclei and the cerebellum (Widmaier *et al*., 2004).

Some memories last for only a few seconds, whereas others last for hours, days, months or years. A common classification of memories divides memories into short-term memory and long-term memory. In the current model of how information is processed and stored into memory, incoming information first enters the CNS and is stored as short-term memory, also known as working memory, which is temporary storage lasting only a few seconds or up to a few hours. Limited space is available for short-term memory, and information placed in short-term memory will be lost if it is not consolidated into long-term memory, which can last years or a lifetime. The mechanisms of consolidation are not well understood, but repetition appears to help. By contrast, repetition is not necessary for certain memories. That most brides and grooms remember their wedding day, even though it only occurred once, suggests that the importance of an experience also play a role in memory (Germann and Stanfield, 2002).

2.3.3 Plasticity in the nervous system

Learning and memory are able to occur because the nervous system is endowed with plasticity, the limited ability to alter its anatomy and function in response to changes in its activity patterns.

One example of plasticity in the nervous system is long-term potentiation (LTP), which in mammals was first discovered in the hippocampus and occurs at preexisting synapses. In LTP, repetitive stimulation of a particular synapse eventually leads to an increase in the strength of that synaptic connection; that is, repetition increases the likelihood that synaptic input will be able to trigger an action potential in the postsynaptic cell. An increase in synaptic strength can be due to an increase in the postsynaptic cell's sensitivity to the neurotransmitter released at the synapse, to an increase in the quantity of neurotransmitter released by the presynaptic cell with each action potential, or to both. LTP is though to be important in the consolidation of long-term memory because it provides a mechanism whereby repetitive activity in particular neural pathways, such as might occur during repetition of a learned fact, can leave a more-or-less permanent "record" of itself once the activity has ceased (Germann and Stanfield, 2002).

One mechanism of LTP is illustrated in figure 5. During normal, low-frequency synaptic transmission glutamate (Glu) is released from the presynaptic terminal and acts on both the NMDA and non-NMDA receptors. The non-NMDA receptors here are the AMPA type. Na^+ and K^+ flow through the non-NMDA channels but not through the NMDA channels, owing to Mg^{2+} blockage of this channel at the resting level of membrane potential (Fig. 5A). In Figure 5B, when the postsynaptic membrane is depolarized by the actions of the non-NMDA receptor-channels, as occurs during a high-frequency tetanus that induces LTP, the depolarization relieves the Mg^{2+} blockage of the NMDA channel. This allows Ca^{2+} to flow through the NMDA channel. The resulting rise in Ca^{2+} in the dendritic spine triggers calcium-dependent kinases (Ca^{2+}/ calmodulin kinase and protein kinase C) and the tyrosine kinase Fyn that together induce LTP. The Ca^{2+}/ calmodulin kinase phosphorylates non-NMDA receptor-channels and increases their sensitivity to glutamate thereby also activating some otherwise silent receptor channels. These

changes give rise to a postsynaptic contribution for the maintenance of LTP. In addition, once LTP is induced, the postsynaptic cell is thought to release (in ways that are still not understood) a set of retrograde messengers, one of which is thought to be nitric oxide, that act on protein kinases in the presynaptic terminal to initiate an enhancement of transmitter release that contributes to LTP (Kandel *et al*., 2000).

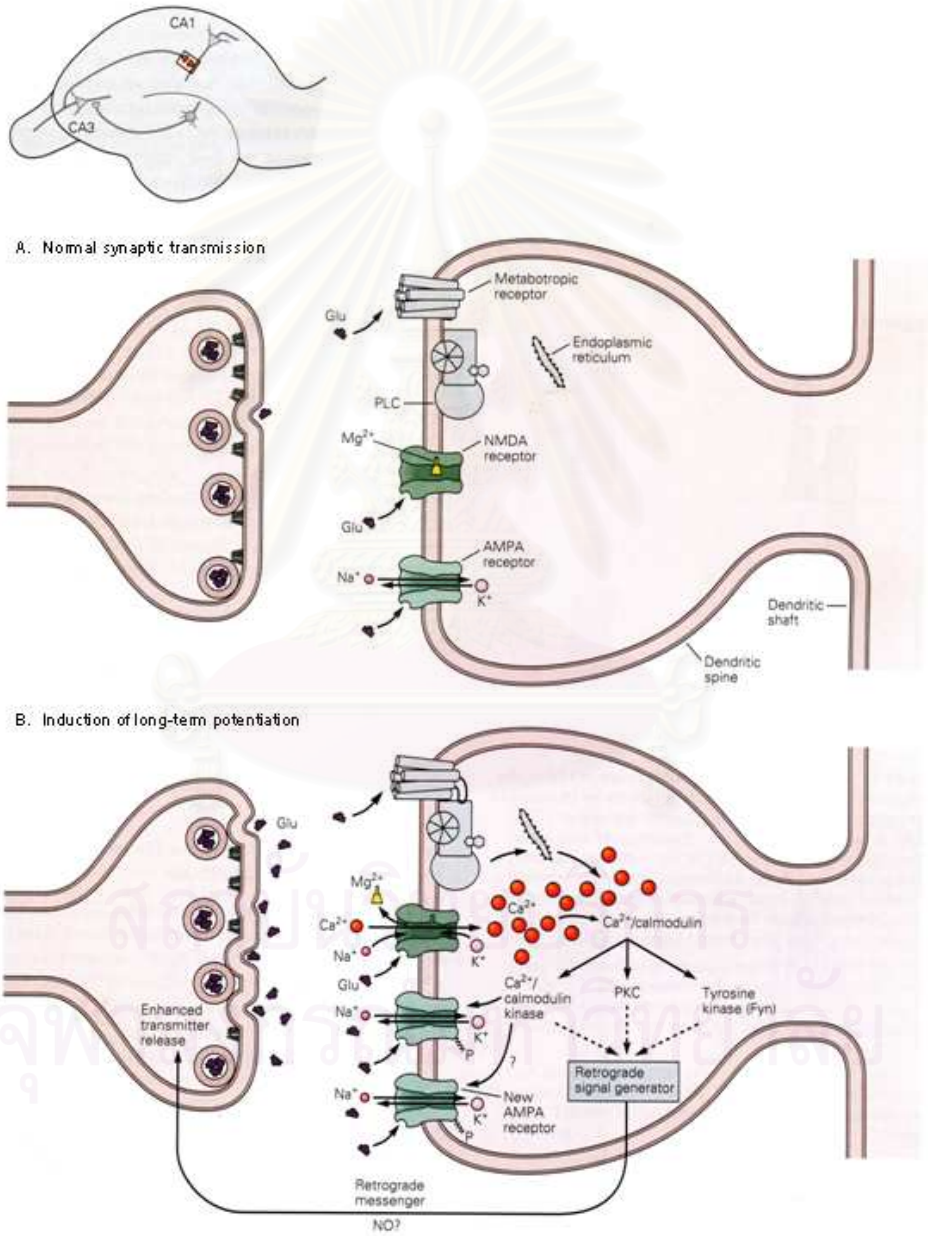


Figure 5 A model for the induction of the early phase of long-term potentiation (Kandel *et al*., 2000).

2.4 Brain oxidative stress and lipid peroxidation

A free radical is an atom or molecule with an unpaired electron in its outer orbit, a state that makes it highly unstable and reactive. Free radicals are formed during normal metabolism, and free radical injury occurs within living cells when the generation of reactive oxygen species exceeds intrinsic antioxidant ability. This situation is also referred to as oxidative or oxidant stress (Pratico and Delanty, 2000).

The brain may be particularly vulnerable to oxidative damage, because it has high energy requirements and a high oxygen consumption rate; is rich in peroxidizable fatty acids; contains high levels of transition metals, which may catalyze the formation of the reactive hydroxyl radical; and has a relative deficit of antioxidant defenses compared with other organs (Floyd, 1999).

Oxidative stress to the central nervous system predominantly manifests as lipid peroxidation because of its high lipid content and unusually high concentration of polyunsaturated fatty acids that are particularly susceptible to oxidation. This may in turn promote the formation of additional reactive oxygen species, and by so doing enhance protein and DNA oxidative damage. Recently, increased activity of the antioxidant enzyme, superoxide dismutase, has been reported in the CSF with aging, suggesting a possible reactive compensatory process secondary to this increased oxidative stress with time (Okabe *et al*., 1996). Apart from aging, other risk factors for the development and clinical expression of Alzheimer's disease (AD) include Down's syndrome, vascular disease, head injury, cigarette smoking and diabetes mellitus (Leibson *et al*., 1997; Stewart, 1998; Nemetz *et al*., 1999; Merchant *et al*., 1999). Interestingly, increasing evidence indicates that these are all associated with increased free radical formation (Pratico and Delanty, 2000).

Lipid peroxidation is the mechanism by which lipids are attacked by reactive oxygen species with sufficient energy to form a carbon radical that reacts with oxygen and results in a peroxyl radical, thus generating lipid peroxides (Gutteridge, 1995). Markers of brain lipid peroxidation have been the most studied indexes of oxidative stress in AD. In quantitative postmortem studies, lipid peroxidation has been assessed by measuring its product malondialdehyde (MDA) levels by the thiobarbituric acid-

reacting substances (TBARS) assay; lipid hydroperoxides; aldehydes and isoprostanes. The majority of the published studies have used the TBARS test. It is easy to perform and inexpensive but also has significant shortcomings when used to assess lipid peroxidation in complex biological systems (Moore and Roberts, 1998).

2.5 Isobolographic analysis

The isobolographic analysis is a universal experimental method applicable to determine pharmacological interaction among drugs exerting the same effect(s) and it may assess some interactions exerted by virtually ineffective drugs as well. This old method has recently been accepted as the "gold standard" in detecting the drug interactions. Theoretically, the isobolographic analysis distinguishes three most important types of interactions: superadditivity (synergy), additivity and subadditivity (antagonism) (Tallarida *et al*., 1997; Luszczki and Czuczwar, 2003).

The present study was aimed to establish

1. The anticonvulsant activity against PTZ test and toxicity testing of *Centella asiatica*'s ethyl acetate fraction (EACA) on
   - the median effective dose (ED_{50})
   - the median neurotoxic dose (TD_{50})
   - the protective index (PI)
   - the median lethal dose (LD_{50})
   - the locomotor activity test
   - the barbiturate potentiation test
   - interaction between EACA and some antiepileptic drugs (AEDs)

2. The possible mechanism of EACA on
   - the level of excitatory and inhibitory amino acid neurotransmitters in the hippocampus of freely moving rats by using microdialysis technique
   - the kinetics of $GABA_A$ receptor-mediated currents of hippocampal pyramidal neuron by using whole cell patch-clamp technique

3. The protective effect of EACA on epileptogenesis
   - the course of kindling development by using a Racine scale
   - learning deficit due to the PTZ-kindling by using step-down test
   - the brain oxidative stress parameter: MDA by using TBARS assay
   - neuronal cell loss by histological examination

CHAPTER III

MATERIALS AND METHODS

3.1 Animals

Male ICR mice weighing 18-25 g were used to evaluate EACA for anticonvulsant activity, some toxicological profile, the course of kindling development, kindling-induced learning deficit, oxidative stress marker in PTZ-kindled mice and isobolographic analysis of drug interaction.

Wistar rats aged 10-20 days were used to study the mechanism of action exerted by EACA on hippocampal membrane receptors.

Male Wistar rats weighing 250-350 g were used to characterize alterations of hippocampal amino acid neurotransmitter levels.

All animals were purchased from the National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand. They were acclimatized in the ventilated room at the ambient temperature of 25°C and on a natural light/dark cycle for at least one week prior to the experiments. Standard food (C.P. mice food) and tap water were provided *ad libitum*.

All animal care and handling were conducted with the approval of the Ethical Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3.2 Plant material and preparation of the extracts

3.2.1 Preparation of the ethanolic total extracts of CA for preliminary study

CA was purchased from Nakornpathom Province which provides pesticide-free CA. The aerial parts were washed with running tap water, dried and coarsely ground. The coarse powder of the plant was macerated with 95 percent ethanol for 7-10 days and filtered. The marc was then remacerated with another portion of 95 percent ethanol until the filtrate was nearly clear. The combined filtrate was concentrated under reduced pressure by rotary evaporator to yield syrupy mass and then evaporated with water bath until no traces of ethanol were left to yield the syrupy crude of the 95 percent ethanol extract (ethanolic total extract).

3.2.2 Preparation of sequential extracts of CA

The ethanolic total extract was dissolved with the combination of ethanol and water (5:1, v/v) to yield the aqueous ethanolic solution. The aqueous ethanolic solution was partitioned with petroleum ether by vigorously shakes and left to separate into upper and lower layers. The upper layer of petroleum ether extract was separated by a separating funnel. The lower layer of residual aqueous ethanolic solution was re-partitioned again and again with petroleum ether until the upper layer is nearly clear. The combined petroleum ether extract was dried over anhydrous sodium sulfate, filtered and then concentrated under reduced pressure by rotary evaporator and water bath until no traces of petroleum ether were left to yield the syrupy mass of petroleum ether fraction. The residual aqueous ethanolic solution was re-extracted with ethyl acetate, and buthanol to yield ethyl acetate, buthanol fraction and also residual aqueous ethanolic fraction, respectively, by the same procedure.

3.3 Administration of tested substance

Each fraction of CA extracts was dissolved in a vehicle (Tween 20:water; 2:5). They were given by the oral administration except for the ethanolic total extract which was also injected intraperitoneally (i.p). A gavage tube was used to deliver the test compound and vehicle by the oral route and the volume of administration was kept at 0.2-0.3 ml/ 25 g BW of the animal. PTZ was dissolved in 0.9% sodium chloride and given by subcutaneous injection. The volume of injection was 0.1-0.2 ml/ 25 g BW of the animal.

The EACA was administered at the optimal pretreated time obtained from 3.4.2 prior to the PTZ injection.

Route of administration and pretreated time of AEDs were selected according to their respective time to peak effect previously reported. Phenytoin, valproate and gabapentin were given intraperitoneally at 90, 60 and 120 min, respectively, prior to the injection of PTZ (Masereel *et al*., 1998; Tantisira *et al*., 1997)

3.4 Anticonvulsant activity of the ethanolic total extract

3.4.1 Anticonvulsant activity against maximal electroshock seizure (MES) test and PTZ-induced seizure test (PTZ test)

The MES test was elicited by the passage of a current of 50 mA, 50 Hz for 0.2 sec from the electroshock apparatus through the brain via corneal electrodes after pretreatment with tested substances. The endpoint of the MES test was generalized seizure with tonic hindlimb extention.

Seizures were also induced by injection of PTZ (70 mg/kg BW; sc). The end point of the PTZ test was a generalized clonic seizure with loss of righting reflex within 60 minutes after injection of PTZ (Tantisira *et al*., 1997).

Intraperitoneally and orally given of a 1,000 mg/kg BW of the ethanolic total extract at the pretreated time of 1 hour were used to verify its anticonvulsant activity in both MES and PTZ tests.

3.4.2 Determination of the optimal pretreated time for anticonvulsant activity against PTZ test

Mice were divided into 3 groups according to pretreated times of 1, 1.5 and 2 hours. The ethanolic total extract (1,000 mg/kg BW) given orally against the PTZ test was used to determine the optimal pretreated time. The pretreated time which given the maximal anticonvulsant activity of CA was selected and used as optimal pretreated time in other experiments.

3.5 Anticonvulsant activity of the sequential extracts of CA

Administrations of the petroleum ether, ethyl acetate, buthanol and also residual aqueous ethanolic fraction at the optimal pretreated time obtained from 3.4.2 were used to verify their anticonvulsant activity in PTZ tests. The dosage of each fraction was calculated by the relation between the ethanolic total extract which was used for sequential extraction and the yield of each fraction delivered (w/w). The fraction which given anticonvulsant activity was selected and used in other experiments.

3.6 Evaluation of anticonvulsant activity against PTZ test and toxicity testing of *Centella asiatica*'s ethyl acetate fraction (EACA)

3.6.1 The median effective dose (ED_{50})

Male ICR mice were divided into five groups. The first group of three mice treated with vehicle was used as control group. The other four groups of EACA (300, 600, 900 and 1,000 mg/kg BW) of eight mice each were used to establish ED_{50} of the EACA to protect against PTZ at the optimal pretreated time obtained from 3.4.2. The ED_{50} value (with their 95% confidence limits) was calculated directly from the respective drug-dose effect curve according to Litchfield and Wilcoxon (1949).

3.6.2 The median neurotoxic dose (TD_{50})

The rotarod test was modified from the one previously described by Cuadrado *et al*. (2002) carried out with a rod of 3.5 cm diameter, rotating at 18 rpm. The end-point to evaluate the minimal neurotoxicity was the inability of the animal to maintain its equilibrium for at least 1 min on the rotating rod in each of three successive trials. Before the experiment, mice were placed on the rotating rod in a training session for 5 minutes. Untreated mice were able to maintain their balance on the rod for several minutes. The EACA were administered to each group of mice and they were tested again after the optimal pretreated time obtained from 3.4.2. Eight mice per dose and five doses were used to determine the TD_{50} (with their 95% confidence limits) by the method of Litchfield and Wilcoxon (1949).

3.6.3 Protective index (PI)

PI, a quantitative measure of the margin between doses producing anticonvulsant (protective) effect and motor toxicity, was calculated by dividing the TD_{50} value by the ED_{50} value.

3.6.4 The median lethal dose (LD_{50})

The EACA were administered to each group of mice. General changes such as ataxia, sedation, respiratory secretion, etc., were observed and lethality within a period of 72 hours was noted. Eight mice per dose and six doses were used to calculate the LD_{50} by the method of Litchfield and Wilcoxon (1949).

3.6.5 Locomotor activity test

An activity cage (UGO Basile, Comerico, Italy) consisting of plexiglass chamber (length 35 cm; width 23 cm; height 20 cm) and counting were used to assess the locomotor activity of mice. The cage floor is made of evenly spaced stainless steel bars (3 mm diameter) that are spaced 11 mm apart. Each of the steel bars is insulated from each other. The odd bars are grounded and the even bars are active and wired out in four sets. The bridge that animal breaks with its paw

disconnects one or more active bars with ground, thereby producing configurations which change as the animal moves. These changes in configuration are converted into pulses and subsequently count by a counter.

Each mouse was placed in the chamber and the activity was established at 10 minutes intervals. A control period of activity was allowing for 30 minutes before and another 120 minutes after the EACA administration. Eight mice per dose and four doses were used to assess the effect of the EACA on the locomotor activity.

3.6.6 Barbiturate potentiation test

The effect of EACA on barbiturate sleeping time was used to evaluate the depressing effect on CNS. In this study, pentobarbital sodium 50 mg/kg BW were intraperitoneally injected immediately to each mouse after the administration of EACA. The sleeping time were measured as the time between the loss and the recovery of righting reflex, the inability and ability, respectively, of the animal to upright itself within five seconds when placed on its back in three successive trials (Thompson, 1990). Eight mice per dose and four doses were used to assess the effect of the EACA on the barbiturate sleeping time.

3.6.7 Interaction between EACA and some antiepileptic drugs (AEDs)

3.6.7.1 Determination of the ED_{50} and TD_{50} of AEDs against PTZ test in mice

Mice were divided into five groups for each AED (phenytoin, valproate and gabapentin) and three groups for each EACA-AED combination. The first group of three mice treated with vehicle and PTZ was used as control group. The other groups of eight mice each were used to establish ED_{50} and TD_{50} of AEDs and their combination with EACA to protect against PTZ at their respective time to peak effect by the method of Litchfield and Wilcoxon (1949).

3.6.7.2 Isobolographic analysis

Isobolographic analysis, the principal method applicable for understanding the real nature of drug interaction, was used to analyze the interactions between EACA and conventional AEDs (phenytoin, valproate and gabapentin) in the PTZ test in mice. The ED_{50} values (with their 95% confidence limits) for each substance administered alone in the PTZ test was calculated according to Litchfield and Wilcoxon (1949). The ED_{50} of each AEDs in the presence of EACA was also calculated in the same manner using three different dose pairs of equi-effective dose of EACA and respective AEDs (Fairbanks *et al*., 2002). In the present study, the mixtures of EACA with an AED were co-administered in a fixed-ratio combination of 1:1. This means that a combination was composed of ½ of the ED_{50} of EACA and ½ of the ED_{50} of AED resulting finally in the full ED_{50} of an EACA-AED combination (Luszczki and Czuczwar, 2003). Substances were delivered in such a way that they were at their time to peak effect during the assessment of effects on the dependent measure.

Isobologram was then constructed from the ED_{50} values of EACA and AEDs when each of them was given alone (Tallarida, 2001). Straight line connecting between two ED_{50} values is the theoretical additive line representing dose pairs of EACA and AEDs that are additives in protecting 50 percent of the animals. Theoretical ED_{50} at the fixed-ratio combination of 1:1 was then compared to the observed experimentally combined ED_{50} to estimate the nature of interaction. If the observed experimentally combined ED_{50} lies on the additive line, then the dose pair having these coordinates is simply additive. On the other hand, the points lying below the line suggests synergistic interaction while the ones above the line would then suggest antagonistic nature of the combination (Porreca, *et al*., 1990; Tallarida, 1992; Tallarida, 2001).

In addition, various combinations of EACA and AEDs used to determine the observed experimentally combined ED_{50} mentioned above were subsequently used for the determination of TD_{50} by rotarod test.

Finally, the PI for each substance administered alone in the PTZ test and each AEDs in the presence of EACA was also calculated

3.7 Possible mechanisms of EACA

3.7.1 Effect of EACA on some hippocampal amino acid neurotransmitter levels relating to convulsion in freely moving rats by microdialysis technique

3.7.1.1 Experimental animals

To determine the effect of EACA on the extracellular levels of glutamate, aspartate, glycine and GABA in the hippocampus following a PTZ administration (60 mg/kg BW), male Wistar rats weighing 250-350 g were randomly divided into four groups of five animals each as below.

Group I	normal saline (p.o.)	+ normal saline (s.c.)
II	vehicle (tween 20/water; 2:5; p.o.)	+ normal saline (s.c.)
III	vehicle (tween 20/water; 2:5; p.o.)	+ PTZ 60 mg/kg BW (s.c.)
IV	EACA 700 mg/kg BW(p.o.)	+ PTZ 60 mg/kg BW (s.c.)

One hour after oral pretreatment with normal saline, vehicle or EACA, normal saline or PTZ 60 mg/kg BW were administered subcutaneously into the animals.

3.7.1.2 Microdialysis experiments

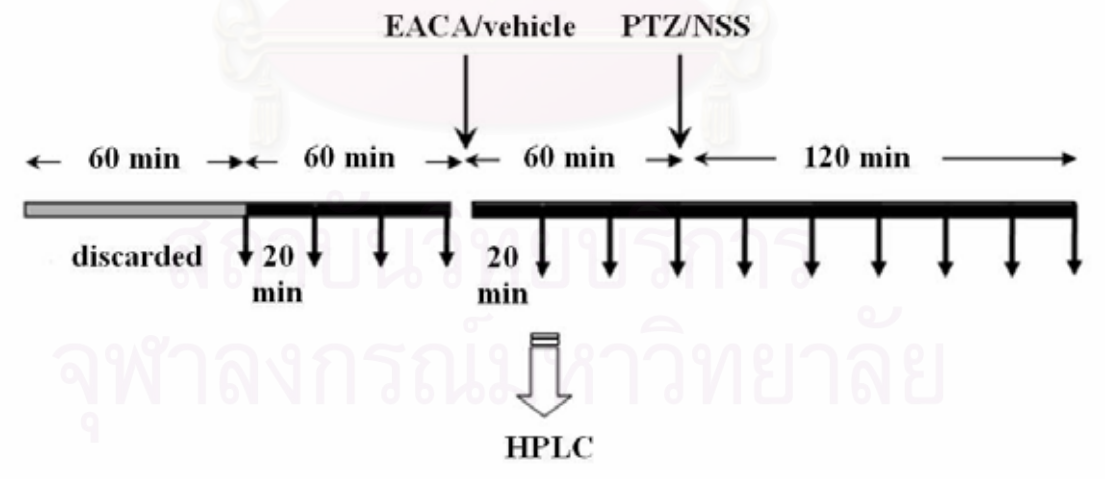
3.7.1.2.1 Microdialysis probe implantation

The rat was anesthetized with chloral hydrate (350 mg/kg BW, i.p.) with supplementary doses as required to maintain surgical anesthesia. The anesthetized rat was then placed in a stereotaxic apparatus (Narishige, Japan). The surface of microdialysis probe (0.2 mm outer diameter, acrylic polymer with 50,000 molecular weight cut off) was totally covered with epoxy resin except the area that contacted the hippocampus of the rat. After the appropriate area of the skull was exposed, the probe was implanted transversely into the hippocampus at coordination according to a stereotaxic atlas of the rat brain which

was referred to bregma; AP -4.5 mm and inferior -3 mm (Pellegrino *et al*., 1979) and then was fixed by polycarboxylate cement. After microdialysis probe implantation, the rat was allowed at least 24 hours for recovery before the experiment was started.

3.7.1.2.2 Collection of CSF samples

The rat was placed in the collecting sample instrument (CMA/120, Carnegie, Sweden) which allowed freely moving. One side of the probe was connected to a constant flow infusion pump (CMA/100, Carnegie, Sweden) by polyethylene tube, and the other side was placed into a collecting tube. The dialysis system was perfused at a rate of 2 µl /min with artificial CSF (aCSF). The composition of aCSF was 120 mM NaCl, 15 mM $NaHCO_3$, 5 mM KCl, 15 mM $CaCl_2$, 1 mM $MgSO_4$ and 6 mM glucose, pH 7.4. As illustrated in the diagram below, the dialysate collected during the equilibrium period of 60 minutes was discarded before the first sample was collected. The dialysate was collected at 20 minutes interval, 60 minutes before and 180 minutes after the vehicle or EACA administration. Amino acid levels were determined by high performance liquid chromatography (HPLC) technique.



At the end of each experiment, the brain was exposed and removed to confirm the appropriate position of microdialysis probe by sectioning the specimen with a sharp blade and then inspected visually. The data are valid only when the right position of probe was confirmed.

3.7.1.3 Analysis of rat hippocampal amino acid neurotransmitter levels

The experimental method, precolumn fluorescence derivatization with O-phthaldialdehyde (OPA) was used to determine the levels of rat hippocampal amino acid neurotransmitters. The mobile phase used was gradient run between 0.05 M phosphate buffer, pH 7.3 in triple distilled water and methanol (HPLC grade). Both of the mobile phases were degassed with continuous helium gas. For gradient run, the mobile phase gradient was increased from 20% to 60% methanol in one linear step at the increment rate of 2%/ minutes for 20 minutes. The rate of mobile phase is 1 ml/min. At the end of run, initial condition was restored by the reversed methanol gradient run from 60% to 20% at the rate of 10%/ minutes. A delay period of about 10 minutes was required for column equilibration.

The solution of OPA was maintained by an addition of 4 µl 2-mercaptoethanol every 4 days. The derivatization procedure was performed by mixing 10 µl of dialysate sample with 10 µl of homoserine solution (internal standard) and adding 50 µl of OPA solution at room temperature. Then 50 µl injection of the mixture to HPLC was made after a precise 2 minutes incubation period.

3.7.2 Effect of EACA on $GABA_A$ receptor current by whole cell patch-clamp technique

3.7.2.1 Dissociation of hippocampal neurons

Experiment was conducted on hippocampal neurons acutely dissociated from Wistar rats aged 10-20 days by a method of Sooksawate and Simmonds (1998). Rat was killed by cervical dislocation and decapitated. Brain was rapidly removed and placed into iced cold physiological salt solution (PSS) for 3 min. The PSS containing (in mM): 140 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11 glucose, 10 HEPES, adjusted to pH 7.4 with Tris-base. The brain was removed and mounted on the stage of a vibroslice using a thin film of α-cyanoacrylate glue. The brain was sliced into 400 µm thick sections with an iced-cold filled vibroslice and then incubated for 30-60 min in oxygen-saturated PSS at 25°C. The hippocampal regions

were carefully dissected out with fine forceps and then were enzymatically treated in PSS containing 0.03% (w/v) protease type XIV (pronase), followed by 0.03% (w/v) protease type X (thermolysin) for 20 minutes each at 31°C and continuously oxygenated throughout the procedure.

After the enzyme treatment, the slices were washed three times with oxygen-saturated PSS. The neurons were in mechanically dissociated by gentle trituration of the chunks through a series of fire-polished glass pipettes of decreasing pore sizes for loosing the intercellular connection and then intact neurons could be dissociated. The neuron suspension was allowed to stand for 5 min before the supernatant containing dissociated neurons was taken off. The supernatant,~1ml, was added with 1 ml of oxygen-saturated PSS and left to stand for 5 min before the supernatant was prompted to use.

The neurons were suitable for electrophysiological recording after being allowed to adhere to the base of recording chamber for 30-40 minutes.

3.7.2.2 Electrophysiological recordings

The effects of EACA on $GABA_A$ receptor-mediated currents were investigated in acutely dissociated hippocampal neurons using the whole cell patch-clamp technique (Gibbs *et al.*, 1997; Roberts and Ramoa, 1999; Sooksawate, 1999). Recordings of $GABA_A$ receptor-mediated currents from the somata of the dissociated pyramidal-shaped hippocampal neurons were conducted at -20 mV (holding potential). The healthy dissociated neuron has a phase-bright smooth surface and internal structure was transparent.

Patch pipettes (3-5 MΩ) were pulled by a two stages vertical microelectrode puller with the use of thin-walled borosilicate glass capillaries without filament (1.5 mm O.D. and 1.17 mm I.D.). The patch pipette was filled with the intrapipette solution containing (in mM): 140 CsCl, 4 $MgCl_2$, 4 Na_2-ATP, 11 EGTA, 1 $CaCl_2$, 10 HEPES, adjusted to pH 7.2 with Tris-base. It was mounted on a suction pipette holder, which connected to amplifier head stage which was mounted on hydraulic micromanipulator. This, in turn, was mounted onto coarse manipulator.

Membrane currents were measured with a patch clamp amplifier and were displayed simultaneously on an oscilloscope and a computer. During recording, neurons were superfused with oxygen-saturated PSS at a rate of 2.5-3.0 ml/min. All experiments were performed at room temperature (22-24°C).

3.7.2.3 Drug application

Rapid drug application was performed with the "U-tube" method (Sooksawate and Simmonds, 1998). A U-shaped glass capillary of diameter ~150 µm with a small circular hole ~50 µm at the tip was placed about 300 µm from the recorded neuron. One end (influx arm) of the U-tube was connected to a reservoir of PSS or drug held about 18 cm above the level of the U-tube tip. The other end (efflux arm) was connected to a vacuum pump which sucked solution from the reservoir through the U-tube and also drew solution in the chamber into the tube.

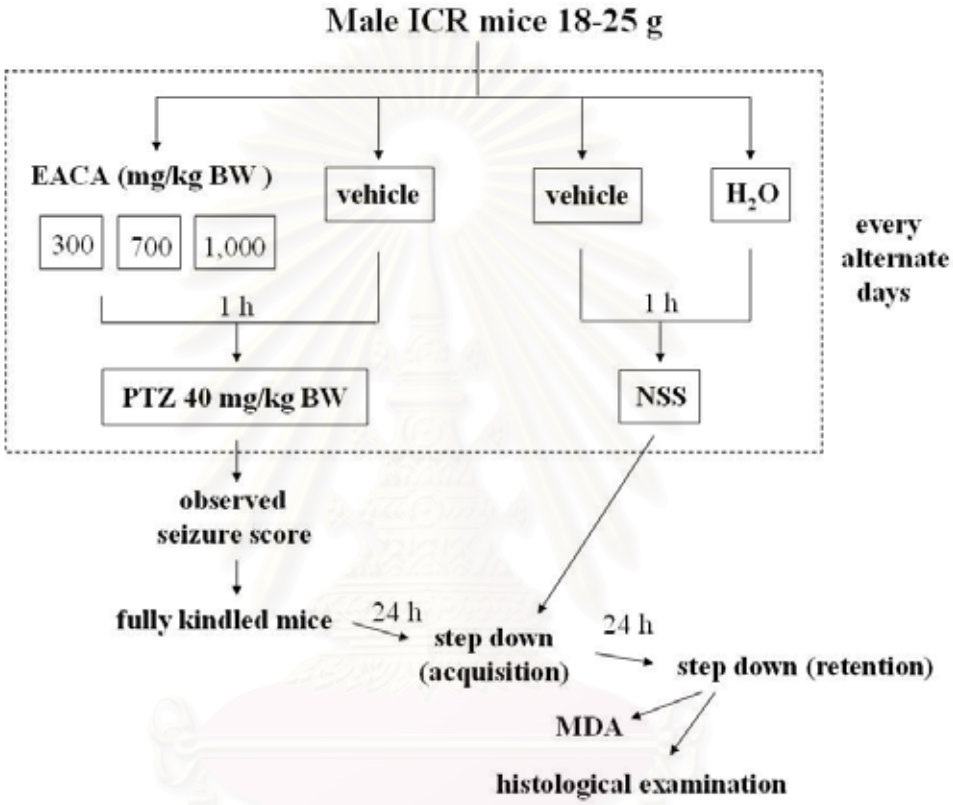
During drug application, the line to the vacuum pump was occluded for 5 sec to allow gravity to drive the solution from the reservoir and superfuse the recorded neuron. Reopening the line caused to be resuctioned the solution surrounding the neuron within 50 ms. The application were separated at least 1-2 min intervals to ensure both adequate wash out of drug from the bath and recovery of the receptors from desensitization (Suthep Jenthep, 2002).

GABA 3 µM was superfused to the recorded neuron in the absence and presence of 0.1, 0.3, 1, 3, 10, 30, and 50 µg/ml EACA in 0.1% dimethyl sulfoxide (DMSO) and 0.1, 0.3, 1 and 3 µM diazepam (DZP) in 0.1% DMSO. At the end of drug application, a 3 µM GABA was applied to monitor for rundown of the $GABA_A$ current. Neurons with current rundown over the lifetime of the recording were excluded.

The data of EACA and DZP on the $GABA_A$ current were expressed as a percent change from GABA alone in each neuron.

3.8 Effect of EACA on epileptogenesis

Effects of EACA on the course of kindling development, kindling-induced learning deficit, oxidative stress marker and survival hippocampal pyramidal neurons in PTZ-kindled mice were evaluated as illustrated in the diagram below.



3.8.1 Kindling induction

Male ICR mice weighing 15-20 g were randomly divided into 6 groups of 15 animals each as below.

Group	I	H_2O (p.o.)	+ normal saline (s.c.)
	II	vehicle (p.o.)	+ normal saline (s.c.)
	III	vehicle (p.o.)	+ PTZ 40 mg/kg BW (s.c.)
	IV	EACA 300 mg/kg BW (p.o.)	+ PTZ 40 mg/kg BW (s.c.)
	V	EACA 700 mg/kg BW (p.o.)	+ PTZ 40 mg/kg BW (s.c.)
	VI	EACA 1000 mg/kg BW (p.o.)	+ PTZ 40 mg/kg BW (s.c.)

One hour after oral administration of distilled water, vehicle or EACA, mice were administered normal saline or PTZ 40 mg/kg BW subcutaneously on every second day (48 h). The PTZ injections were stopped when the animals in the third group showed fully kindled, seizure score of 5 on three consecutive injections (Gupta *et al*., 2003).

After each PTZ injection the convulsive behavior was observed for 30 minutes and the resultant seizures were scored according to a Racine scale (Racine, 1972) as follows: stage 0 no response

1 mild facial clonus and eye blinking

2 severe facial clonus, head nodding, chewing

3 unilateral or alternating forelimb clonus

4 bilateral forelimb clonus and rearing

5 bilateral forelimb clonus with rearing and falling

6 tonic hindlimb extension.

3.8.2 Passive avoidance with negative (punishment) reinforcement : step-down

One of the most common animal tests in memory research is the inhibition to imitate activities or learned habits. The term “passive avoidance” is usually employed to describe experiments in which the animal learns to avoid a noxious event by suppressing a particular behavior (Vohora *et al*., 2000). Step-down test is one of the most frequently used tests for passive avoidance.

Memory deficit was evaluated using the step-down type of passive avoidance test. The apparatus consists of plexiglass chamber (23x35x20 cm) with a stainless-steel grid floor and a wooden platform (5 cm diameter, 4 cm height) fixed in one corner. Electric shock (1 Hz, 1 ms, 36 V DC) was delivered to the grid floor with an isolated pulse stimulator. Twenty-four hours after the last administration of PTZ, mice were placed in the chamber to get adapted to environment for 3 minutes without electric shock. When electric shock was delivered, mice escaped from the grid floor back up onto the platform. The duration of training test was 5 minutes and the shock was maintained for this period. Twenty-four hours after training, mice were placed

on the platform for the retention test. The electric shock was still delivered for 5 minutes. The step-down latency (time taken to descent) and number of errors (number of descent) in 5 minutes were recorded (Veerendra and Gupta, 2002; Luo *et al*., 2003).

3.8.3 Biochemical estimation of marker of oxidative stress: MDA

After the behavioral testing, 10 animals per each group were decapitated and the brains were quickly removed, cleaned with ice-cold saline and stored at -80°C.

3.8.3.1 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 rat brain were separated and used to determine lipid peroxidation and protein content.

3.8.3.2 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°C for 60 minutes. The mixture was cooled under tap water and 5 ml of *n*-buthanol: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 and absorbance was measured at 532 nm using a spectrophotometer (Gupta *et al*., 2003). The concentration of MDA was expressed as nmol/mg protein.

3.8.3.3 Measurement of protein content

Protein measurement was determined by the method of Lowry *et al*. (1951) and was modified by Miller (1959) by using bovine serum albumin as standard. The homogenated brain tissue sample (from 3.8.3.1) 10 µl was diluted with 3 ml of distilled water (1:300). The alkaline copper reagent 1 ml was added to 1 ml of the diluted brain tissue sample and then added with 3 ml of 1:10 diluted Folin-phenol

reagent after 10 minutes of reaction. The mixture was warmed in water bath (50 ºC) for 10 minutes and then cooled in room temperature. The absorbance was measured at 540 nm using a spectrophotometer compared with standard bovine serum albumin. The concentration of protein was expressed as mg protein/ml.

3.8.4 Histological examination

After the behavioral testing, 5 animals per each group were used to investigate neuronal damage in hippocampal formation (CA1 and CA3) with Cresyl violet staining method. Animals were decapitated. The whole brains were removed and quickly frozen in dry ice. Coronal sections (10 µm thick) were taken at the level of hippocampus (approximately 1.5 mm caudal to the bregma) by using a cryostat and stained with 1% Cresyl violet for the microscopic observation. Photograph (x40) of the CA1 and CA3 subfields of the hippocampus were taken and then the numbers of surviving pyramidal neurons (the neuron with a distinct nucleus) per 0.068 mm^2 in CA1 and CA3 subfields were counted. Average surviving cell numbers were counted over consistent fields, over both hemispheres and over three sections in each brain. The degree of surviving pyramidal neurons at the hippocampal CA1 or CA3 areas were expressed as the density 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).

3.9 Data analysis

For the determination of the ED_{50} and TD_{50}, the dose response curve was plotted between doses (log scale) and probits, which were transformed from percentage of protection. Three to five different doses of each substance were used to construct the dose response curve. The linear regression method was used to fit the curve and the value with confidence limits for 95% probability in parentheses was then calculated by the method of Litchfield and Wilcoxon (1949).

All data were presented as mean $\pm$ standard error of the mean (S.E.M.) except for ED_{50} and TD_{50} presented as the value with confidence limits for 95% probability. Student's unpaired *t* test was used to determine statistical significant difference between the ED_{50} and TD_{50} of AEDs in the presence and in the absence of EACA. One way analysis of variance (one-way ANOVA), followed by Duncan's multiple range test were used for multiple comparisons. Differences of $P < 0.05$ were considered statistically significant.

CHAPTER IV

RESULTS

4.1 Anticonvulsant activity of ethanolic total extract and sequential extracts of CA

In our study, the crude ethanolic total extract of CA, given orally, was able to protect the animals exclusively in the PTZ (70 mg/kg BW) but not MES (50 mA, 50 Hz for 0.2 sec) model at the optimal pretreated time of 1 hour (Table 2 and 3). When four fractions (petroleum ether, ethyl acetate (EACA), buthanol and residual aqueous ethanolic fraction) obtained from sequential extraction of CA was tested, only EACA but not the other fractions demonstrated anticonvulsant activity (Table 4). Subsequently, the EACA was further investigated for its toxicity testing, possible mechanisms and also effect on epileptogenesis.

4.2 Evaluation of anticonvulsant activity against PTZ test and toxicity testing of EACA

4.2.1 The median effective dose (ED_{50})

EACA in the doses of 300, 600, 900 and 1,000 mg/kg BW, given orally at the pretreated time of 1 h, was able to protect the animals against PTZ-induced convulsion exhibiting the ED_{50} of 673(299-1515) mg/kg BW (Fig. 6).

4.2.2 The median neurotoxic dose (TD_{50}) and PI

Both normal and vehicle treated mice were able to maintain their equilibrium for at least 1 min on the rotating rod in each of three successive trials. Neurological impairment indicated by the inability of the animal to maintain their equilibrium for at least 1 min was observed gradually increased in mice after the administration of EACA at the doses of 30, 100, 300, 600 and 1,000 mg/kg BW, respectively. The median neurotoxic dose (TD_{50}) of EACA was found to be 415(147-

1169) mg/kg BW (Fig. 7). Therefore, the protective index (TD_{50}/ED_{50}) of EACA was 0.62.

4.2.3 The median lethal dose (LD_{50})

The most frequent adverse effects observed in mice receiving high doses of EACA (600-5,000 mg/kg BW) were ataxia and hypnosis. Lethality was not observed within 72 hours, even though in the highest dose of 5,000 mg/kg BW (Table 5). Therefore, the LD_{50} of EACA appeared to be higher than 5,000 mg/kg BW.

4.2.4 Effect on locomotor activity

As illustrated in Figure 8, no significant different was observed between four groups at each time point. However, on total horizontal counts as illustrated in Figure 9, both groups of EACA (700 and 1,000 mg/kg BW) depressed locomotor activity of mice significantly different from those receiving vehicle ($P < 0.05$). While, no significant different was observed between NSS and vehicle treated groups.

4.2.5 Effect on barbiturate sleeping time

In comparison to vehicle, co-administration of pentobarbital sodium 50 mg/kg BW with EACA either 700 or 1,000 mg/kg BW did not prolong barbiturate sleeping time as showed in Figure 10 ($P > 0.05$). In addition, no significant different was observed between NSS and vehicle treated groups.

Table 2 Anticonvulsant activity of ethanolic total extract of CA 1,000 mg/kg BW in both MES and PTZ test following intraperitoneal and oral administration to mice.

Test model	Route of administration	Treatments (number of animal treated)	Number of animal protected
MES	IP	Vehicle (3)	0
		Ethanolic total extract (5)	0
	PO	Vehicle (3)	0
		Ethanolic total extract (5)	0
PTZ	IP	Vehicle (2)	0
		Ethanolic total extract (5)	0
	PO	Vehicle (3)	0
		Ethanolic total extract (5)	3

Table 3 The optimal pretreated time for anticonvulsant activity of ethanolic total extract of CA 1,000 mg/kg BW following oral administration against PTZ test.

Pretreated time (h)	% anticonvulsant
1.0	60
1.5	40
2.0	40

Table 4 Percent of animals in each group which showed anticonvulsant activity for each sequential extract of CA. Dose in the 2nd column was calculated by the relation between the ethanolic total extract which was used for sequential extraction and the yield of each fraction delivered (w/w), Dose X 3 in the 4th column represents triple of dose in the 2nd column, ~ Dose X 6 in the 6th column represents double of dose in the 4th column

Fraction	Dose (mg/kg BW)	Results	Dose X 3 (mg/kg BW)	Results	~Dose X 6 (mg/kg BW)	Results
Petrolium ether	40	0	120	0	250	0
Ethyl acetate	80	0	240	20	500	40
Buthanol	110	0	330	0	600	0
Residual aqueous ethanolic	260	0	780	0	1000	0

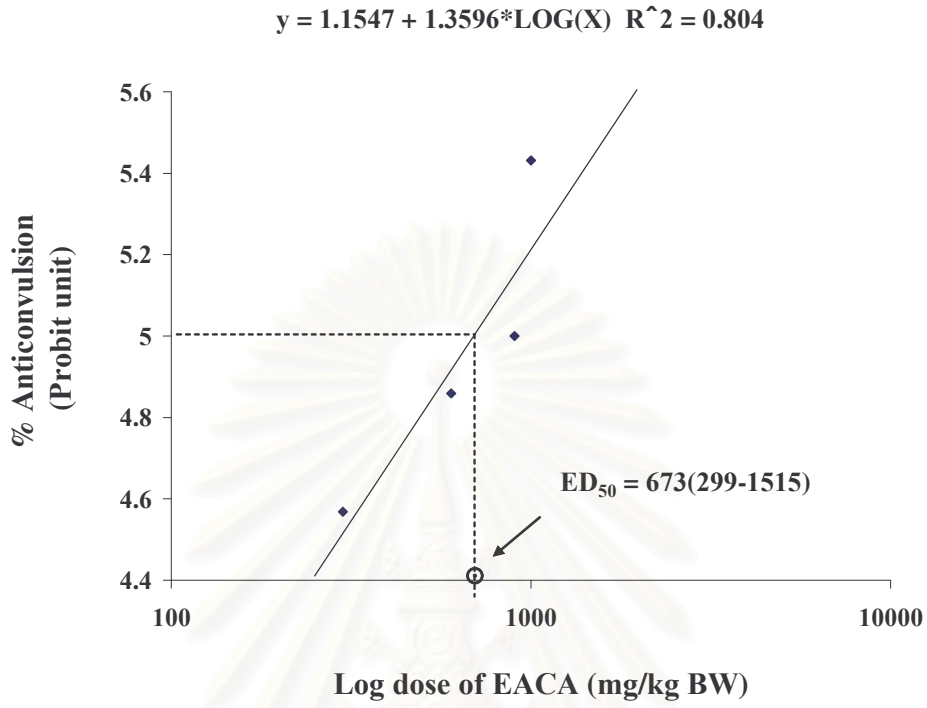


Figure 6 Log dose-response curve of EACA against PTZ-induced seizures in mice at pretreated time of 1 hour (n=8 in each group).

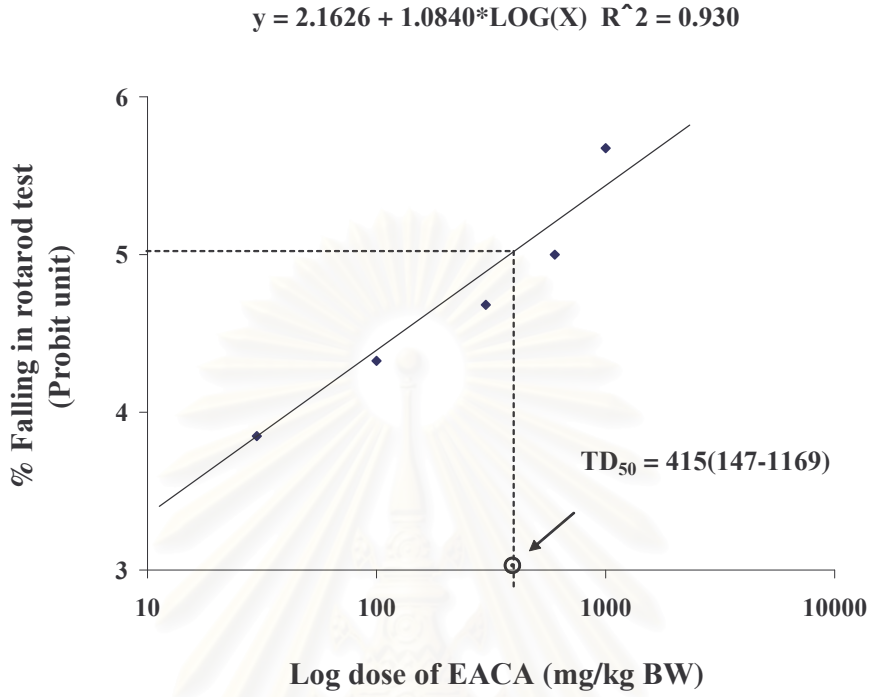


Figure 7 Log dose-response curve of neurotoxicity on rotarod test exhibited by EACA in mice at pretreated time of 1 hour (n=8 in each group).

Table 5 Effect of EACA on %survival of mice in 72 hours, value in brackets represents number of animal treated.

EACA (mg/kg BW)	% Survival in 72 h
30 (n=8)	100
100 (n=8)	100
300 (n=8)	100
600 (n=8)	100
1,000 (n=8)	100
5,000 (n=4)	100

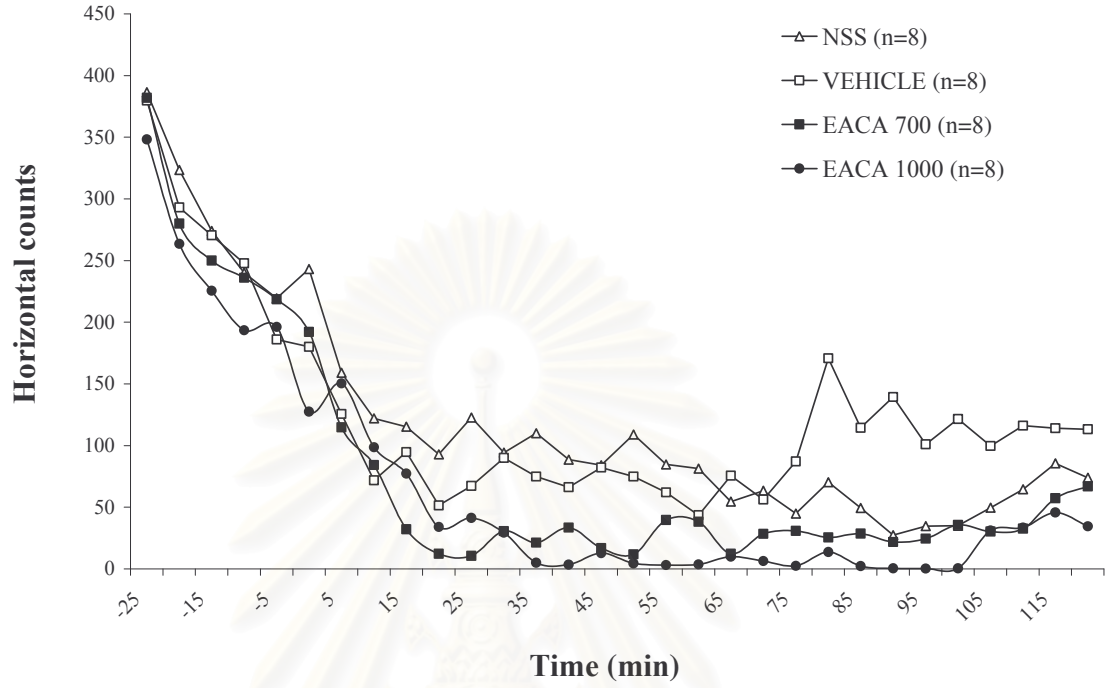
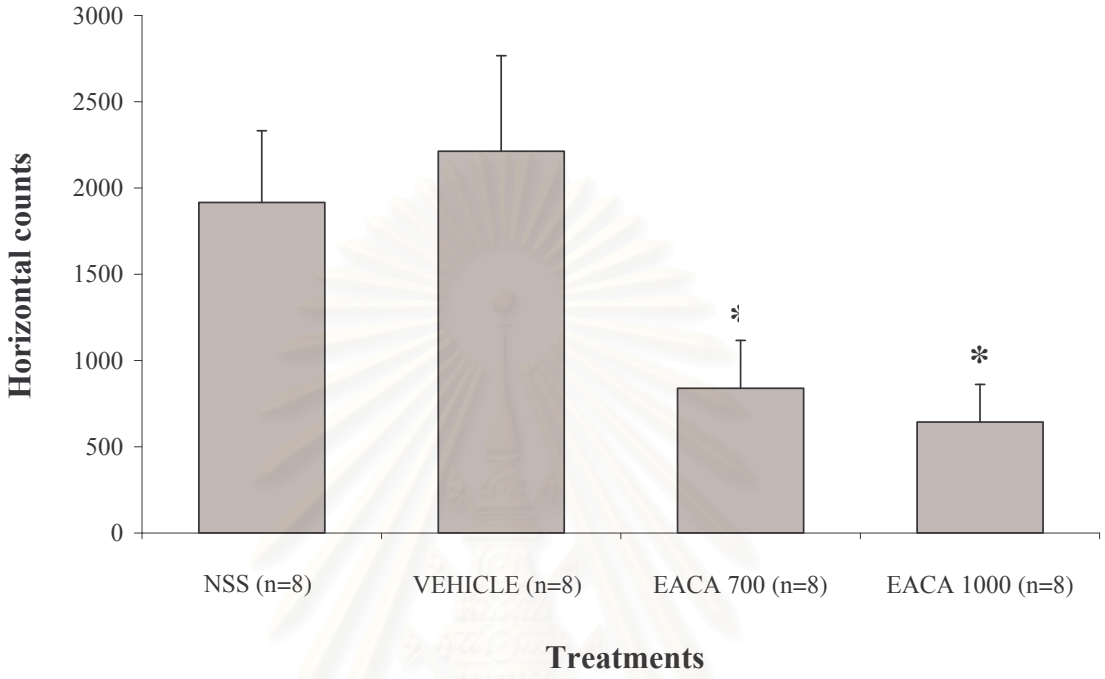


Figure 8 Effects of an oral administration of EACA 700 and 1,000 mg/kg BW on horizontal counts of locomotor activity in mice at various times. Value in brackets represents number of animal treated.



* $P < 0.05$ denotes statistically significant difference from vehicle

Figure 9 Effects of an oral administration of EACA 700 and 1,000 mg/kg BW on total horizontal counts within 120 min of locomotor activity in mice (MEAN $\pm$ S.E.M.). Value in brackets represents number of animal treated.

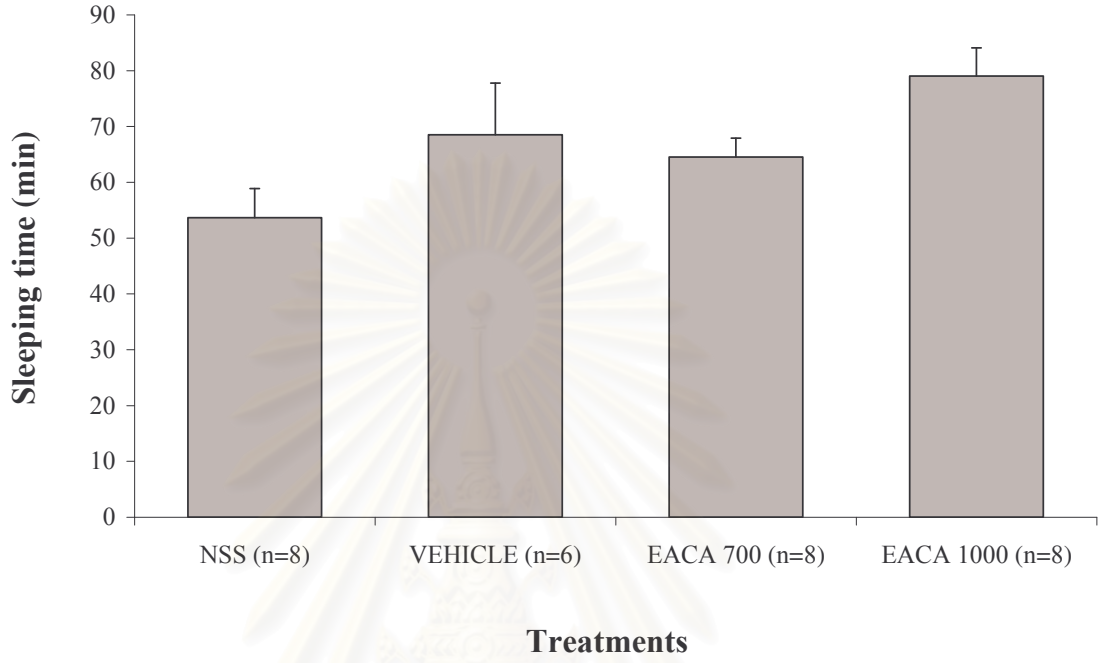


Figure 10 Effects of an oral administration of EACA 700 and 1,000 mg/kg BW on barbiturate sleeping time in mice (MEAN $\pm$ S.E.M.). Value in brackets represents number of animal treated.

4.2.6 Interaction between EACA and some AED

4.2.6.1 EACA and phenytoin

ED_{50} of intraperitoneally given phenytoin (3, 5, 10 and 20 mg/kg BW) was found to be 13(7-25) mg/kg BW when given alone and it was decreased to 5(3-8) mg/kg BW in the presence of orally given EACA (Fig. 11A and Table 6). The combination also decreased ED_{50} of EACA from 673(299-1515), when given alone, to 277(187-409) mg/kg BW (Fig. 11A). The isobolographic representation of the interaction between EACA and phenytoin (Fig. 11B) illustrated that the observed combined ED_{50} value, constructed from the experimentally calculated ED_{50} of EACA (X axis) and phenytoin(Y axis), lay below the additive line. Thus, synergistic interaction of the combination was suggested. However, no statistical difference was noted between the ED_{50} values of phenytoin in the presence and in the absence of EACA (P = 0.7015). Therefore, the interaction of phenytoin and EACA was simply additive.

As illustrated in Figure 11C, the combination also decreased TD_{50} of EACA from 415(147-1169), when given alone, to 99(11-854) mg/kg BW. The TD_{50} of phenytoin in the absence of EACA, 55(6-491) mg/kg BW, was not statistically different (P = 0.9203) from its corresponding value in the presence of EACA, 2(1-6) mg/kg BW. Taken together with the visual assessment of isobologram in Figure 11D, the neurotoxicity of EACA and phenytoin was also additive in nature resulting in the PI of 0.4 for the combination (Table 6).

4.2.6.2 EACA and valproate

Similarly, ED_{50} of intraperitoneally given valproate (70, 85, 100 and 150 mg/kg BW) was found to be 104(88-121) mg/kg BW when given alone and it was decreased to 29(21-40) mg/kg BW in the presence of orally given EACA (Fig. 12A and Table 6). The combination also decreased ED_{50} of EACA from 673(299-1515), when given alone, to 201(144-282) mg/kg BW (Fig. 12A). The isobolographic representation of the interaction between EACA and valproate (Fig. 12B) illustrated that the observed combined ED_{50} value lay below the additive line. Thus, synergistic interaction of the combination was likely. However, no statistical difference was

noted between the ED_{50} values of valproate in the presence and in the absence of EACA (P = 0.3399). Therefore, like the results of EACA and phenytoin previously mentioned, the interaction of valproate and EACA was also additive.

In the presence of valproate, the TD_{50} of EACA was decreased from 415(147-1169), when given alone, to 231(142-378) mg/kg BW (Fig. 12C). The TD_{50} of valproate in the absence of EACA, 247(107-568) mg/kg BW, was not statistically different (P = 0.5524) from its corresponding value in the presence of EACA, 33(20-54) mg/kg BW. Taken together with the visual assessment of isobologram in Figure 12D, the neurotoxicity of EACA and valproate was also additive in nature resulting in the PI of 1.14 for the combination (Table 6).

4.2.6.3 EACA and gabapentin

In line with the results of phenytoin and valproate, ED_{50} of intraperitoneally given gabapentin (100, 300, 700 and 1000 mg/kg BW) was found to be 310(150-638) mg/kg BW when given alone and it was decreased to 79(41-153) mg/kg BW in the presence of orally given EACA (Fig. 13A and Table 6). The combination also decreased ED_{50} of EACA from 673(299-1515), when given alone, to 183(94-354) mg/kg BW (Fig. 13A). The isobolographic representation of the interaction between EACA and gabapentin (Fig. 13B) illustrated that the observed combined ED_{50} value lay below the additive line. Thus, synergistic interaction of the combination was suggested. However, no statistical difference was noted between the ED_{50} values of gabapentin in the presence and in the absence of EACA (P = 0.6846). Therefore, the additive interaction between gabapentin and EACA was indicated.

In contrast to a decrease of TD_{50} of EACA when it was given in a combination with phenytoin or valproate, the combination between EACA and gabapentin increased TD_{50} of EACA from 415(147-1169), when given alone, to 1449(205-10198) mg/kg BW (Fig. 13C). The TD_{50} of gabapentin in the absence of EACA, 719(141-3660) mg/kg BW, was not statistically different (P = 0.9952) from its corresponding value in the presence of EACA, 622(89-4345) mg/kg BW. Therefore, the antagonistic interaction of neurotoxicity between gabapentin and EACA which was visually suggested from isobologram in Figure 13D was not

accepted. The neurotoxicity of EACA and gabapentin could be just as additive as the other's. However, in contrast to the results of previously described combination, the combination between gabapentin and EACA increased protective index of gabapentin about 3 times from 2.32 in monotherapy to 7.87 in combination (Table 6).

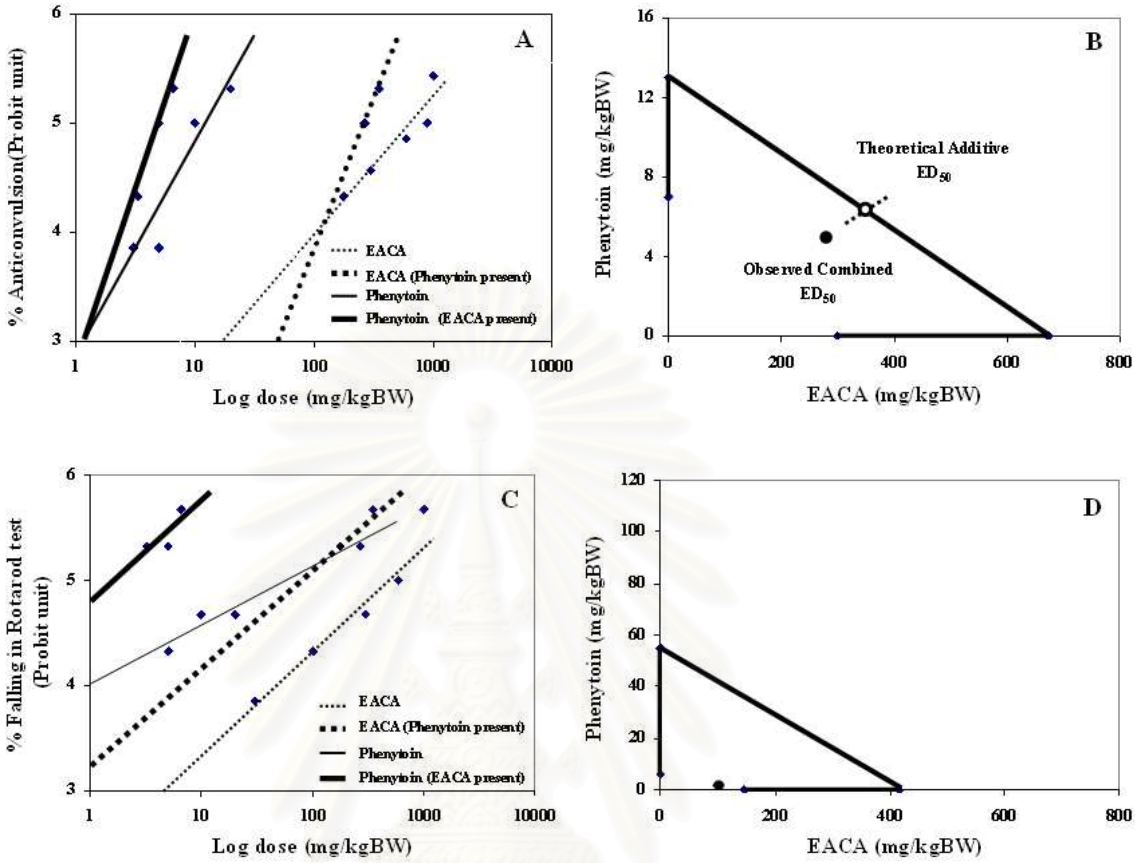


Figure 11 Dose-response curves and isobolographic representation of EACA and phenytoin on ED_{50} and TD_{50} in the pentylenetetrazole model in mice. **A**, dose-response curves of anticonvulsant effect of EACA [ED_{50}= 673(299-1515);·····], phenytoin [ED_{50}= 13(7-25); ——], EACA in the presence of phenytoin [ED_{50} = 277(187-409);▪ ▪ ▪] and phenytoin in the presence of EACA [ED_{50}= 5(3-8); ▬]. **B**, isobolographic representation of the interaction between EACA and phenytoin. In this graph the ED_{50} values of EACA and phenytoin are plotted as the x- and y-axis intercepts, respectively. The thicker lines directed from each ED_{50} value toward zero represent the lower 95% confidence limit of each ED_{50} value. The straight line connecting these two points is the theoretical additive line. The open circle that lies on the theoretical additive line represents the calculated theoretical ED_{50} value of the combination, were the interaction additive. The closed circle represents the experimentally observed ED_{50} value of the combination of EACA-phenytoin. In this experiment, the ED_{50} value of the combination of EACA-phenytoin fall below and inside the lower confidence limits of the theoretical additive, suggesting the interaction is additive. Consistent with this, the experimental ED_{50} value was not significantly different from the theoretical additive ED_{50} value (Student's *t* test, *P*=0.7015), indicating that the interaction was additive. **C**, dose-response curves of minimal neurotoxic effect of EACA [TD_{50}=415(147-1169);·····], phenytoin [TD_{50}=55(6-491); ——], EACA in the presence of phenytoin [TD_{50}= 99(11-854);▪ ▪ ▪] and phenytoin in the presence of EACA [TD_{50} = 2(1-6); ▬]. **D**, isobolographic representation of the interaction between EACA and phenytoin. The experimental TD_{50} value was not significantly different from the theoretical additive TD_{50} value (Student's *t* test, *P*= 0.9203), indicating that the interaction was additive.

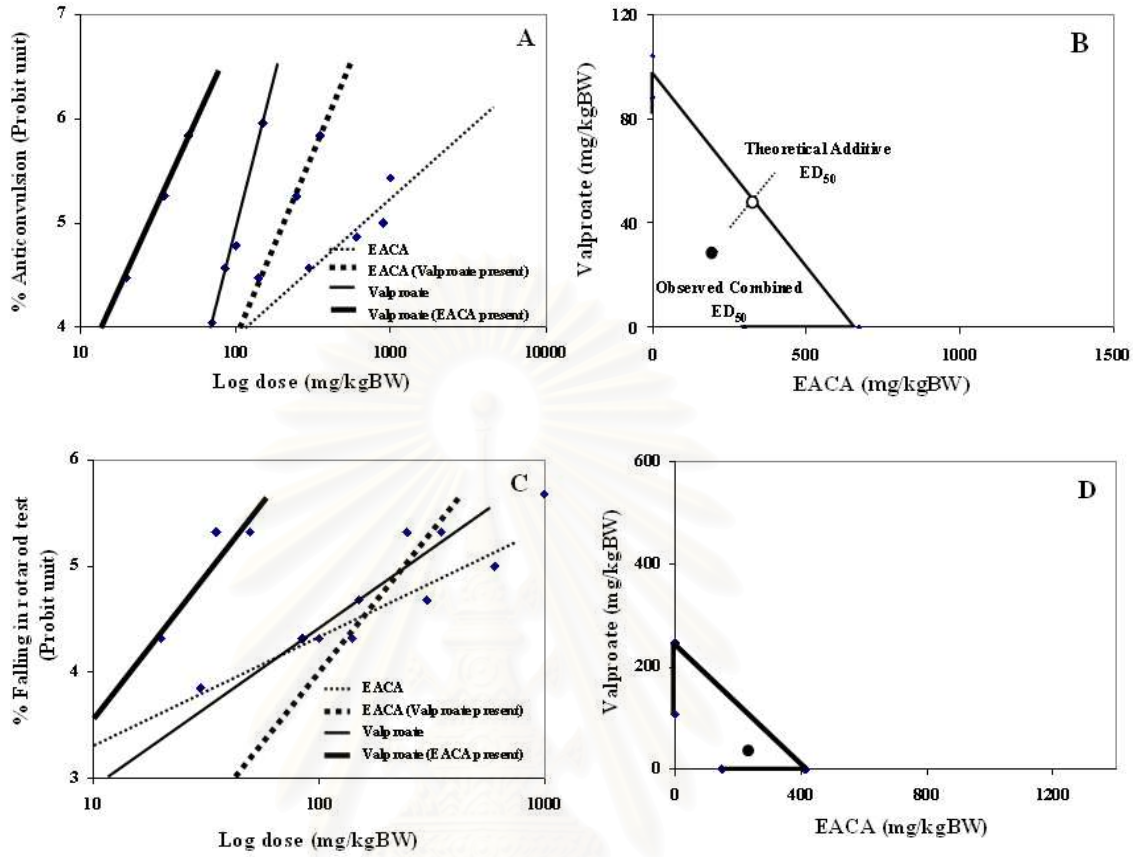


Figure 12 Dose-response curves and isobolographic representation of EACA and valproate on ED_{50} and TD_{50} in the pentylenetetrazole model in mice. **A**, dose-response curves of anticonvulsant effect of EACA [ED_{50}= 673(299-1515);·····],valproate [ED_{50}=104(88-121);——], EACA in the presence of valproate [ED_{50}= 201(144-282);▪ ▪ ▪] and valproate in the presence of EACA [ED_{50}= 29(21-40);▬]. **B**, isobolographic representation of the interaction between EACA and valproate. The experimental ED_{50} value was not significantly different from the theoretical additive ED_{50} value (Student's *t* test, *P*=0.3399), indicating that the interaction was additive. **C**, dose-response curves of minimal neurotoxic effect of EACA [TD_{50}= 415(147-1169);·····], valproate [TD_{50}= 247(107-568); ——], EACA in the presence of valproate [TD_{50} = 231(142-378); ▪ ▪ ▪] and valproate in the presence of EACA [TD_{50} = 33(20-54); ▬]. **D**, isobolographic representation of the interaction between EACA and valproate. The experimental TD_{50} value was not significantly different from the theoretical additive TD_{50} value (Student's *t* test, *P*=0.5524), indicating that the interaction was additive.

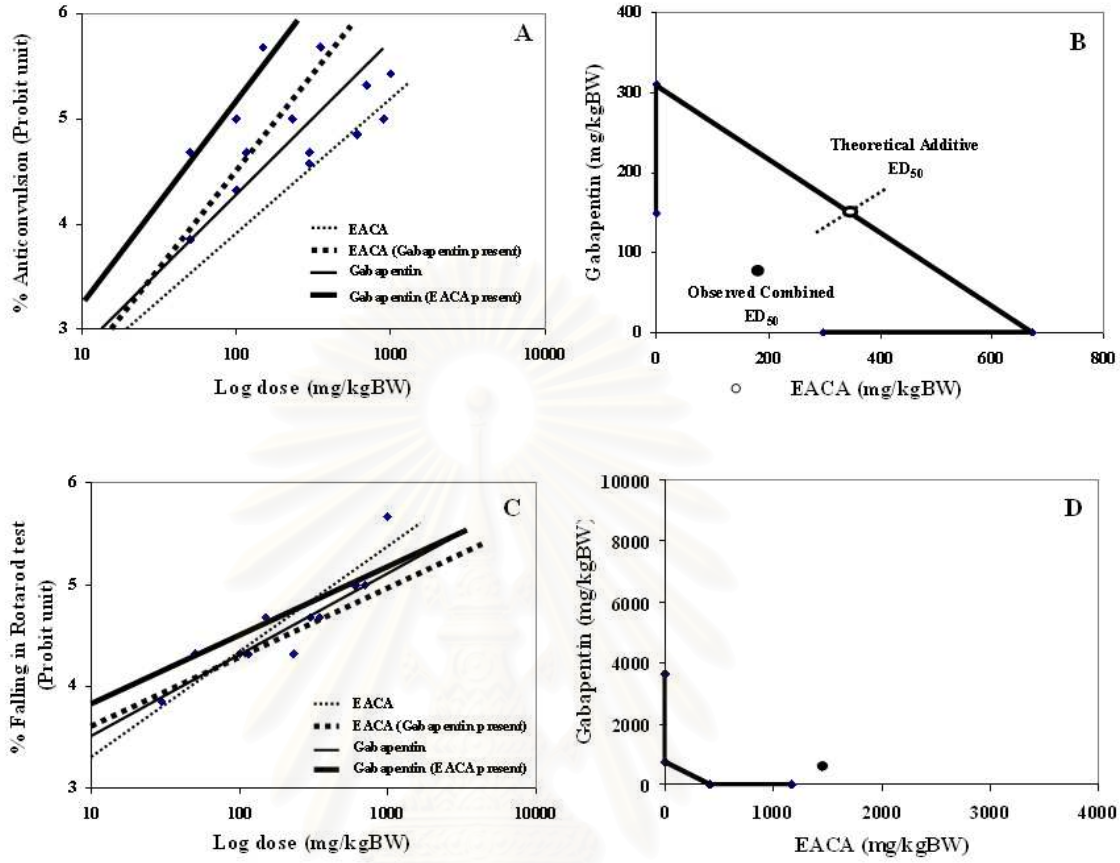


Figure 13 Dose-response curves and isobolographic representation of EACA and gabapentin on ED_{50} and TD_{50} in the pentylenetetrazole model in mice. **A**, dose-response curves of anticonvulsant effect of EACA [ED_{50}= 673(299-1515); ·····], gabapentin [ED_{50}= 310(150-638); ——], EACA in the presence of gabapentin [ED_{50}= 183(94-354); ▪▪▪] and gabapentin in the presence of EACA [ED_{50}=79(41-153); ▬▬]. **B**, isobolographic representation of the interaction between EACA and gabapentin. The experimental ED_{50} value was not significantly different from the theoretical additive ED_{50} value (Student's *t* test, *P*=0.6846), indicating that the interaction was additive. **C**, dose-response curves of minimal neurotoxic effect of EACA [TD_{50}= 415(147-1169); ·····], gabapentin [TD_{50}= 719(141-3660); ——], EACA in the presence of gabapentin [TD_{50}= 1449(205-10198); ▪▪▪] and gabapentin in the presence of EACA [TD_{50}= 622(89-4345); ▬▬]. **D**, isobolographic representation of the interaction between EACA and gabapentin. The experimental TD_{50} value was not significantly different from the theoretical additive TD_{50} value (Student's *t* test, *P*=0.9952), indicating that the interaction was additive.

Table 6 The median effective doses (ED_{50}), median neurotoxic doses (TD_{50}) and protective indices (PI) of phenytoin, valproate and gabapentin given intraperitoneally either alone or in combination with EACA in mice.

Groups	ED_{50} (mg/kg BW)	TD_{50} (mg/kg BW)	PI (TD_{50}/ED_{50})
EACA	673(299-1515)	415(147-1169)	0.62
Phenytoin	13(7-25)	55(6-491)	4.23
Phenytoin (with EACA)	5(3-8)	2(1-6)	0.4
Valproate	104(88-121)	247(107-568)	2.38
Valproate (with EACA)	29(21-40)	33(20-54)	1.14
Gabapentin	310(150-638)	719(141-3660)	2.32
Gabapentin (with EACA)	79(41-153)	622(89-4345)	7.87

4.3 Possible mechanisms of EACA

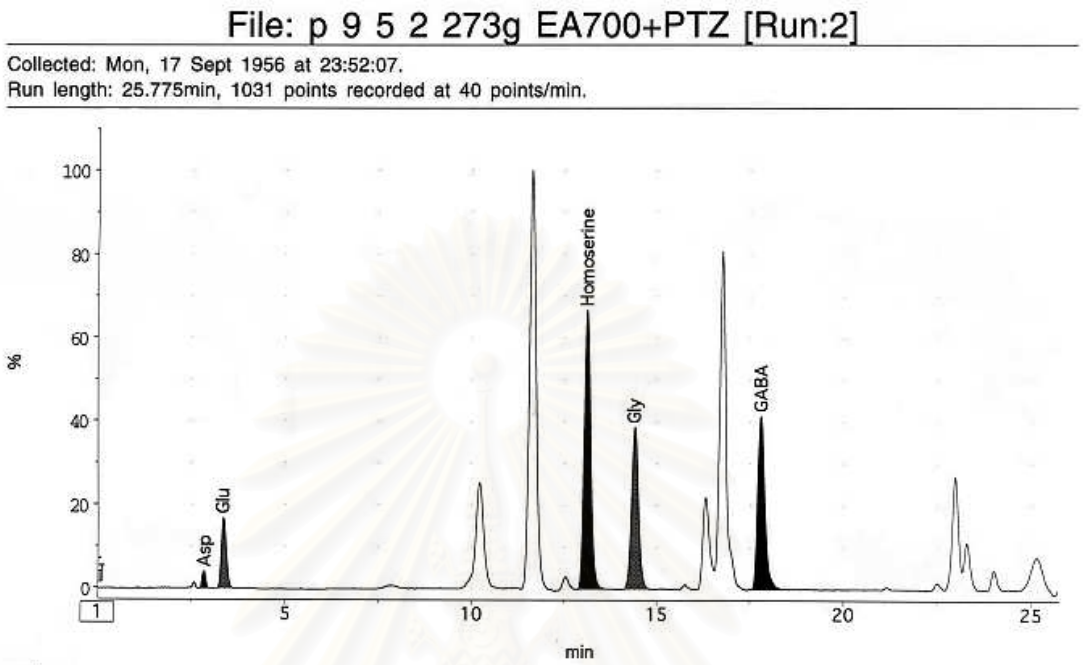
4.3.1 Effects of EACA on some hippocampal amino acid neurotransmitter levels relating to convulsion in freely moving rats by microdialysis technique

The effects of EACA on the extracellular levels of the excitatory amino acid neurotransmitters (aspartate and glutamate) and the inhibitory amino acid neurotransmitters (glycine and GABA) in the hippocampus following a PTZ administration (60 mg/kg BW) were determined in freely moving rats. The data were expressed as a percent change from basal value which was determined from an average of the three consecutive samples before the administration of NSS, vehicle or EACA. Qualitative and quantitative determination of the amino acid neurotransmitters were achieved by HPLC as exemplified by HPLC chromatogram in Figure 14.

In normal rats, as shown in Figure 15, EACA 700 mg/kg BW tended to decrease but did not show significant effect upon the extracellular levels of aspartate, glutamate, glycine and also GABA in the dialysate collected from the hippocampus when compared with control. On the contrary, EACA at the dose of 1,000 mg/kg BW significantly increased the level of aspartate in comparison to the control group or those receiving lower dose of EACA ($P < 0.05$).

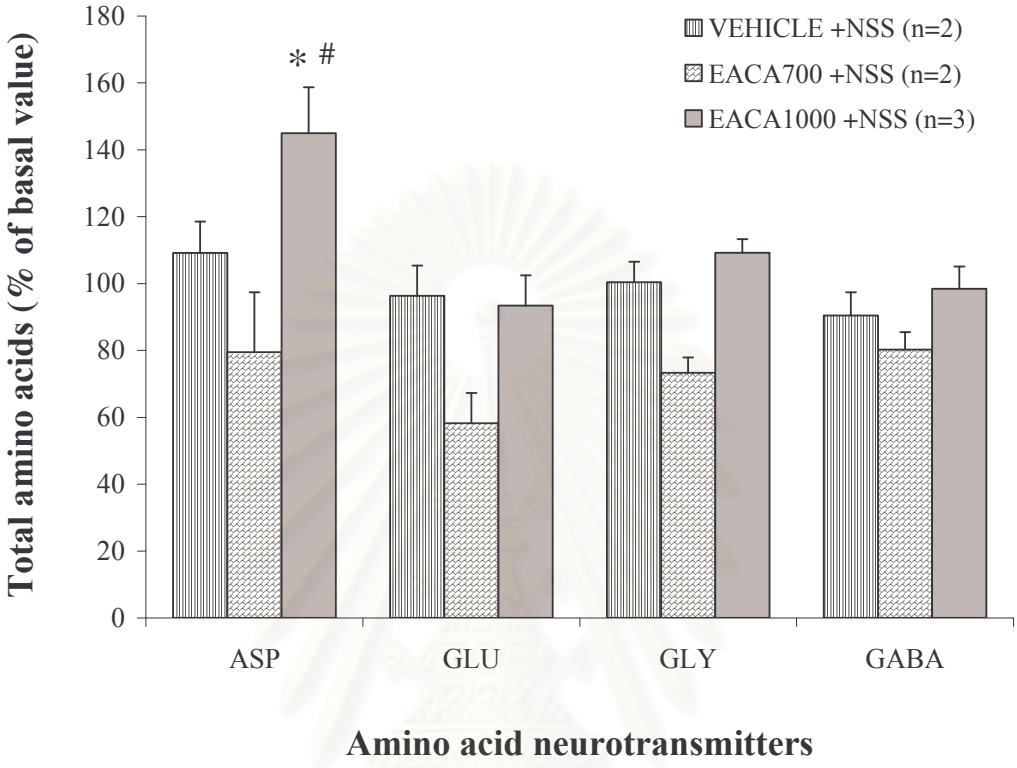
In control groups (Fig. 16), the effect of vehicle on spontaneous release of aspartate, glutamate, glycine and GABA was not statistically different from those of NSS. The administration of PTZ 60 mg/kg BW that demonstrated convulsion in all of the animal in vehicle+PTZ-treated group showed significantly increased aspartate level in the collected dialysate when compared with vehicle alone ($P < 0.05$), while other amino acids were not affected. Pretreatment of EACA 700 mg/kg BW could protect most of the animal against PTZ. In this group, the excitatory amino acid neurotransmitters (both aspartate and glutamate) were gradually decreased while the inhibitory amino acid neurotransmitters (glycine and GABA) tended to increase when compared with vehicle+PTZ-treated group, however, none of them was statistically significant.

The dialysate collected from convulsive animal of EACA 700+PTZ treated group showed gradually increment of aspartate in comparison with the protected animal of EACA700+PTZ treated groups. In addition, small decrement of glycine and GABA were noted when compared with non-convulsive animal of EACA 700+PTZ treated group. Similarly, none of them was statistically significant.



Peak		Name	t_R (min)	Start (min)	End (min)	Area	Height (%)	Norm (%)	Peak Type	Amount	F
1		Asp	2.850	2.750	2.950	0.413	4.074	1.26	BB		
2		Glu	3.350	3.200	3.600	2.457	16.775	7.52	BB		
3		Homoserine	13.125	12.900	13.600	12.878	66.601	39.38	BB		
4		Gly	14.400	14.150	14.800	8.084	38.550	24.72	BB		
5		GABA	17.800	17.575	18.350	8.867	41.371	27.12	BB		
						32.70	167.371	100.00			

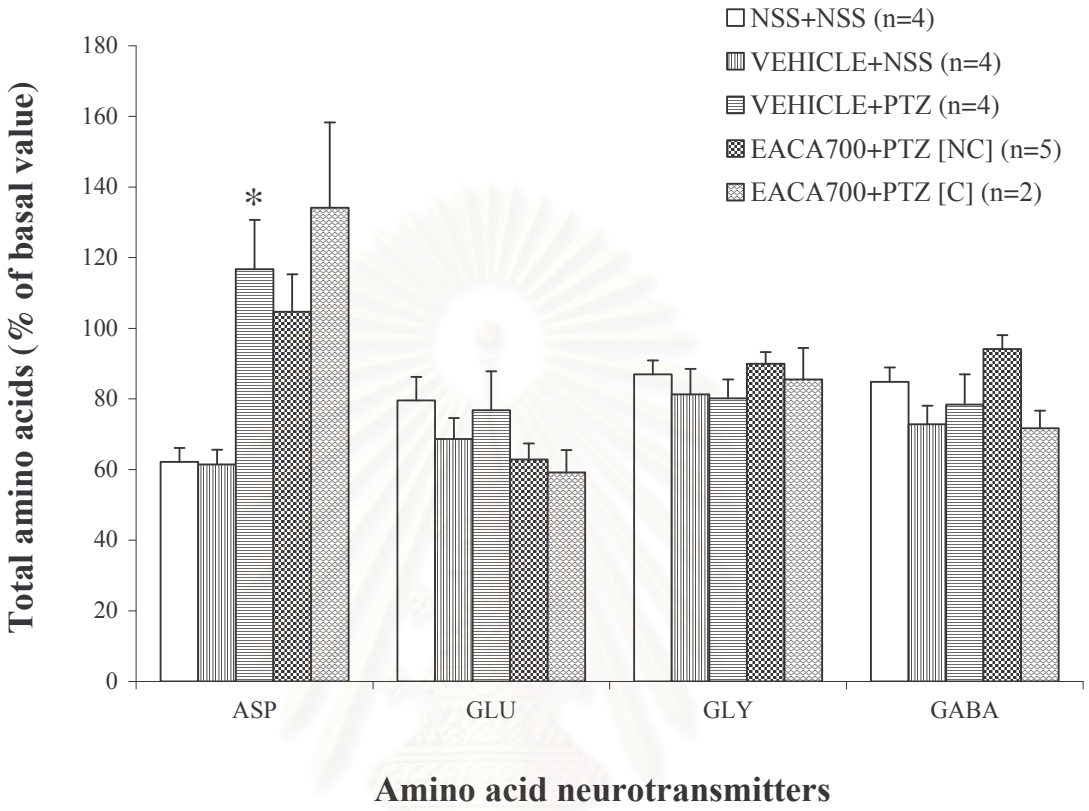
Figure 14 HPLC chromatogram of OPA-derivatized amino acid neurotransmitter from rat hippocampus



* $P < 0.05$ denotes statistically significant difference from vehicle+NSS

\# $P < 0.05$ denotes statistically significant difference from EACA700+NSS

Figure 15 Effects of EACA 700 and 1,000 mg/kg BW on the total amount of hippocampal amino acid neurotransmitter levels in the dialysate collecting from normal rats for 180 minutes (MEAN ± S.E.M.). Value in brackets represents number of animal treated.



* $P < 0.05$ denotes statistically significant difference from vehicle+NSS

Figure 16 Effect of EACA 700 mg/kg BW on the total amount of hippocampal amino acid neurotransmitter levels in the dialysate collecting from normal and PTZ-treated rats for 180 minutes (MEAN $\pm$ S.E.M.). Value in brackets represents number of animal treated.

4.3.2 Effect of EACA on $GABA_A$ receptor current by whole cell patch-clamp technique

Because, one of the important mechanisms in which the agents provide protection against generalized and focal seizures was the direct action on the GABA receptor-chloride channel complex (as with benzodiazepine, barbiturate) (Kwan, 2001). Thus, the effects of EACA on $GABA_A$ receptor current were investigated.

In this experiment, GABA 3 µM that did not show desensitization was selected for studying the effect of EACA on $GABA_A$ receptor current (Suthep Jenthep, 2002). The EACA (10, 30 and 50 µg/ml) did not induce any alteration of hippocampal membrane current in the absence of GABA (Fig. 17). When co-application with GABA 3 µM, lower concentration of EACA, 0.1 up to 3 µg/ml, produced a slightly potentiation of $GABA_A$ current with maximal potentiation at 3 µg/ml (Fig. 18). On the contrary, the higher concentration of EACA (10, 30 and 50 µg/ml) the currents were partially blocked during co-application with GABA in the concentration-dependent manner. Statistically significant difference was denoted at 50 µg/ml when compared to the other groups ($P < 0.05$).

To confirm this experimental setup, the effect of DZP on $GABA_A$ receptor current was also investigated. The potentiation of the GABA-induced current was also observed during co-application with DZP at the concentration of 0.1, 0.3, 1 µM in the concentration-dependent manner. Whereas, increasing concentration, the potentiation was decreased (Fig. 19 and 20). However, statistically significant increase was observed at the concentration 0.3, 1 and 3 µM when compared to the GABA alone ($P < 0.05$).

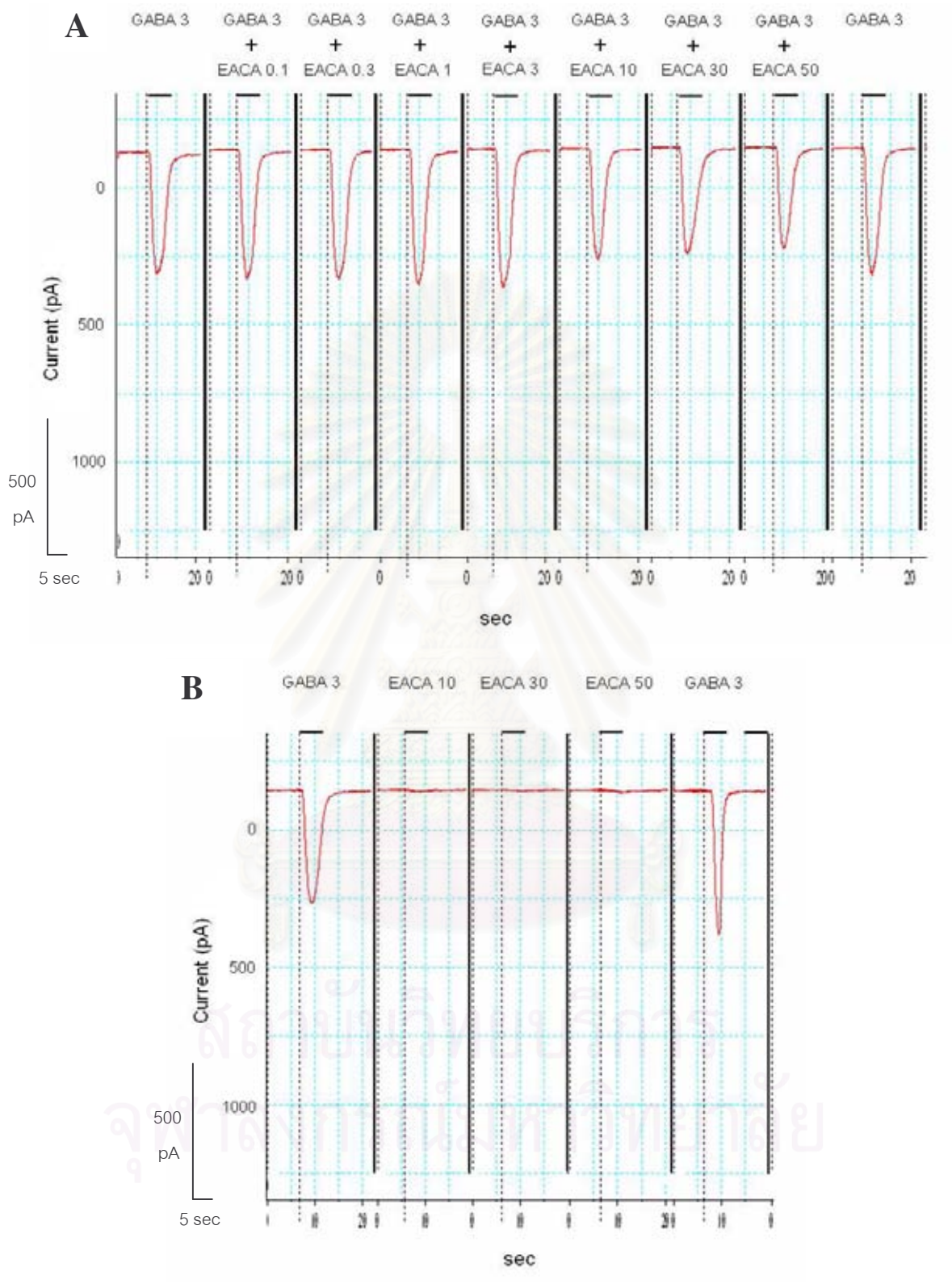
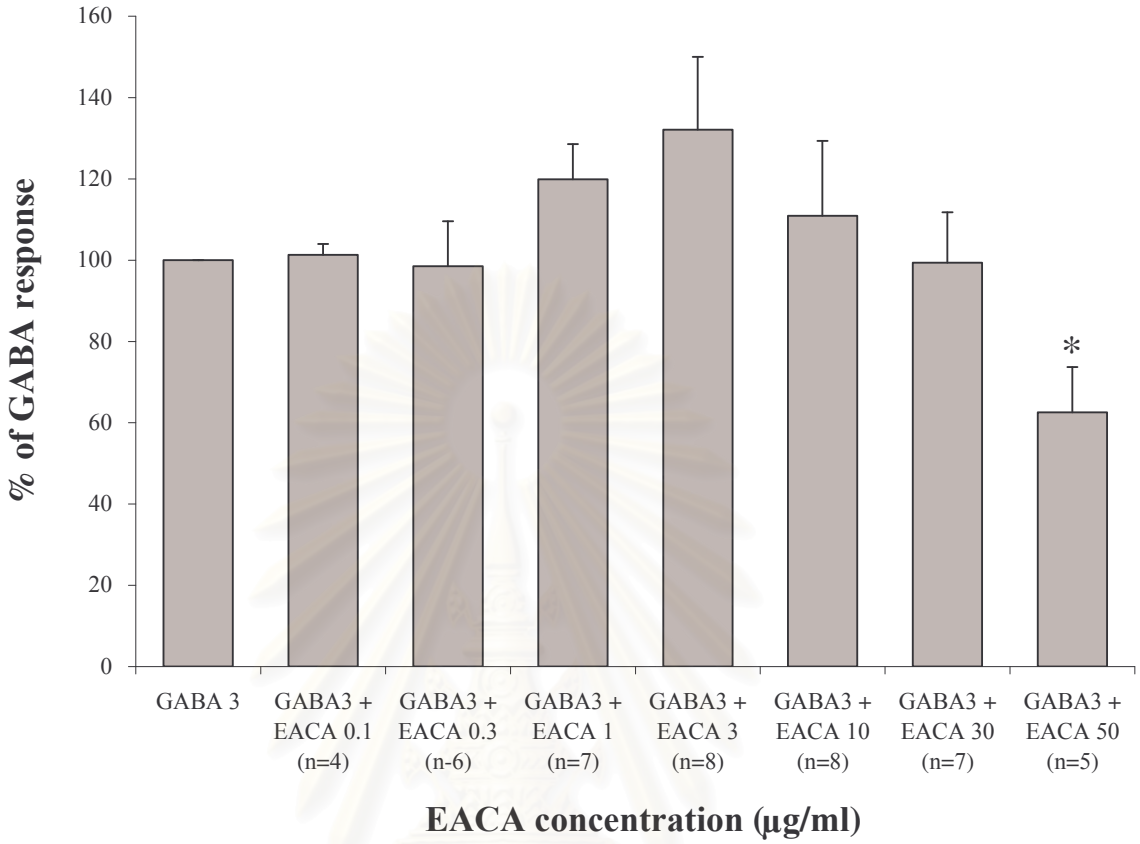


Figure 17 Representative tracing demonstrated the effect of EACA on hippocampal $GABA_A$ receptor current. (A), EACA 0.1 - 50 µg/ml during co-application with GABA 3 µM. (B), EACA 10, 30 and 50 µg/ml in the absence of GABA. Each horizontal bar above the trace indicates the period of a continuous application of drugs (~5 sec).



* $P < 0.05$ denotes statistically significant difference from the other group

Figure 18 Effects of EACA 0.1 up to 50 µg/ml on hippocampal $GABA_A$ receptor current (MEAN $\pm$ S.E.M.). Value in brackets represents numbers of recorded cells.

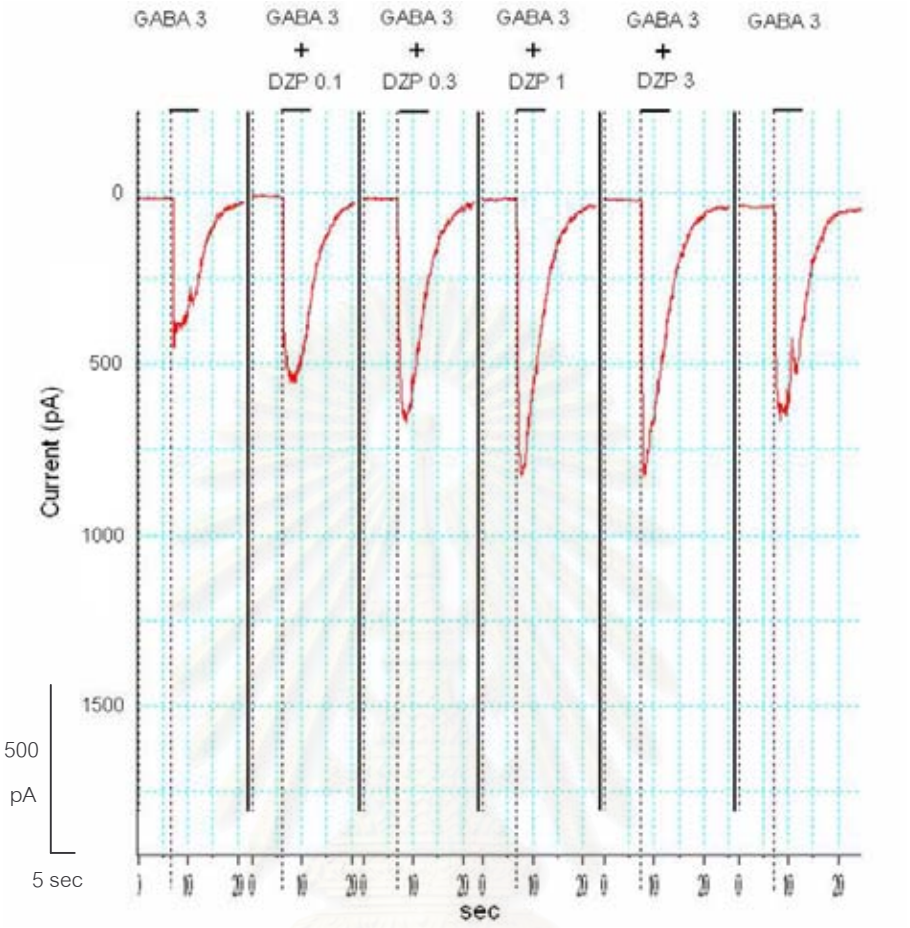
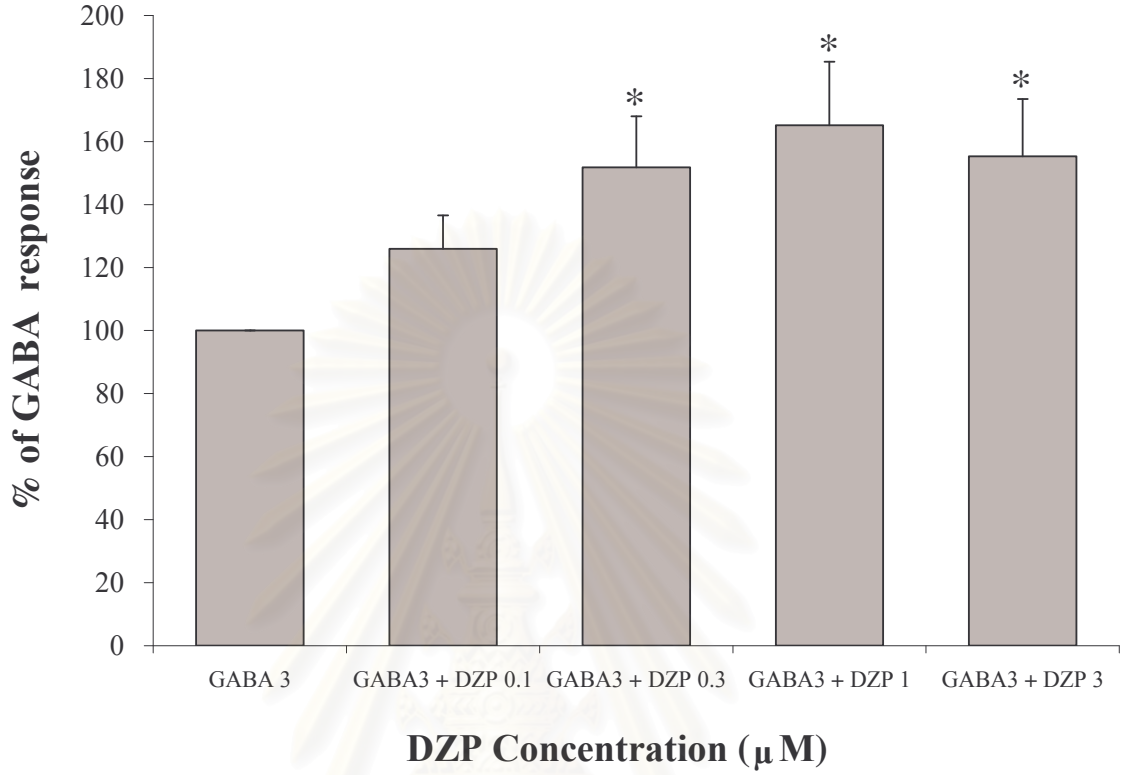


Figure 19 Representative tracing demonstrated the effect of DZP 0.1, 0.3, 1 and 3 μM on hippocampal $GABA_A$ receptor current. Each horizontal bar above the trace indicates the period of a continuous application of drugs (~5 sec).



* $P < 0.05$ denotes statistically significant difference from GABA3

Figure 20 Effects of DZP 0.1, 0.3, 1 and 3 μM on hippocampal $GABA_A$ receptor current (MEAN ± S.E.M.) (n=6 in each group).

4.4 Effect of EACA on epileptogenesis

4.4.1 Effect on kindling induction by PTZ

In vehicle+PTZ-treated group, repeated administration of subconvulsant dose of PTZ (40 mg/kg BW) on every alternate days (for 76 days, 38 injections) resulted in increasing convulsive activity leading to generalized clonic-tonic seizures score of 5 (Racine, 1972). Administration of EACA in the doses of 300, 700 and also 1000 mg/kg BW did not modify the course of kindling induced by PTZ, as all of the animal could achieve stage 5 within 38 injections of PTZ (Fig. 21). In addition, as shown in Figure 22, the number of PTZ injection for fully kindle induction was not significantly different when compared between EACA+PTZ-treated groups and the group of PTZ alone.

4.4.2 Effect of EACA on step-down test in normal and fully-kindled mice.

In step-down test, the step-down error did not differ significantly between the vehicle+PTZ and EACA (300, 700 and 1,000 mg/kg BW)+PTZ- treated groups in both initial and retention tests (24 and 48 h after the last administration of PTZ, respectively) (Fig. 23).

In the step-down latency, the average initial latency at 24 h after the last administration of PTZ in all of EACA+PTZ-treated groups were not significantly different when compared with the vehicle+PTZ-treated group. However, the retention latency at 48 h after the last administration of PTZ in vehicle+PTZ-treated group tended to decrease when compared with saline and vehicle treated groups. The larger doses of EACA showed reversal effect on PTZ-induced cognitive deficit in which significant increase was observed in the group of 1,000 mg/kg BW in comparison to the vehicle+PTZ-treated group ($P<0.05$) (Fig. 24).

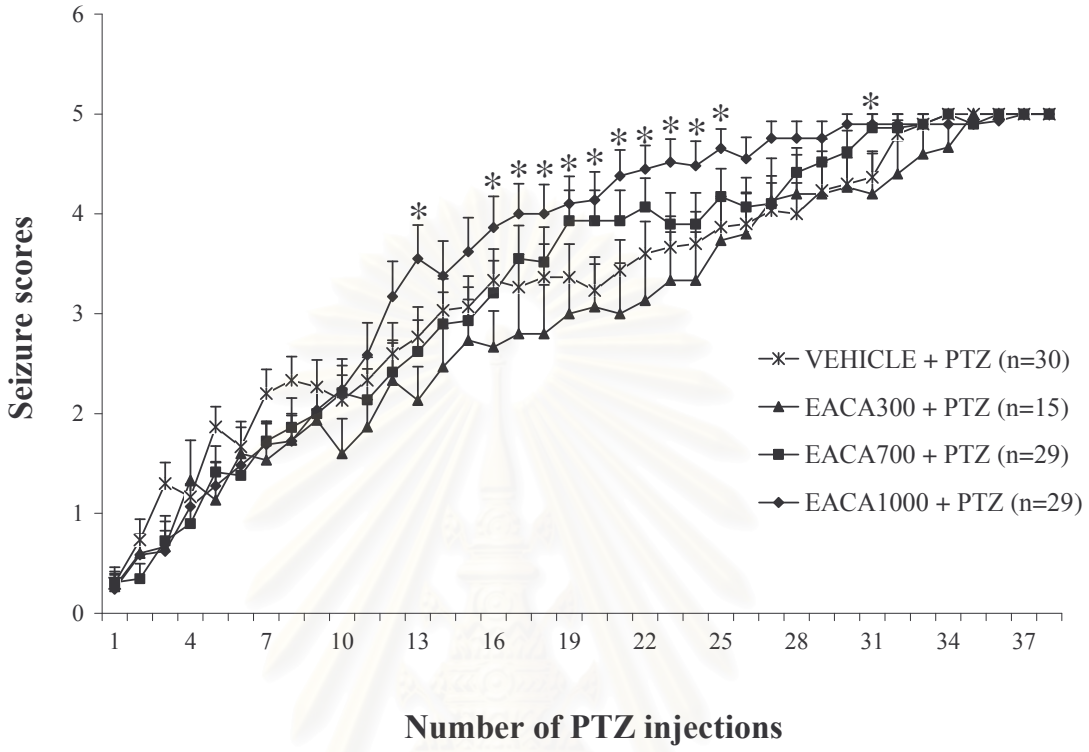
4.4.3 Effect of EACA on MDA level in fully-kindled mice.

Effects of EACA administration at the doses of 300, 700 and 1000 mg/kg BW on cerebral cortex MDA levels in normal and PTZ-kindle mice are shown in Figure 25. EACA did not change MDA level in PTZ-induced kindled mice as compared with the normal and vehicle+PTZ-treated group.

4.4.4 Effect of EACA on survival CA1 and CA3 pyramidal neurons in fully-kindled mice

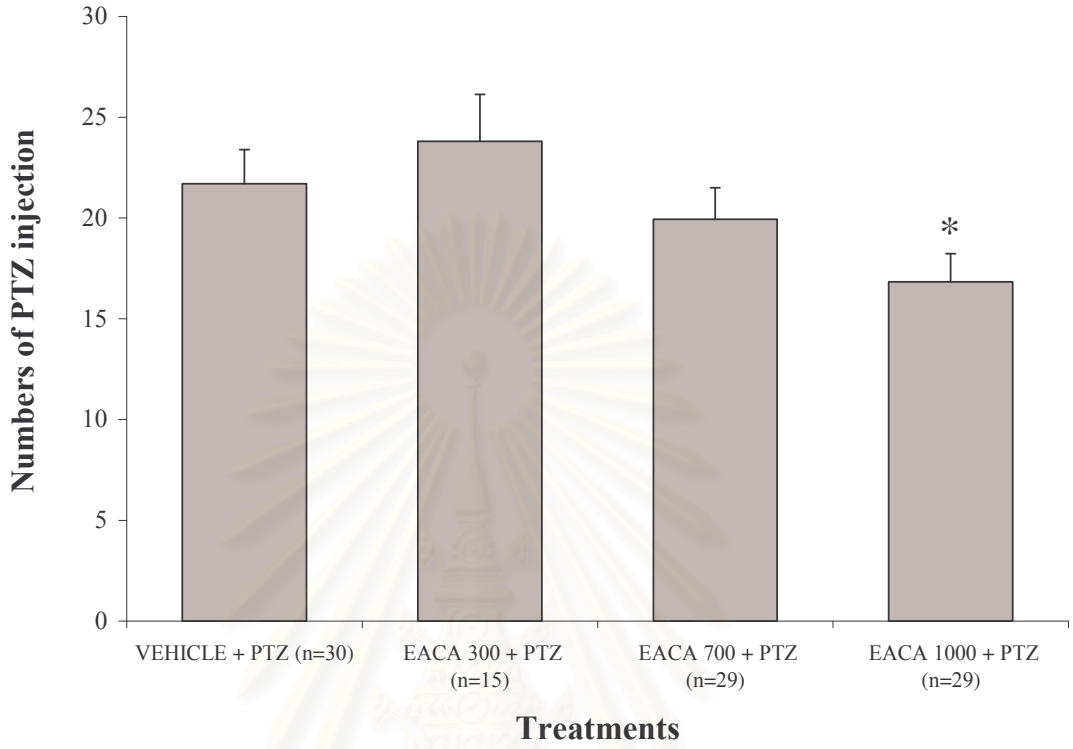
To determine the relative survival of pyramidal neurons in the CA1 and CA3 regions after EACA administration in the course of kindling induction by PTZ, an average surviving cell numbers were counted over consistent fields, over both hemisphere, and over three sections in each brain.

No obvious cell loss was noted in microscopic examination of the specimens (Fig. 26). However, as seen in Figure 27, the number of pyramidal neurons in both regions of both sides was gradually decreased in vehicle+PTZ-treated group, while the administration of EACA 300 and 700 mg/kg BW every alternate day before PTZ injection can increase survival pyramidal neurons population and showed significantly increased ($P<0.05$) in EACA 700+PTZ-treated group when compared with vehicle+PTZ-treated group in both regions of both sides. In contrast to an increase survival pyramidal neurons of the EACA 700 mg/kg BW, the higher dose of EACA 1,000 mg/kg BW seemed to show the lower protection.



* $P < 0.05$ denotes statistically significant difference from EACA300+PTZ

Figure 21 Effects of EACA 300, 700 and 1,000 mg/kg BW pretreatment on the development of PTZ-kindled seizures in mice. The EACA was injected 1 h prior to PTZ challenge. The ordinate represents seizure score (MEAN $\pm$ S.E.M.). The abscissa represents number of PTZ injection. Value in brackets represents number of animal treated.



* $P < 0.05$ denotes statistically significant difference from EACA300+PTZ

Figure 22 Numbers of PTZ injection for fully kindled induction in normal and EACA 300, 700 and 1,000 mg/kg BW treated mice (MEAN $\pm$ S.E.M.). Value in brackets represents number of animal treated.

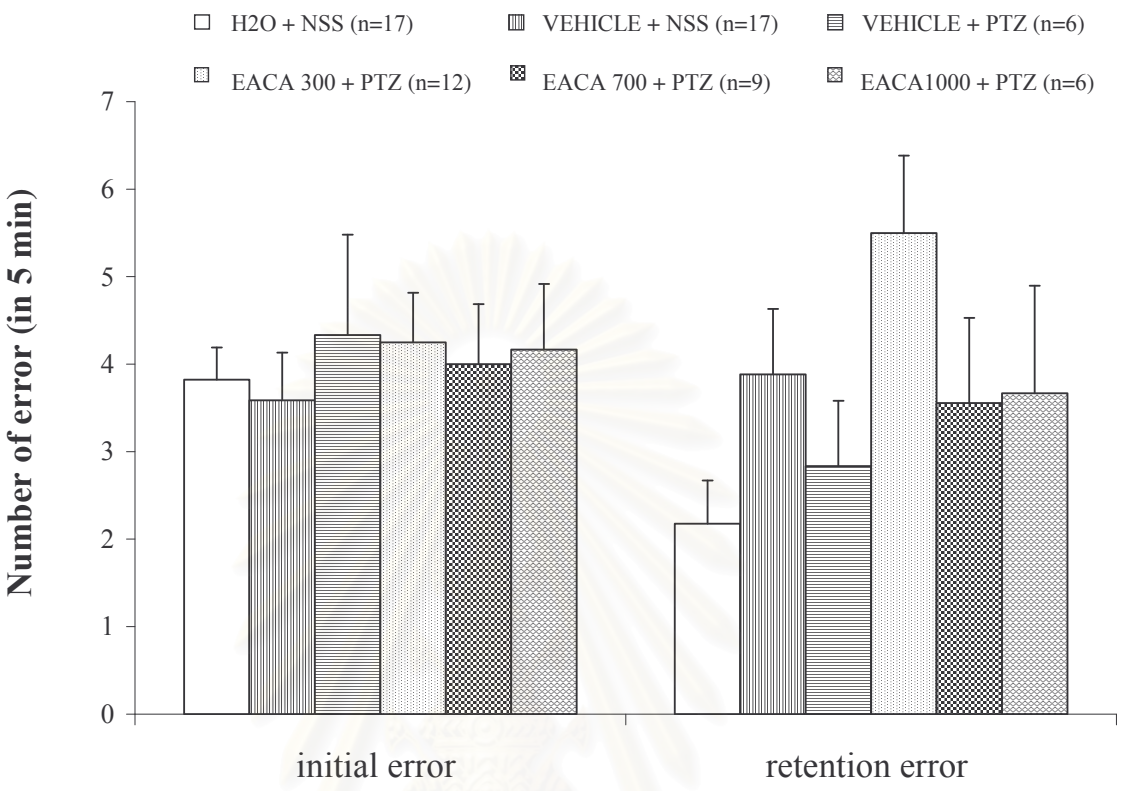
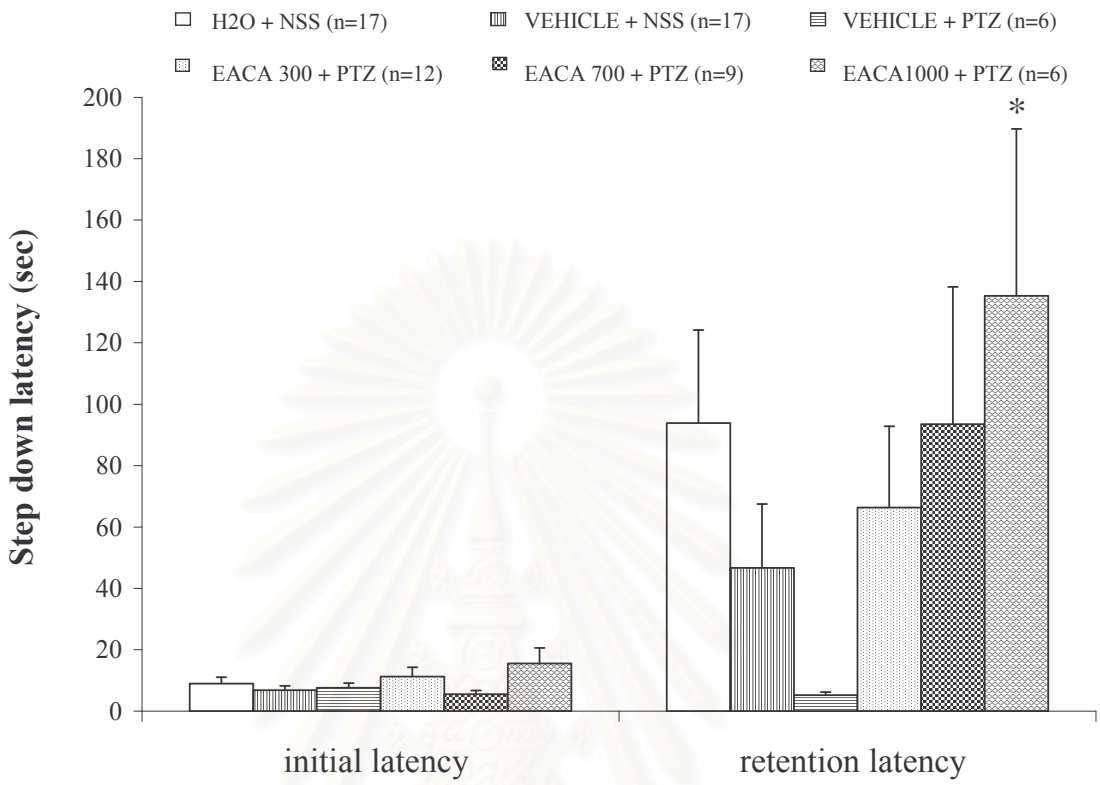


Figure 23 Effects of EACA 300, 700 and 1,000 mg/kg BW on step-down error in normal and PTZ-kindled mice (MEAN $\pm$ S.E.M.). The ordinate represents number of error in 5 minutes. The abscissa represents initial error (24 h) and retention error (48 h) after the last injection of PTZ. Value in brackets represents number of animal treated.



* $P < 0.05$ denotes statistically significant difference from vehicle+PTZ (in each group)

Figure 24 Effects of EACA 300, 700 and 1,000 mg/kg BW on step-down latency in normal and PTZ-kindled mice. The ordinate represents step-down latency in seconds (MEAN $\pm$ S.E.M.). The abscissa represents initial latency (24 h) and retention latency (48 h) after the last injection of PTZ. Value in brackets represents number of animal treated.

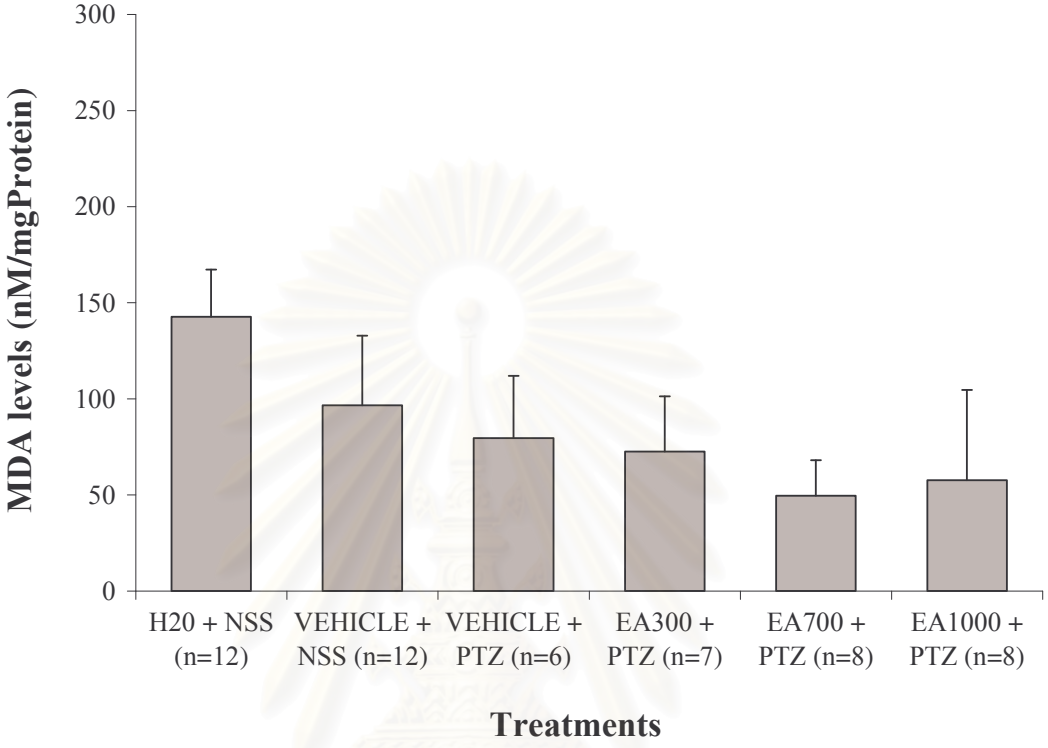


Figure 25 Effects of EACA 300, 700 and 1,000 mg/kg BW on MDA level in normal and PTZ-kindled mice cerebral cortex. The ordinate represents MDA level in nM/mgProtein (MEAN $\pm$ S.E.M.) after the 48 h of last injection of PTZ. Value in brackets represents number of animal treated.

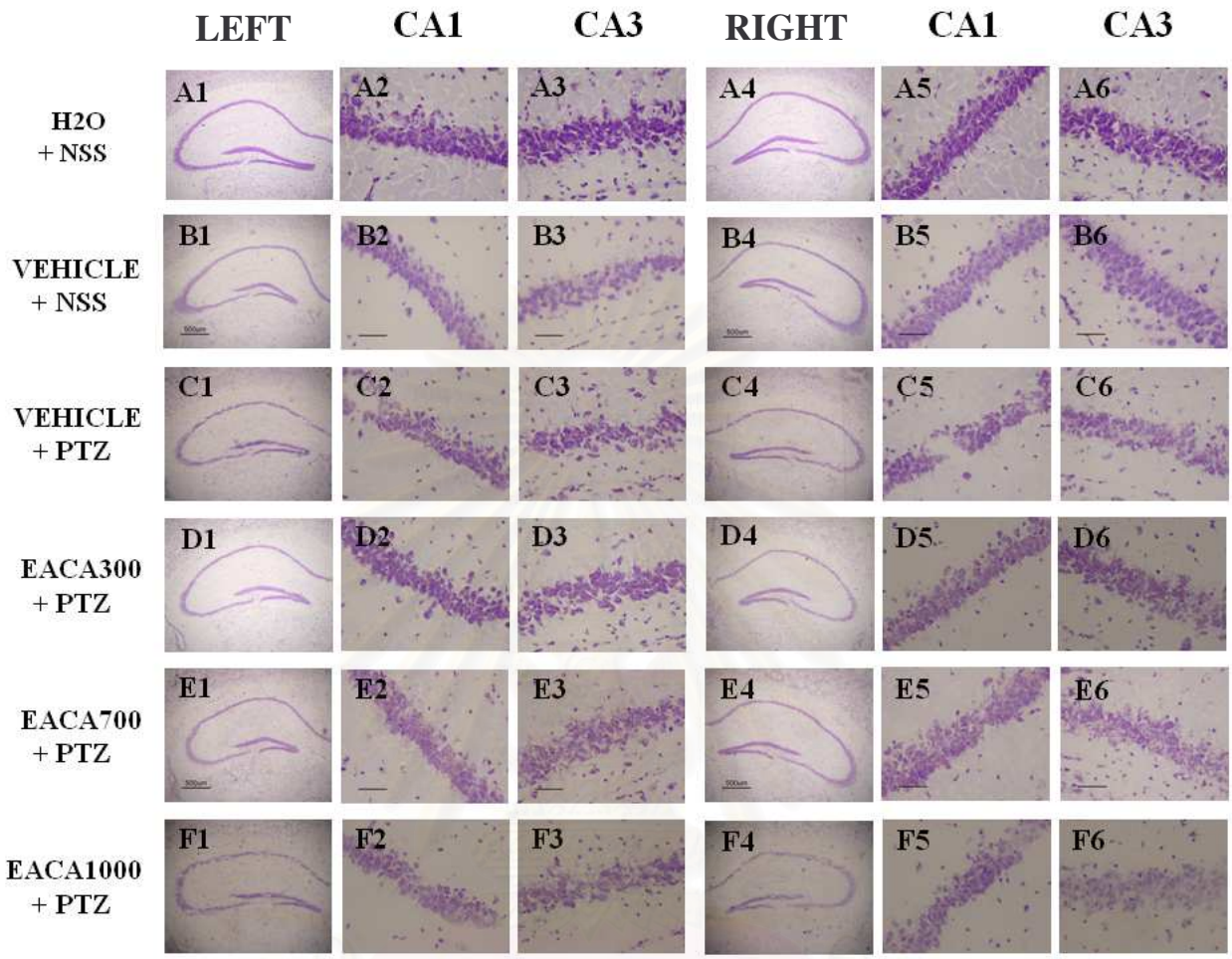


Figure 26 Nissl staining with cresyl violet of CA1 and CA3 neurons in transverse left and right hippocampal slices. *A1-A6*: Nissl staining in a H_2O+NSS-treated group, *B1-B6*: in a vehicle+NSS-treated group, *C1-C6*: in a vehicle+PTZ-treated group, *D1-D6*: in an EACA300+PTZ-treated group, *E1-E6*: in an EACA700+PTZ-treated group and *F1-F6*: in an EACA1000+PTZ-treated group. Scale bars are 500 µm for column 1 and 4 and 50 µm for column 2,3,5 and 6

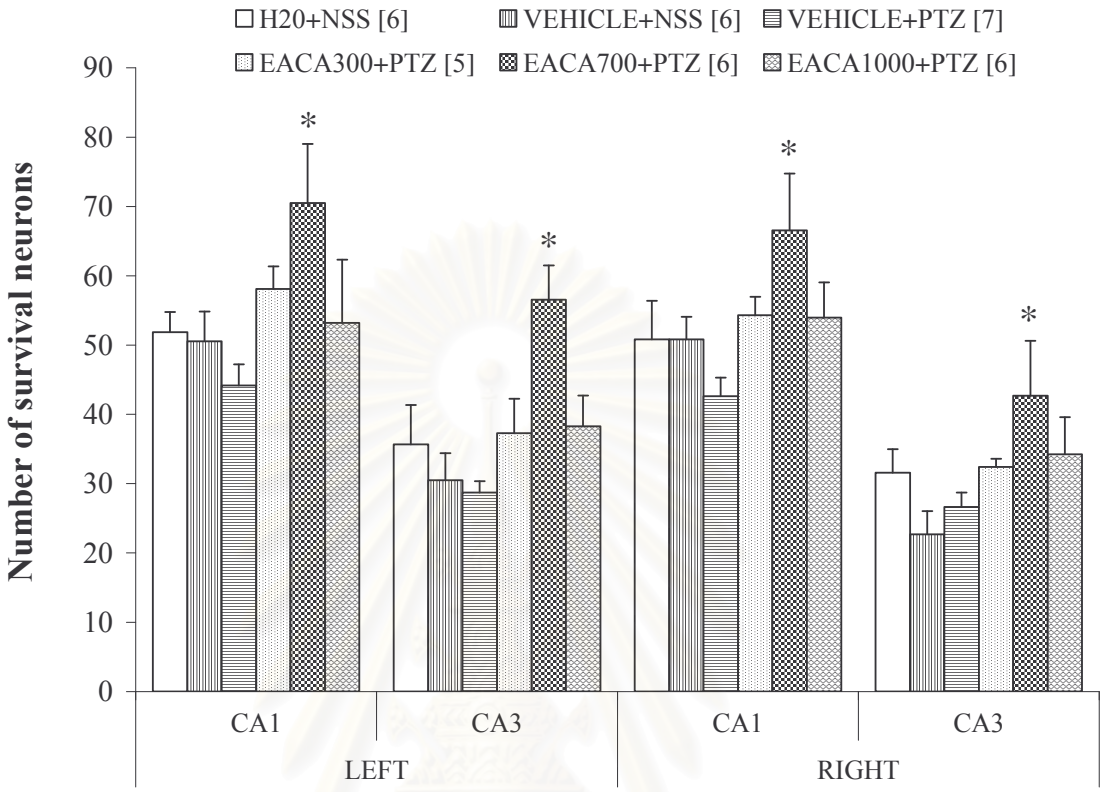


Figure 27 Effects of EACA 300, 700 and 1,000 mg/kg BW on numbers of survival CA1 and CA3 hippocampal pyramidal neurons in normal and PTZ-kindled mice (MEAN $\pm$ S.E.M.). Value in brackets represents number of animal treated.

CHAPTER V

DISCUSSION

Seizures represent the most common neurological disturbance. Up to almost 10% of the population will experience at least one seizure in their lifespan. Of these, nearly half will be subject to recurrent seizures (epilepsy). Epilepsy is particularly troublesome for patients because of its unpredictable occurrence and the associated abrupt loss of competence. This often results in severe social and personal morbidity with attendant loss of self confidence, personal safety, financial and recreational independence. Treatment is generally very successful and promising new modalities of treatment are rapidly being developed (Gillham *et al*., 1990; Nichols *et al*., 1993; Filloux, 2005).

Complex partial seizures demonstrate impairment of consciousness, automatisms and focal epileptiform discharges on EEG recordings. Patients with temporal lobe epilepsy may be remarkably resistant to treatment with AEDs. Poorly controlled seizures may result in treatment with two or more AEDs. The use of chemotherapeutic drugs in epilepsy therapy involves the risk of life threatening host toxicity (Tatum *et al*., 2000). Another issue complicating drug therapy of epilepsy is that most of AEDs have a narrow therapeutic range (Ketter *et al*., 1999). The concentrations required for effective seizure control may be close to the concentrations that cause side effects. Medications that have a broader therapeutic index will be easier to use and more likely to be taken as prescribed (Leppik, 2001). Therefore, effort has been put on both the search for new AEDs with better pharmacological profiles as well as an establishment of adjunctive medication or other non pharmacological treatment to achieve a stage of seizure free.

The fact that *Centella asiatica* (CA) has been proposed to possess a broad profile of pharmacological activity including anticonvulsant (Brinkhaus *et al*., 2000) has leaded us to investigate its effect in animal models of epilepsy.

The present results showed that the crude ethanolic total extract of CA, given orally, was able to protect the animals exclusively in the PTZ but not MES model at

the optimal pretreated time of 1 hour. Actually, the MES test is an excellent animal model for the identification of new AEDs that block seizure spread and are likely to be effective for the management of generalized tonic-clonic seizures in human. It is highly reproducible with consistent endpoints. While, the subcutaneous PTZ test is an effective model that identifies the AEDs that raise seizure threshold and are likely to couple with AEDs that are effective in the treatment of absence seizures. PTZ-induced seizures are potentiated by GABA agonist (White, 1997; Löscher, 1998; Stables and Kupferberg, 2005). Accordingly, the PTZ-induced clonic seizure is blocked by drugs acting at the $GABA_A$ receptor such as benzodiazepine and phenobarbital (White, 1997). The alcoholic extract of CA has been reported to increase brain GABA levels in a dose dependent manner (Chatterjee *et al*., 1992). Its stimulatory effect on this inhibitory transmitter in the CNS was inhibited by the specific GABA-antagonist, bicuculline (Brinkhaus *et al*., 2000). Therefore, it was suggested that anticonvulsant activity of CA might involve GABAergic system which could probably be a part of anticonvulsant activity of CA observed in the present studies.

Based on the finding that the crude ethanolic total extract of CA was able to protect the animals in the PTZ test only when it was given orally but not intraperitoneally, it is suggested that the active compound was ingested as a prodrug and subsequently being activated by metabolic process. The same phenomenon was illustrated by nitrated phenolic compounds which exerted a marked stimulating effect upon metabolism (Pugsley, 1935). Thus, further investigations have to be carried out in order to identify the constituents of the EACA and their respective pharmacokinetic profiles.

Anticonvulsant activity against PTZ test and toxicity testing of EACA

It has been demonstrated that unwanted effects such as sedation, dizziness, ataxia, headache, nausea and impairment of motor function are common during the treatment with AEDs (Deckers *et al*., 1997; Browne and Holmes, 2001; Kwan, 2004). Furthermore, Leppik (2001) demonstrated that the pharmacologically related side effects of AEDs usually involve the CNS. Phenytoin and carbamazepine, both active

at the sodium channel, have ataxia as a prominent feature. Barbiturates and benzodiazepines cause sedation and somnolence. The rotarod test is routinely used to assess such neurotoxicity of the AEDs (Stables and Kupferberg, 2005). When four fractions obtained from sequential extraction of CA were tested, only EACA demonstrated anticonvulsant activity exhibiting the ED_{50} of 673(299-1515) mg/kg BW. Respective TD_{50} of EACA assessed by rotarod test was found to be 415(147-1169) mg/kg BW resulting in the PI of 0.62. EACA was practically safe exhibiting the median lethal dose (LD_{50}) that was higher than 5,000 mg/kg BW. Thus, the relative safety margin (LD_{50}/ED_{50}) was more than 7.43. The depressant effect of EACA at 700 and 1,000 mg/kg BW, on total horizontal count of locomotor activity was significantly different from that of vehicle. However, the prolongation of barbiturate sleeping time exhibited by EACA was not significantly different from the effect of vehicle. Therefore, it can be anticipated that the therapeutic dose of EACA could depress the central nervous system and produce some neurotoxicity on motor coordination as those exhibited by most AEDs, though, in terms of the relative safety margin, it seemed to be comparatively safer.

Clinical combination of AEDs to control refractory epilepsy is advantageous if it fully controls the seizure and simultaneously causing no synergy of adverse effects. There is increasing evidence suggesting that in addition to a consideration of mechanism of AED, animal experiments using isobolographic analysis could also be beneficial to predict clinical outcome (Luszczki and Czuczwar, 2003).

Isobolographic analysis in the present study indicates that the combination of EACA can enhance anticonvulsant effect of all AEDs tested. A distinct additive effect was observed in all combinations; ED_{50} of phenytoin, valproate and gabapentin in combination with EACA were approximately 38%, 28% and 25% of their corresponding value, when being given alone.

The adverse effects of respective combination on motor coordination, estimated by rotarod test, were also increased as all the combined TD_{50} values were decreasing. However, interestingly, the protective index (PI) which is the ratio between the neurotoxic dose and effective dose of gabapentin in combination with EACA was markedly increased (7.87 vs 2.32), whereas respective values for

phenytoin and valproate were decreased. Thus, a combination of gabapentin and EACA seemed to offer not only a higher protection of animals against PTZ induced convulsion but also a broader margin between anticonvulsant dose and neurotoxic dose as well. Though in the present study, gabapentin was given intraperitoneally, it can be anticipated that an addition of EACA into patients taking clinically available gabapentin tablets would result in better control of the seizure in parallel with a lesser degree of motor impairment than those exhibited by gabapentin alone. Additive effect of EACA was also demonstrated when it was combined with phenytoin or valproate. However, the advantage in these cases seemed to be offset by the finding that their respective protective indices were also decreased.

It is difficult to explain the underlying mechanism of the interaction observed. Firstly, this is the first evidence to demonstrate the additive anticonvulsant effect of currently available AEDs with CA's extract in which the active principles accounted for its anticonvulsant were not yet identified. Secondly, drug interaction of concurrently administered AEDs can occur by pharmacodynamic as well as pharmacokinetic mechanisms (Anderson, 1998) and none of them could be ruled out by isobolographic analysis (Tallarida, 2001). Though, different routes of administration of EACA and AEDs used in the present study make the interaction by enhancing absorption of AEDs unlikely, some other pharmacokinetic interaction should be further investigated. Furthermore, the fact that additivity of EACA was observed on phenytoin, valproate and gabapentin which are AEDs of different mechanisms of action and different pharmacokinetic profiles (Jacobs *et al*., 2001), thus, no clues on the possible mechanism of interaction can be anticipated.

Possible mechanisms of EACA

In vivo microdialysis experiments using PTZ-treated freely moving rats were performed to search for the possible effects of EACA on hippocampal amino acid neurotransmitter levels.

In general, epileptic syndromes have very diverse primary causes, which may be genetics, developmental or acquired (Chapman, 2000). Regardless of these primary causes, an imbalance between excitatory and inhibitory amino acid

neurotransmission appears to play a major role in hyperactivity of the brain in initiation and spread of seizure.

In normal rats, EACA 700 mg/kg BW tended to decrease the extracellular levels of aspartate, glutamate, glycine and also GABA in the dialysate collected from the hippocampus when compared with control. On the contrary, EACA at the higher dose of 1,000 mg/kg BW significantly increased the level of aspartate in comparison to the control group or those receiving lower dose of EACA. The results observed could hypothetically be explained by the existence of at least two CNS active compounds which differ in pharmacological action and potency. One with anticonvulsant activity exerted its effect at lower dose of EACA, whereas, the other which seemed to be a proconvulsant via an increase in aspartate, exerted its effect at higher dose.

In another group of the animals in microdialysis study, convulsion was induced by the administration of PTZ 60 mg/kg BW after pretreatment of vehicle or EACA 700 mg/kg BW for 1 hour. Out of 7, EACA could protect 5 rats from PTZ-induced convulsion. Two demonstrated stage 5 convulsion showing significantly increased aspartate level and slightly increased glutamate (not significant) in the collected dialysate from hippocampus when compared with vehicle alone. This is consistent with the finding that the extracellular levels of glutamate and aspartate in amygdale and frontal cortex of freely moving rats were elevated following a single administration of PTZ (Rocha *et al*., 1996). The mechanisms underlying convulsion by PTZ from neurochemical evidence suggested that PTZ binds to the picrotoxin site of the GABA receptor complex (Ramanjaneyulu and Ticku, 1984), impairing GABA/benzodiazepine (BDZ)-coupled chloride channel activity (Corda *et al*., 1990) and blocking GABA-mediated inhibition (MacDonald and Barker, 1977). In addition, it is known that PTZ does not competitively interact with BDZ receptors (Hantraye *et al*., 1987). Considering the GABA modulated BDZ sites (Tallman *et al*., 1978), the decreased BDZ binding was observed following PTZ can be explained by the impairment of GABA function (Corda *et al*., 1991). The other explanation of these results involves the accumulation of Ca^{2+} during seizure activity (Griffiths *et al*., 1983), an effect that involved the activation of NMDA receptors (MacDermott *et al*.,

1986). If intracellular Ca^{2+} suppressed the GABA activated-Cl^- conductance (Inoue *et al*., 1986), the increased glutamate and aspartate found after PTZ administration could be a critical step that may block the GABAergic inhibition (Stelzer *et al*., 1987).

In protected group, the profile of excitatory amino acid neurotransmitters (both aspartate and glutamate) were gradually decreased while the inhibitory amino acid neurotransmitters (glycine and GABA) tended to increase when compared with vehicle+PTZ-treated group, however, the difference was not statistically significant. Generally, diminution of excitatory neurotransmission and/or potentiation of inhibitory neurotransmission are potential targets of AEDs development (Schwartzkroin, 1997). On the other hand, animals that exhibited convulsion while receiving the same dose of EACA (700 mg/kg BW) and PTZ, implied that the EACA could not overcome the increment of aspartate induced by PTZ. Furthermore, small decrement of glycine and GABA that were also observed, when compared with non-convulsive animal of EACA 700+PTZ-treated group, might potentiate excitation

As a matter of fact that the extract of CA was able to protect the animals exclusively in convulsion induced by PTZ which is a selective blocker of the chloride ionophore coupled to $GABA_A$ receptor (Sejima *et al*., 1997). The molecular mechanism of action of EACA on the synaptic inhibition mediated by $GABA_A$ receptor was also investigated in the dissociated hippocampal pyramidal neurons of the rat using whole cell patch-clamp technique.

The result obtained demonstrated that EACA alone at the concentration 10 up to 50 µg/ml (a limitation of its solubility in 0.1% DMSO), did not induce any current. A slight potentiation of the GABA-induced current was observed when EACA was co-applied at concentration of 0.1-3 µg/ml with maximal potentiation at 3 µg/ml. At higher concentration, the potentiation was lower and a partial blockade on the GABA-induced current was observed at 50 µg/ml.

As the crude ethanolic total extract of CA was found to be able to protect the animals in the PTZ test exclusively when being given by oral route, therefore, first pass metabolism is needed to activate anticonvulsant activity of EACA. This notion may provide an explanation for the finding from patch clamp experiment in which a

slight potentiation on $GABA_A$ current (that underlying its anticonvulsant activity) was observed. In addition, the result on $GABA_A$ current observed is well corresponds to our results in the microdialysis experiment in which the bell-shaped effect of this EACA fraction was observed.

Effects of EACA on epileptogenesis

In this experiment, the protective effect of EACA on the course of kindling development, learning deficit, the brain oxidative stress and the neuronal density parameters were investigated in the PTZ-kindled mice.

The kindled model of partial epilepsy is perhaps the most studied model of epileptogenesis (Pitkanen, 2002). The changes that take place with each stimulation do lead to changes in plasticity that contributes to a permanent state of hyperexcitability. In this regard, the development of a stage 5 seizure can be viewed as epileptogenesis (White, 2002). It is likely, therefore, that the kindling model has been used to assess whether a drug administered during the kindling process will prevent or delay the development of kindling. To study the antiepileptogenic potential of an investigational drug, the substance has to be administered before the kindling stimulation (White, 2002). An increased susceptibility of animals to PTZ seizures after repeated injections of this drug was first observed in 1941 (Sacks and Glaser, 1941). PTZ kindling therefore provides a means for studying a persistent decrease in seizure thresholds.

Previous investigation showed that the aqueous extract of CA decreased the PTZ-kindled seizures and improved the learning deficit induced by PTZ kindling as evidence by decreased seizure score and increased latencies in passive avoidance behavior (Gupta *et al*., 2003). On the contrary, EACA did not affect the development or expression of PTZ-kindled seizures. All animals receiving EACA achieve stage 5 within 38 injections of PTZ. Actually, the lower dose, 300 mg/kg BW, could slightly delay seizure scored as the numbers of PTZ injection was gradually increased as compared to vehicle+PTZ-treated group. On the contrary, in higher doses (1,000 mg/kg BW) of EACA, potentiations of PTZ induced convulsion was observed, as evidence by significant increment of seizure scores during kindled induction and a

significant decrease in the numbers of PTZ injection when compared with lower dose of 300 mg/kg BW. These findings are in good agreement with results in other part of our study that the higher dose of EACA promotes convulsion, though, the number of PTZ injection for fully kindle induction was not significantly different when compared to the group of PTZ alone.

The fully kindled mice were further investigated for memory deficit by step-down test. In accordance with the results observed by Gupta *et al*., (2003), EACA can significantly prolong the retention latency in step-down test at the dose of 1,000 mg/kg BW when compared with vehicle+PTZ-treated group, whereas step-down error did not differ. However, based on the statistical analysis that there was no significance between vehicle+PTZ-treated group and vehicle treated group, therefore the effect of EACA (1,000 mg/kg BW) on memory cannot be concluded. Moreover, in contrast to previous reports, PTZ which induced fully kindled mice did not increase the level of MDA and its effect was not modified in the presence of EACA.

The PTZ-induced kindling involved the activation of excitatory amino acid system from the beginning of the process (Rocha *et al*., 1996). Excessive activation of NMDA receptors induces the neuronal cell death mediated by intracellular Ca^{2+} overload. In addition, NMDA subtype of glutamate receptors in the cerebral cortex and hippocampus plays an important role in learning and memory. Such excitotoxic neuronal death appears to contribute to various neurological disorders such as cerebrovascular dementia and Alzheimer's disease (Bliss and Collingridge, 1993; Parsons *et al*., 1998). In the present studies, the number of surviving cells in PTZ-treated group, though decreasing, was not significantly different from saline and the rest of the experimental animal except in the animal receiving EACA 700 mg/kg BW. Therefore, neuroprotective effect at certain dose of EACA can be observed. Fluctuation of imbalance between excitatory and inhibitory amino acids exerted by various dose of EACA in microdialysis experiment agrees well with inconsistency of the results observed. Furthermore, in the dose of 1,000 mg/kg BW of EACA, the protective effect might be overwhelm by excitotoxicity resulted from the partial blockade of $GABA_A$ receptor observed in the patch-clamp experiment.

Non-significant results on MDA level and number of surviving cells of vehicle+PTZ and vehicle-treated groups observed in mice after the step-down test could be interpreted as no differences between these two groups. However, if one takes into consideration that PTZ could definitely induced fully-kindled mice, some other explanations might account for the discrepancy observed. They are a) treatment on every alternate days (48 h) may not enough to affect all of these parameters in mice; (b) since, all treatments were stopped when animals showed seizure score of five on three consecutive injections, they did not receive any treatment on the day of step-down test (along with 24 and 48 h), and on the days of MDA and number of surviving cells measurements (along with 48 h); and finally (c)it should be pointed that number of surviving cells were quantified only at CA1 and CA3, therefore number of neurons in some other area, such as dentate gyrus, which might be more affected than hippocampus, were not included.

CHAPTER VI

CONCLUSION

The present studies demonstrated the anticonvulsant activity of EACA. A small reduction of hippocampal excitatory amino acid neurotransmitters (both aspartate and glutamate) with a slight increase of inhibitory amino acid neurotransmitters (glycine and GABA), in conjunction with a gradual potentiation on $GABA_A$ receptor current might play the important roles on its anticonvulsant activity. In PTZ-kindling models, EACA had no significant effect on either the course of epileptogenesis or passive avoidance task or brain MDA level. Though neuroprotective effect of EACA was evident in the dose of 700 mg/kg, it is unlikely that EACA could produce any significant effect on the course of epileptogenesis. However, its additivity of efficacy on some currently used AEDs obtained suggested the potential of EACA to be further developed as adjunctive medication.

Since, anticonvulsant activity of the EACA was clearly observed at the lower concentration whereas proconvulsant activity was predominating in higher dose. Therefore, identification and separation of active substances with different pharmacological profiles in EACA have to be carried out in order to develop a safe adjunctive medication from CA.

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