CHAPTER IV

RESULTS

Part I Anticonvulsant activity and Neuroprotective effect of VPU and VPA on pilocarpine-induced seizure

Anticonvulsant activity

Anticonvulsant activity of VPU was observed at pretreated time of 30 min in pilocarpine-induced seizure model. Whereas 0.5 % CMC, a vehicle for the tested substances, exhibited no protection. Intraperitoneal administration of pilocarpine produced status epilepticus in eight rats (8/9). However, one rat displayed only gustatory automatism, piloerection and head nodding. Motor limbic seizures started after 18.25 $\pm$ 6.71 min (10-30 min; min-max) after pilocarpine administration.

VPU, given intraperitoneally, in the dose of 40, 50, 70 and 90 mg/kg BW protected the rats from behavioral features of seizures produced by pilocarpine, with an ED_{50} of 49 mg/kg BW (Fig. 4.1). Similar results with lower degree of protection (ED_{50} = 322 mg/kg BW) were observed in VPA- treated animals (250, 300, 400 and 500 mg/kg BW) (Fig. 4.2).

Both VPU and VPA prolonged the onset of motor limbic seizures and decreased severity of seizure induced by pilocarpine (Table 1 and 2). Application of pilocarpine in rat pretreated with VPA (250 mg/kg BW) resulted in motor limbic seizures in 2/6 and 3/6 animals during 30 min and 60 min, respectively. Most of the rats (5/6) receiving high dose of VPA (500 mg/kg BW) displayed gustatory automatism, piloerection and head nodding. Furthermore one rat was found to develop motor limbic seizures within 30 min. VPU exhibited a marked suppressant action on the seizures induced by pilocarpine. In low dose group of VPU (40 mg/kg BW), rats exhibited motor limbic seizures in 3/6 and 1/6 animals during 30 min and 60 min, respectively. Two rats in this group elicited scratching and head nodding. VPU in the dose of 90 mg/kg BW, protected rats (5/6) against seizures induced by pilocarpine. Only one rat developed motor limbic seizures within 60 min.

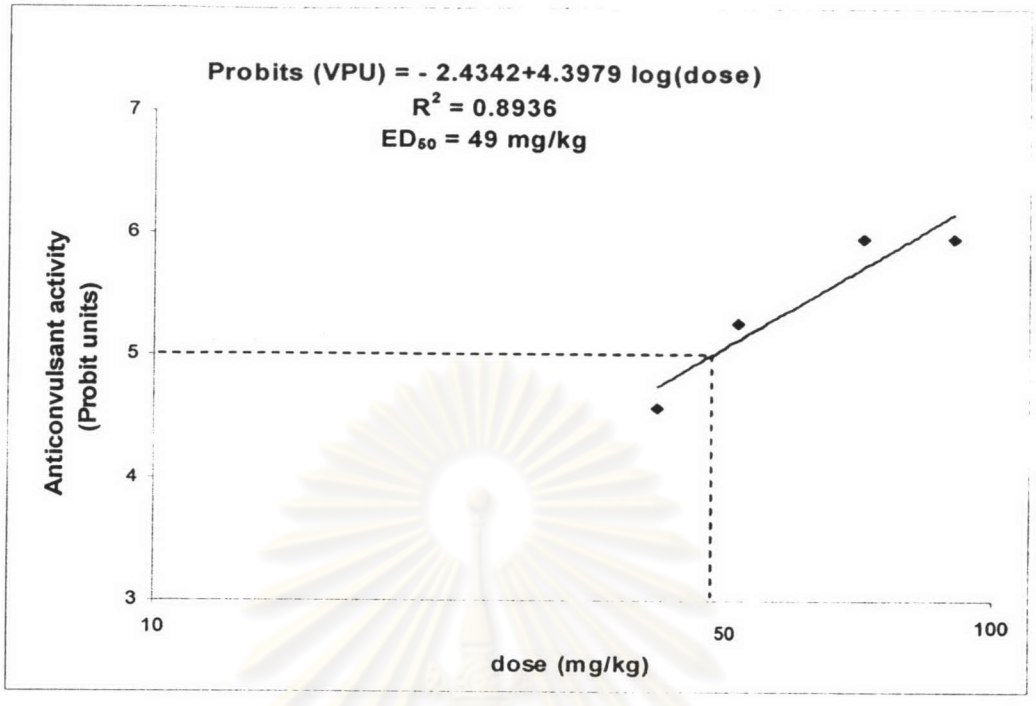


Figure 4.1 Log dose-response curve of VPU on pilocarpine-induced seizure in rats at 30 min pretreated time (N=6 in each dose)

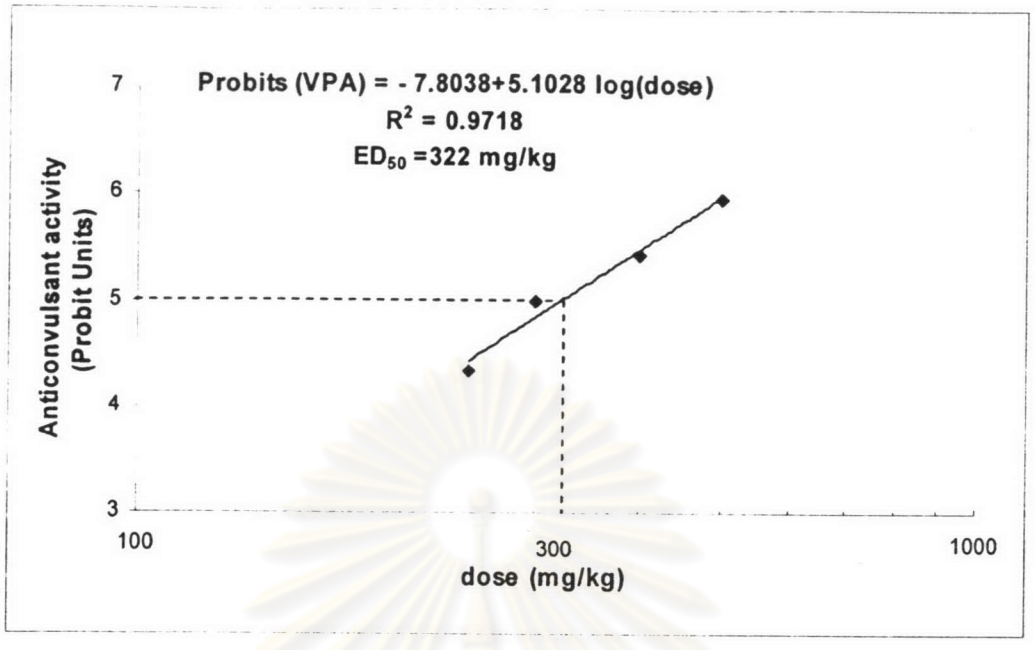


Figure 4.2 Log dose-response curve of VPA on pilocarpine-induced seizure in rats at 30 min pretreated time (N=6 in each dose)

Table 4.1 Effects of VPA and VPU on the onset of motor limbic seizures induced by pilocarpine (380 mg/kg BW, i.p.)

	Onset of motor limbic seizures (min)		
	0-30 min	31-60 min	> 60 min (protected)
Pilocarpine (n=9)	8	-	1
Pilo-VPU 40 mg/kg (n=6)	3	1	2
Pilo-VPU 50 mg/kg (n=6)	1	1	4
Pilo-VPU 70 mg/kg (n=6)	1	-	5
Pilo-VPU 90 mg/kg (n=6)	-	1	5
Pilo-VPA 250 mg/kg (n=6)	2	3	1
Pilo-VPA 300 mg/kg (n=6)	1	2	3
Pilo-VPA 400 mg/kg (n=6)	2	-	4
Pilo-VPA 500 mg/kg (n=6)	1	-	5

Values given are the number of animals displaying motor limbic seizures ($\geq$ stage 4 of Racine scale) induced by pilocarpine.

Table 4.2 Effects of VPA and VPU on the severity of seizures induced by pilocarpine (380 mg/kg BW, i.p.)

	Severity of seizures induced by pilocarpine				
	stage1	stage2	stage3	stage4	stage5
Pilocarpine (n=9)	-	1	-	-	8
Pilo-VPU 40 mg/kg (n=6)	1	-	1	-	4
Pilo-VPU 50 mg/kg (n=6)	-	2	2	-	2
Pilo-VPU 70 mg/kg (n=6)	1	2	2	-	1
Pilo-VPU 90 mg/kg (n=6)	2	1	2	-	1
Pilo-VPA 250 mg/kg (n=6)	1	1	-	3	1
Pilo-VPA 300 mg/kg (n=6)	1	1	1	2	1
Pilo-VPA 400 mg/kg (n=6)	1	2	1	1	1
Pilo-VPA 500 mg/kg (n=6)	3	1	1	-	1

Values given are the number of animals displaying various degree of seizures induced by pilocarpine.

Neuronal morphological determinations

Figure 4.3 illustrated histological changes observed in the CA1 and CA3 regions of the hippocampus. Cresyl violet staining showed no histological lesion in the control group. Neuronal loss, shrinkage and dark staining of neurons were observed both in the CA1 and CA3 regions of the hippocampus in pilocarpine-treated rats (Fig. 4.3). In this group, the number of cells was massively decreased in CA1 region while CA3 region was less extensively damaged (Fig. 4.4-4.5). In comparison to control group the number of surviving neurons in both CA1 and CA3 regions in pilocarpine-treated rats was significantly decreased ($P < 0.01$).

Administration of VPU (70 mg/kg BW) and VPA (500 mg/kg BW) attenuated neuronal damage caused by pilocarpine (Fig. 4.3). Numbers of neuronal cells in CA1 (Fig. 4.4) and CA3 (Fig. 4.5) which were drastically decreased by pilocarpine were restored by VPU and VPA (Fig. 4.4-4.5). Surviving neurons in VPU- and VPA-treated groups were comparable to those in control group receiving 0.5% C.M.C. in NSS.

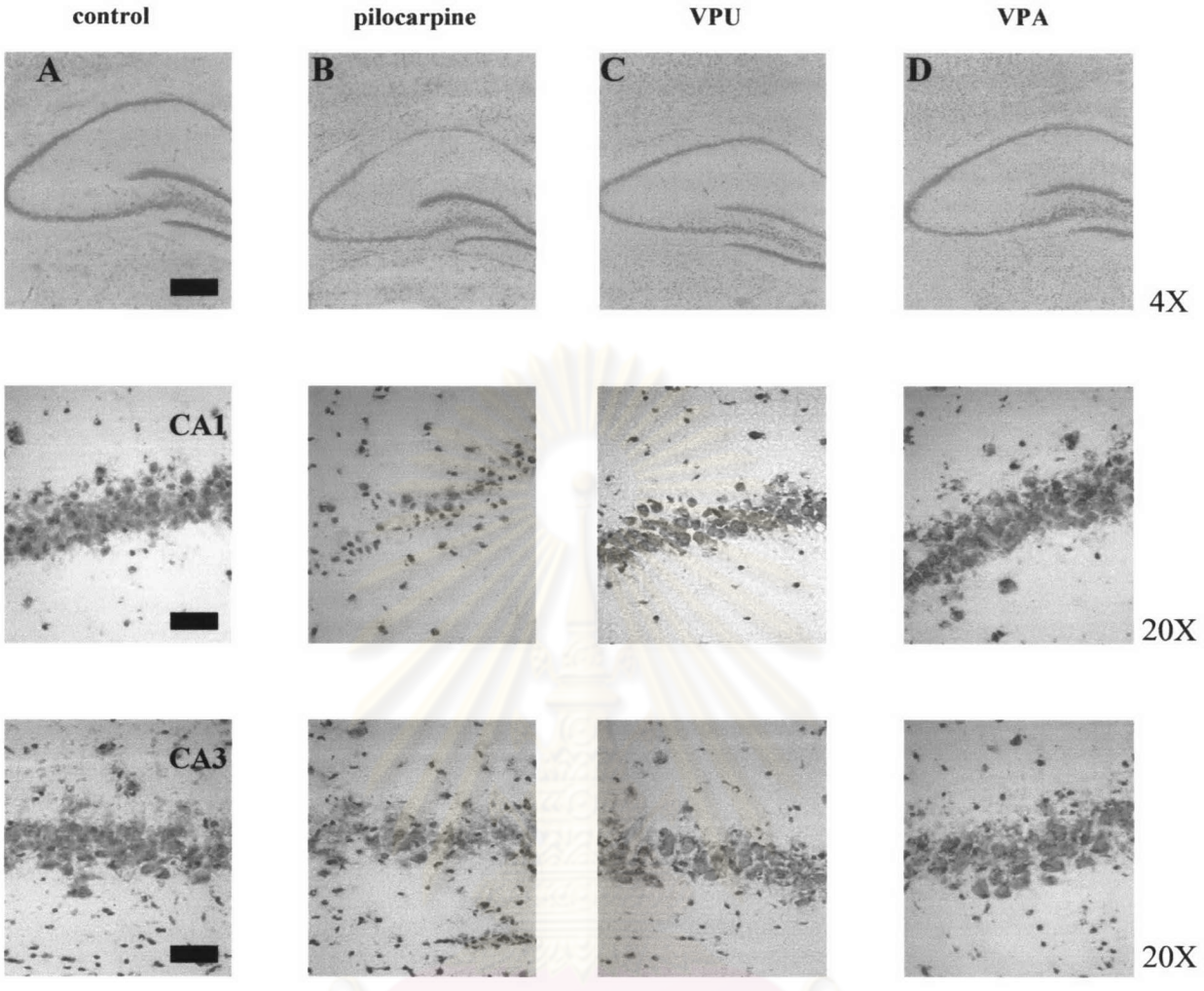


Figure 4.3 Representative photographs of hippocampal coronal sections of Cresyl violet-stained at low magnification in the upper panel (A, B, C, D), higher magnification of CA1 regions in the middle panel and CA3 regions in the lower panel.

A: NSS-0.5%CMC

B: pilocarpine-0.5%CMC

C: pilocarpine-VPU, 70 mg/kg

D: pilocarpine-VPA, 500 mg/kg

Scale bar represent 500 μm in upper panel.

Scale bars represent 100 μm in middle and lower panels.

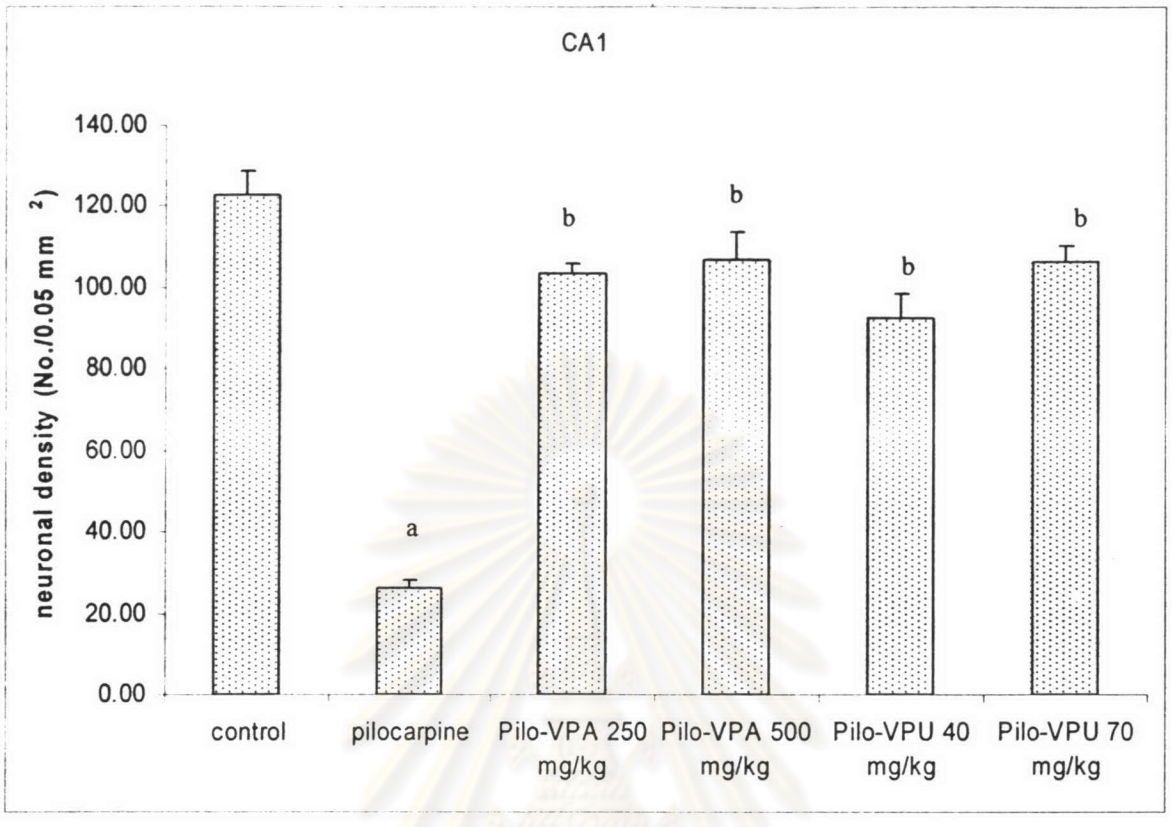


Figure 4.4 Effects of VPA and VPU on the number of CA1 pyramidal neurons of the hippocampus exposed to pilocarpine (n=6)

Each bar represents the (mean±SEM) of the neuronal density (No./0.05 mm^2)

a $P< 0.01$ compared to the control group

b $P< 0.01$ compared to the pilocarpine-treated group

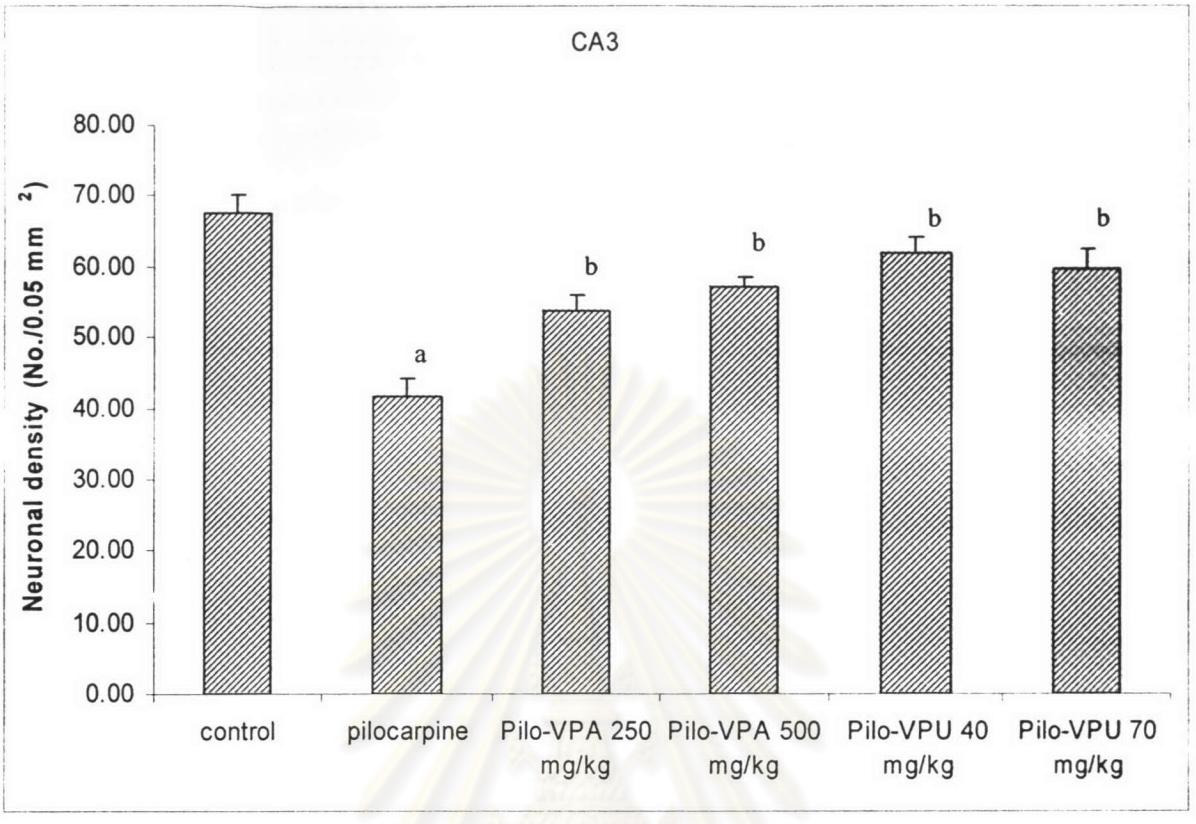


Figure 4.5 Effects of VPA and VPU on the number of CA3 pyramidal neurons of the hippocampus exposed to pilocarpine (n=6)

Each bar represents the (mean±SEM) of the neuronal density (No./0.05 mm^2)

a $P< 0.01$ compared to the control group

b $P< 0.01$ compared to the pilocarpine-treated group

Part II Effects of VPU and VPA on the level of hippocampal amino acid neurotransmitters in freely moving rats

Alterations of amino acid neurotransmitters

The effects of VPU (50 and 100 mg/kg BW, i.p.) and VPA (300 and 600 mg/kg BW, i.p.) on hippocampal glutamate, aspartate, glycine and GABA were investigated in pilocarpine-induced seizure in freely moving rats. Alterations in amino acid neurotransmitter levels were expressed as a percent change from three consecutive samples before the administration of the tested substances.

As shown in Figure 4.6-4.9, the level of hippocampal glutamate, aspartate, glycine and GABA in normal saline-treated rats remained unchanged from baseline throughout the experiments. In contrast, the administration of pilocarpine resulted in a sustained increase of glutamate and aspartate levels (Fig. 4.6-4.7). Though the increment of aspartate was not so pronouced as that of glutamate, significant changes were observed in the total amount of both glutamate and aspartate. Comparatively small alterations were noted on the level of total amount of glycine and GABA, however, none of them was statistical significance (Fig. 4.10-4.11).

Different doses of VPU (50 and 100 mg/kg BW) and VPA (300 and 600 mg/kg BW) significantly abolished pilocarpine-evoked increases in extracellular glutamate levels (Fig. 4.10-4.11). VPU did not demonstrate the dose-dependent manner to reduce the elevation of glutamate level (Fig. 4.10) whereas VPA significantly did (Fig. 4.11). Pilocarpine-induced increases in hippocampal aspartate were significantly reduced to normal level by low and high doses of VPU and VPA (Fig. 4.10-4.11).

In addition hippocampal glycine and GABA which were not significantly affected by pilocarpine were significantly reduced by VPU and VPA in a dose dependent manner (Fig 4.10-4.11).

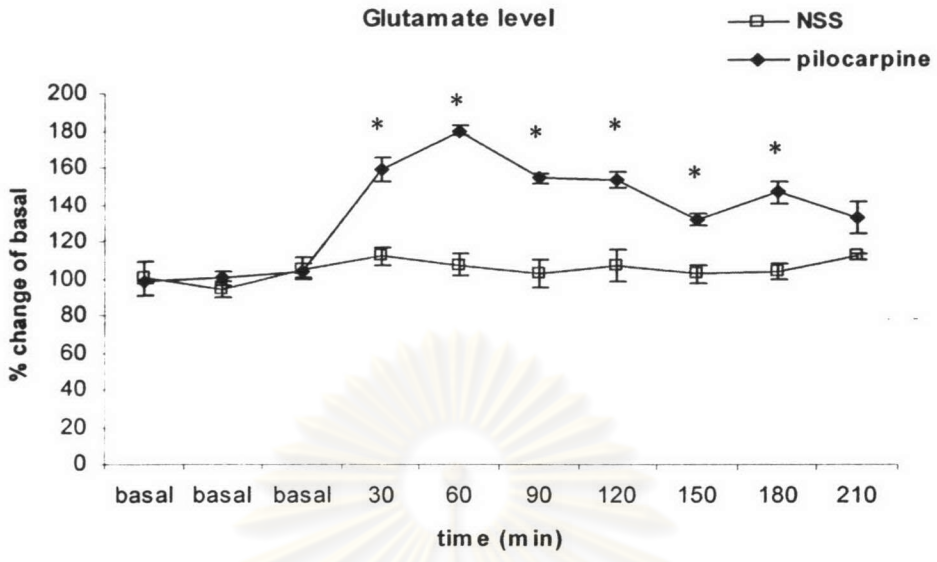


Figure 4.6 Effect of an intraperitoneal injection of pilocarpine and normal saline on the extracellular levels of glutamate (in percentage of the basal level) (mean±S.E.M). The asterisks denote the values significantly different from the corresponding baseline values ($P < 0.05$).

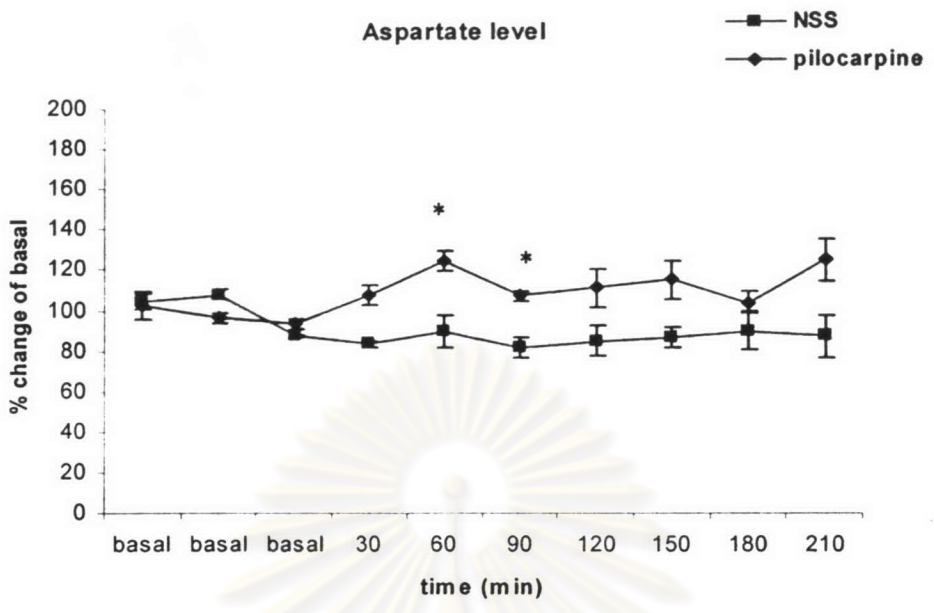


Figure 4.7 Effect of an intraperitoneal injection of pilocarpine and normal saline on the extracellular levels of aspartate (in percentage of the basal level) (mean±S.E.M). The asterisks denote the values significantly different from the corresponding baseline values ($P < 0.05$).

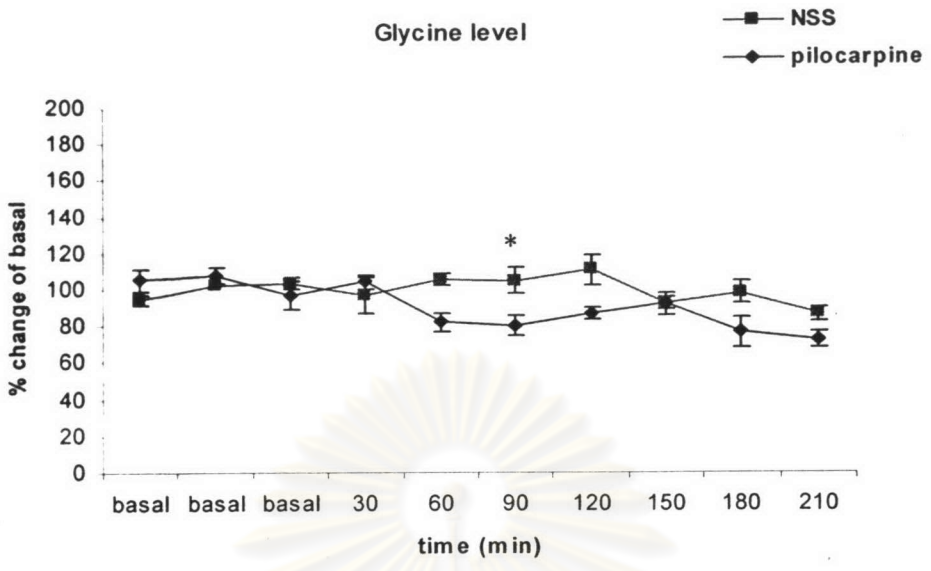


Figure 4.8 Effect of an intraperitoneal injection of pilocarpine and normal saline on the extracellular levels of glycine (in percentage of the basal level) (mean±S.E.M).The asterisk denotes the value significantly different from the corresponding baseline values ($P < 0.05$).

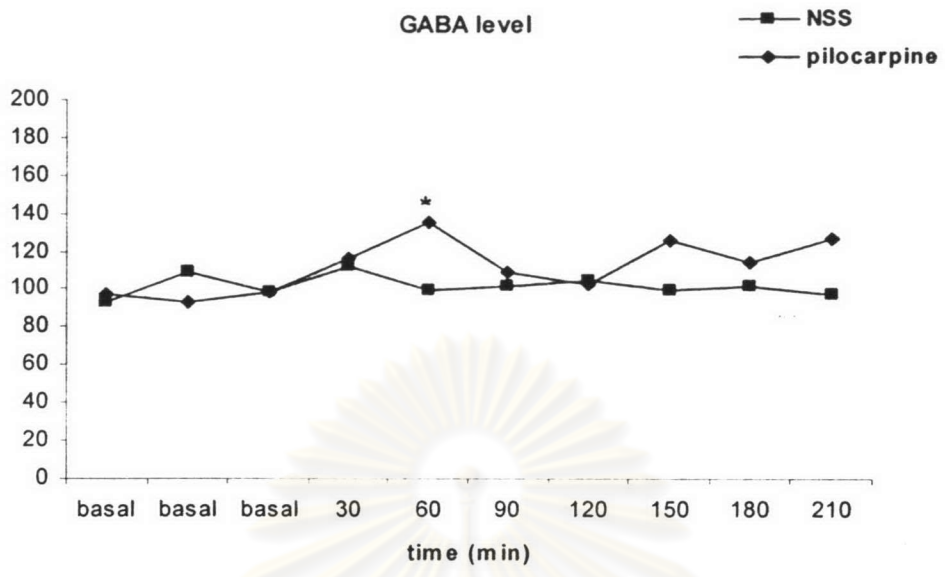
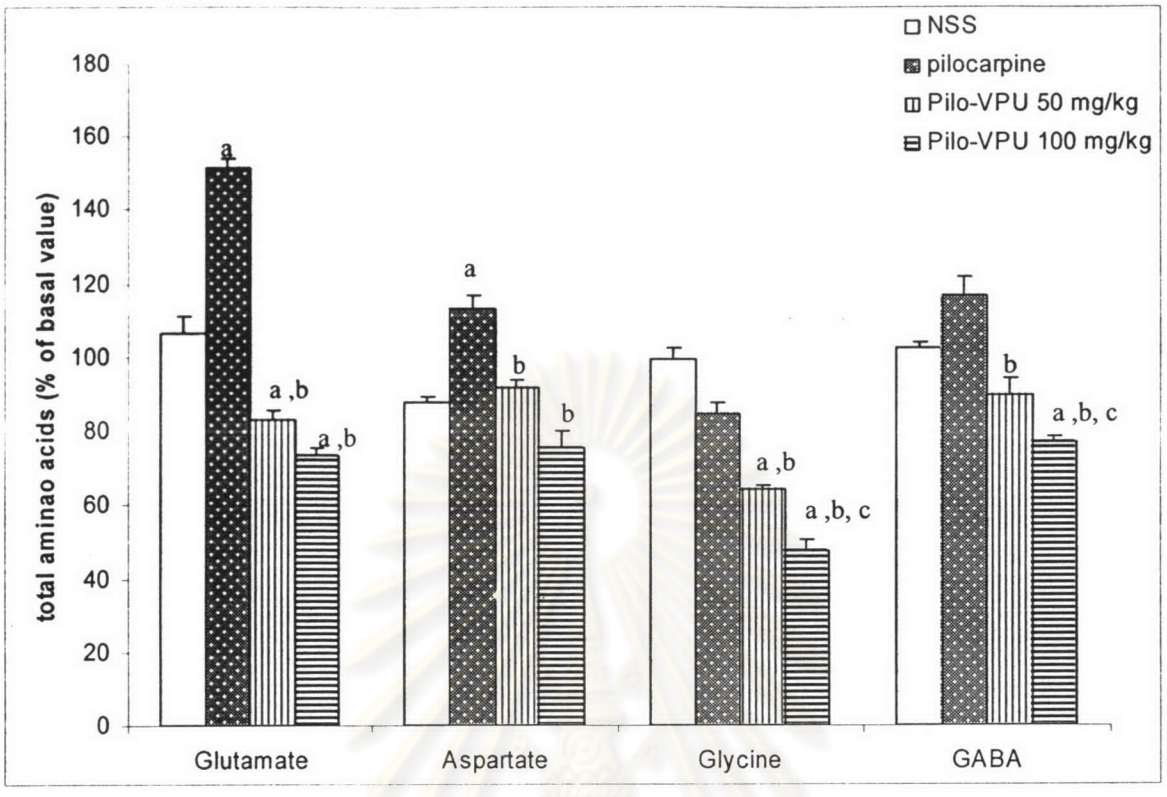


Figure 4.9 Effect of an intraperitoneal injection of pilocarpine and normal saline on the extracellular levels of GABA (in percentage of the basal level) (mean±S.E.M). The asterisk denotes the value significantly different from the corresponding baseline values ($P < 0.05$).

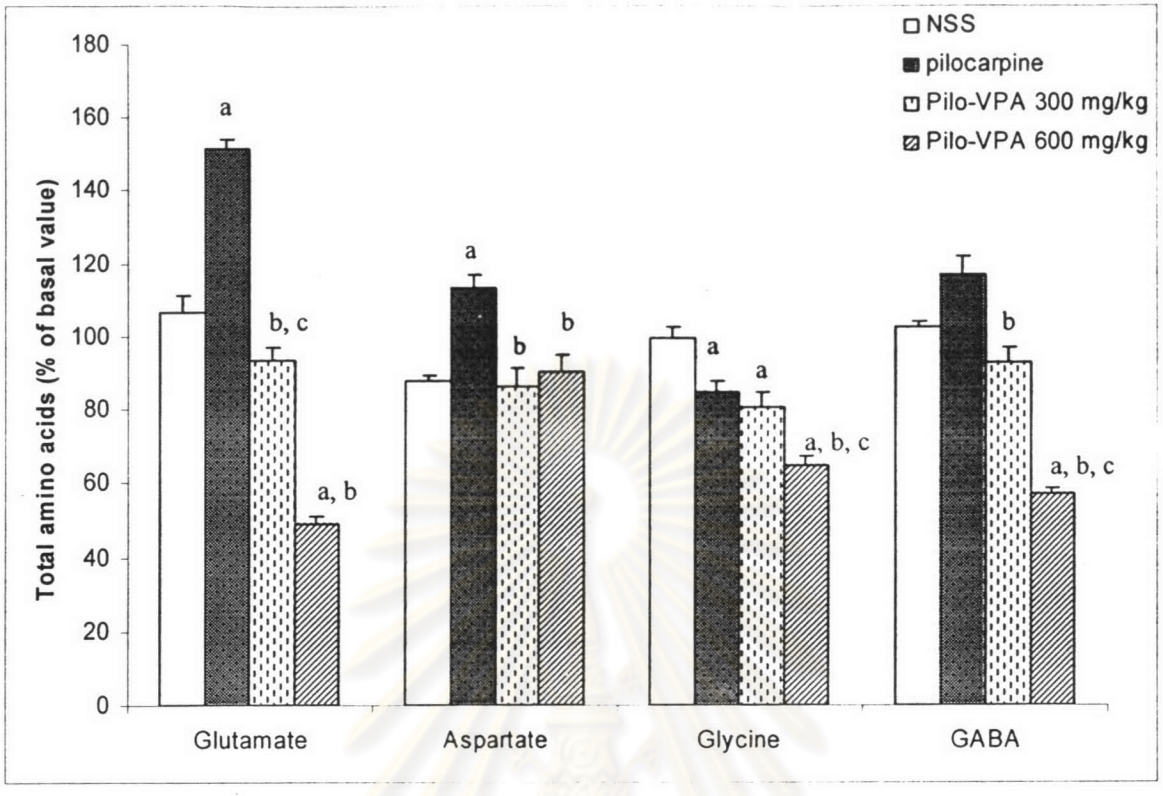


a $P< 0.05$ denotes statistically significant difference from NSS

b $P< 0.05$ denotes statistically significant difference from pilocarpine

c $P< 0.05$ denotes statistically significant difference from VPU 50 mg/kg BW

Figure 4.10 Effects of an intraperitoneal administration of VPU (50 and 100 mg/kg BW) on the total amount of the rat hippocampal amino acid levels in the dialysate collected for 210 min



a $P< 0.05$ denotes statistically significant difference from NSS

b $P< 0.05$ denotes statistically significant difference from pilocarpine

c $P< 0.05$ denotes statistically significant difference from VPA 300 mg/kg BW

Figure 4.11 Effects of an intraperitoneal administration of VPA (300 and 600 mg/kg BW) on the total amount of the rat hippocampal amino acid levels in the dialysate collected for 210 min

Part III *Ex vivo* determination of lipid peroxidation and neuronal mitochondrial activity

Lipid peroxidation

As shown in Figure 4.12 pilocarpine significantly induced an increase in MDA level. Intraperitoneal administration of either VPU or VPA significantly abolished the effect of pilocarpine. High dose of VPU (100 mg/kg BW) and VPA (600 mg/kg BW) seemed to produce more effect than lower dose but the results were not statistically significant (Fig. 4.12).

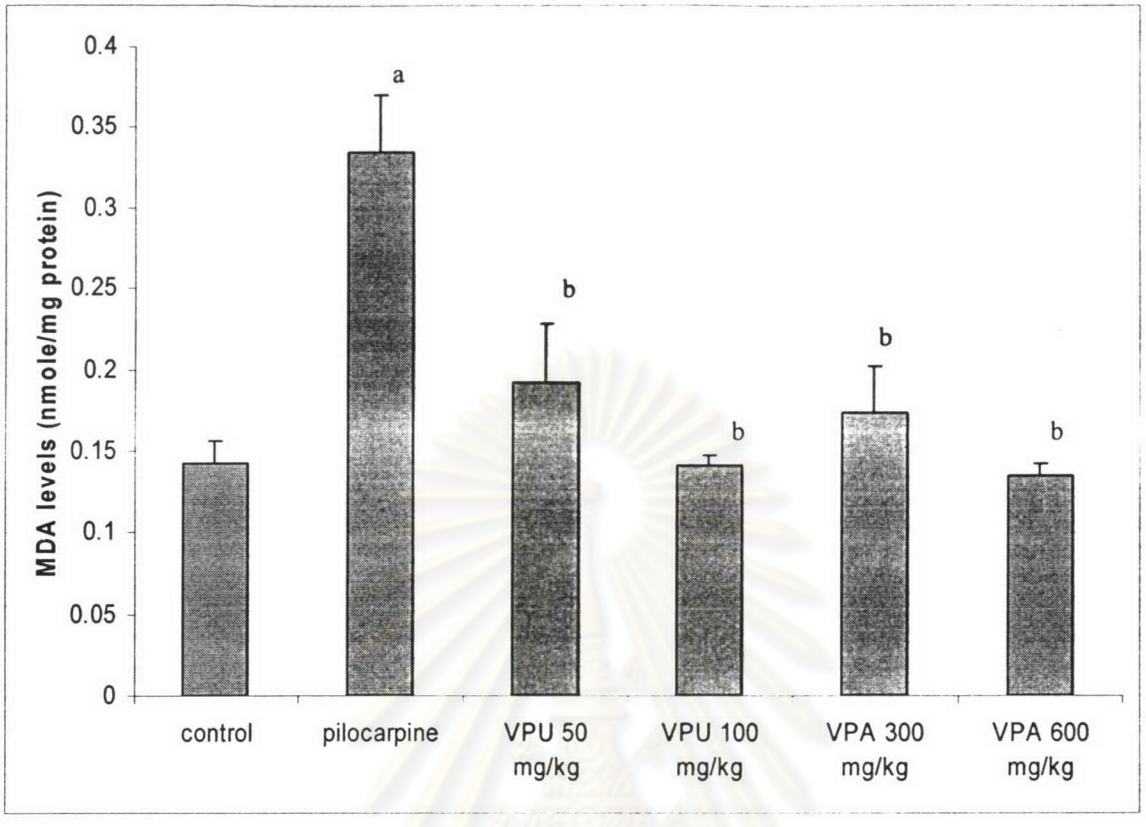


Figure 4.12 The effects of VPU (50 and 100 mg/kg BW) and VPA (300 and 600 mg/kg BW) on the MDA levels in hippocampus of adult rats after pilocarpine-induced seizure.

Each bar represents the mean ± S.E.M.

[a] compare to the control group ($P<0.05$)

[b] compare to the pilocarpine-treated group ($P<0.05$)

Neuronal Mitochondrial Activity

Mitochondrial respiration in 0.5% CMC treated rats

Mean rates of active ADP-linked state 3 respiration and resting state 4 respiration with glutamate plus malate as substrates were 60.17±3.18 and 27.97±1.4 nanoatoms of oxygen / min/mg protein, respectively, in untreated brain mitochondria (n = 5 ; mean ± SEM).

It is well known that glutamate plus malate are NAD^+-linked substrates which donate electrons to mitochondrial respiratory chain via complex I. Further experiments were performed with succinate which donates electron to the respiratory chain via complex II, as substrate. When the respiratory chain was activated by succinate, the state 3 and state 4 respiratory rates were 135.38±2.13 and 89.83±1.964 nanoatoms of oxygen / min/mg protein, respectively, in untreated brain mitochondria (n = 5 ; mean ± SEM).

The ratio of state 3 to state 4 (the respiratory control ratio: RCR) with glutamate plus malate and succinate used as substrates in control group were 2.17±0.14 and 1.51±0.01, respectively.

The efficiency of oxidative phosphorylation is determined by the P/O ratio. When glutamate plus malate and succinate were electron donors, the P/O ratio were 2.89±0.09 and 1.8 ± 0.05, respectively.

Effect of pilocarpine on rat brain mitochondrial respiration

When glutamate plus malate were used as substrates, significant inhibition of state 3 respiration rate was demonstrated while state 4 respiration rate was unaffected in pilocarpine-treated rats (Table 4.3). Similar results were observed when succinate was used as well (Table 4.4). The RCR and P/O ratio that glutamate plus malate and succinate were used as substrates were significantly decreased in pilocarpine group (Table 4.3-4.4).

Effects of VPU and VPA on brain mitochondrial respiration of pilocarpine treated rats

When glutamate plus malate were used as the substrates, pilocarpine-induced inhibition of state 3 respiration rate was successfully restored to the control level by VPU (50 and 100 mg/kg BW) while state 4 respiration rate which was unaffected by pilocarpine, was significantly increased in the presence of VPU (50 and 100 mg/kg BW). The state 3 and state 4 respiration rates were unaffected by VPA. Furthermore the RCR and P/O ratio were unaffected by VPU and VPA (Table 4.3).

When succinate was used as substrate, both VPU and VPA did not have any effects on stage 3, stage 4 respiration rates, the RCR and the P/O ratio in pilocarpine-treated rats (Table 4.4).

Table 4.3 Respiration of rat brain mitochondria with glutamate plus malate as substrates

	state 3	state 4	RCR	P/O
Control (n=4)	60.17±3.18	27.97±1.40	2.17±0.14	2.89±0.09
Pilocarpine (n=4)	36.65±2.16 [a]	27.97±1.66	1.31±0.02 [a]	1.78±0.11 [a]
Pilo-VPU 50 mg/kg (n=4)	53.38±1.79 [b]	37.50±1.76 [a, b]	1.43±0.03 [a]	1.96±0.17 [a]
Pilo-VPU 100 mg/kg (n=4)	60.59±2.38 [b]	39.90±2.30 [a, b]	1.57±0.05 [a]	2.16±0.06 [a]
Pilo-VPA 300 mg/kg (n=4)	39.83±1.97 [a]	26.68±1.48	1.50±0.04 [a]	1.95±0.20 [a]
Pilo-VPA 600 mg/kg (n=4)	41.31±2.68 [a]	26.69±2.10	1.55±0.02 [a]	2.25±0.11 [a]

[a] compare to the control group [b] compare to the pilocarpine-treated group

($P < 0.05$)

RCR = respiratory control ratio (a ratio of oxygen consumption in state 3 to state 4 that indicates the functional integrity of the mitochondria), P/O = P/O ratio (an index of oxidative phosphorylation which indicates the efficiency of mitochondrial ATP synthesis)

Table 4.4 Respiration of rat brain mitochondria with succinate as substrate

	state 3	state 4	RCR	P/O
Control (n=5)	135.38±2.13	89.83±1.96	1.51±0.01	1.8±0.05
Pilocarpine (n=5)	116.31±4.35[a]	95.97±4.91	1.22±0.04[a]	1.21±0.12[a]
Pilo-VPU 50 mg/kg (n=5)	114.19±2.08[a]	93.85±4.31	1.23±0.07[a]	1.22±0.04[a]
Pilo-VPU 100 mg/kg (n=5)	111.44±1.72[a]	91.52±2.54	1.22±0.05[a]	1.31±0.16[a]
Pilo-VPA 300 mg/kg (n=5)	105.08±0. 70[a]	87.07±3.57	1.21±0.05[a]	1.34±.07[a]
Pilo-VPA 600 mg/kg (n=5)	119.49±2.45[a]	93.22±1.80	1.28±0.03[a]	1.19±.03[a]

[a] compare to the control group

($P < 0.05$)

RCR = respiratory control ratio (a ratio of oxygen consumption in state 3 to state 4 that indicates the functional integrity of the mitochondria), P/O = P/O ratio (an index of oxidative phosphorylation which indicates the efficiency of mitochondrial ATP synthesis)