Effect of Chronic Ethanol-Treated On Spatial and Non-Spatial Leaning in Mice

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4837440538 PHIRIYACHATR KANANURACK: EFFECT OF CHRONIC ETHANOL-TREATED ON SPATIAL AND NON-SPATIAL LEARNING IN MICE, Advisors: Assist. Prof. PANRAPEE SUTTIWAN, Co-Advisor: Assoc. Prof. NAIPHINICH KOTCHABHAKDI, Assoc. Prof. SOMPOCH IAMSUPASIT, 26 pp.

Ethanol can produce detectable impairments in memory after only a few drinks and, as the amount of ethanol increases, so does the degree of impairment. Long-term ethanol treatment can induce structural changes in central nervous system (Adolf Pfefferbaum, 1997., Clive Harper and Izuru Matsumoto, 2003, Wright et al., 2003, Cohen, N. J. & Eichenbaum, H., 1993). However, the effect of role of chronic ethanol treatment on spatial and non-spatial learning and memory remains unclear. This study aims to investigate effect of chronic ethanol-treated on spatial and non-sptial learning in mice. Mice were divided into 3 major groups: ethanol treated group, vehicle control group and the control group, 10 animals per group. The major groups were divided into two groups, spatial learning group and non-spatial learning group. To determine effect of chronic ethanol on learning, in the ethanol-treated group, mice were injected 4.4 g/kg/day of ethanol (20% v/v diluted from 90% ethanol in an isotonic sterile 0.9% saline solution) and vehicle control group were injected normal saline (isotonic sterile 0.9% saline solution) via intraperitoneal (*i.p.*) for 21 days. In contrast, control group was housed in same condition but do nothing with them. 24 hours after that, mice were behavioral trained in Morris water maze for 4 days and probe trial will be done after last training on Day4. Control group and vehicle control group were found to be significantly decreased escape latency time than ethanol-treated group only in the spatial learning condition, but not in the non-spatial learning condition. The results indicated that: (1) ethanol impaired learning and memory of spatial learning, (2) ethanol doesn't impaired learning and memory of non-spatial learning.

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CHAPTER 1

INTRODUCTION

Alcohol-related diseases are the third most important risk factor behind unsafe sex, tobacco consumption, crime and violence. The national GDP decrease about 2-3% from traffic accidents alcohol-related or about 100 billion baht per year.

The alcohol consumption rate among Thai population has been rapidly increasing, In 1989 was 20.2 liters and 58.0 liters per capita consumption in 1 year in 2003. Thais have the fifth highest rate of alcohol consumption in the world. Consumption of alcohol is particularly common among males (www.thaihealth.or.th) and the age of drinking has declined to young people drinking (NSO, 2006).

World Health Organization (WHO) has reported that 1.8 million deaths from unintentional injuries alone, account for about one third of the 1.8 million deaths and a loss of 58.3 million of Disability-Adjusted Life Years (DALY) while neuro-psychiatric conditions account for close to 40% from alcohol consumption. There are about 2 billion people consume alcoholic beverages, 76.3 million with alcohol use disorders (WHO, 2004).

Chronic alcohol intoxication was found to lead the impairment in many brain areas that related with intellectual and behavior impairment. Many previous studies suggest and indicate that alcohol plays a major role on hippocampus in both acute and chronic alcohol intoxication. Alcohol was reported that alcohol attenuates the acquisition of new information by reduce the hippocampal formation to process new information and faster to forget.

Hippocampus play importance role in learning and memory. Hippocampus is the main entrance of alcohol, which is known to be impaired in alcoholic. The only input of the principle receptor in hippocampus is *N*-methyl-D-aspartate (NMDA) receptor. Many previous studies suggest that NMDA receptor was the main areas damage by making it more receptive to the neurotransmitter glutamate (Aaron M. White, *et al.*, 2000) that makes NMDA receptor reduce by

overactivate. Injury in NMDA receptor leads to loss in learning and memory (Harper & Matzumoto, 2005).However, the effect of role of chronic ethanol treatment on spatial and non-spatial learning and memory remains unclear. In this study, we used alcohol as a toxic substance to examine learning and memory loss, role of chronic alcoholism on spatial and non-spatial learning will be evaluated and we hypothesize that alcohol treated mice will show poorer performance in both spatial and non-spatial tasks.

CHAPTER 2

OBJECTIVE

The hippocampal formation has been reported to be one of the regions most sensitive to prolonged alcohol administration. The deficit in ability of hippocampus leads to loss in learning and memory by act as an intermediate storage information between its initial acquisition and consolidate to last memory.

Since hippocampus is the major area that is damage by ethanol, when hippocampus is attracted from toxicity of alcohol administration and lead to spatial and non – spatial learning become poorer performance following by hippocampus dysfunction.

1. To observe and measure spatial task in mice

2. To observe non – spatial task in mice

CHAPTER 3

REVIEW LITERATURE

3.1 Ethanol

Ethanol (EtOH) is the simplest and most commonly used alcohol. Ethanol is consisted of two carbon (C) atoms as backbone which surrounded by five hydrogen (H) atoms and one hydroxyl group (OH), functional group of an alcohol molecule (figure 3.1). The carbon atom is bound to hydrogen atoms and other carbon atom(s) to form a carbon chain. Ethanol is known as ethyl alcohol, drinking alcohol, grain alcohol, pure alcohol, hydroxyethane, and ethyl hydrate. Alcohol is referred to common name and best known as type in alcoholic beverage. Evaporative, inflammable, colorless liquid and miscible in organic solvents is defined as properties. Alcohol can show either acidic or basic properties at the O-H group.

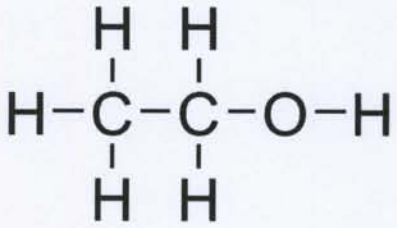


Figure 3.1 Ethanol structure

3.2 Hippocampus

3.2.1 Anatomy and Physiology of Hippocampus

In the veterbrates, hippocampus located in the medial temporal lope that is the part of forebrain. Hippocampus is the part of limbic system. Hippocampus is the most essential for human and animals, for which short term memory and spatial learning and memory task (Bird, C.M., and Burgess, M. 2008). Mammal has 2 hippocampi in each side of brain. Hippocampi consist of a ventral and dorsal portion, both of which share similar composition but are parts of completely different neural circuits in mammalian species. Hippocampus's shape is like seahorse that derived from Greek word for seahorse.

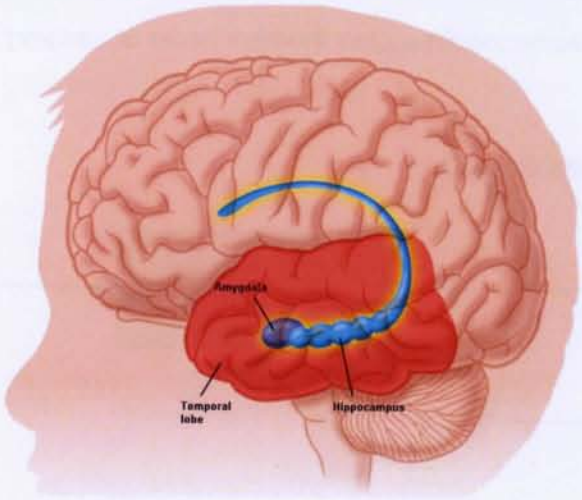


Figure 3.2 Location of Human Hippocampus (www.BrainConnection.com)

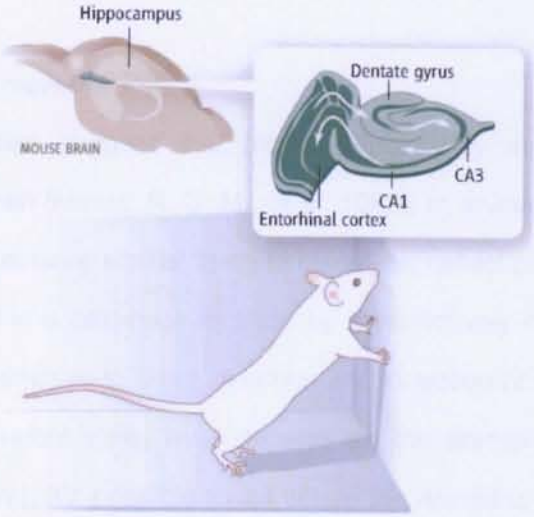


Figure 3.3 Location of Rodent Hippocampus (http://dericbownds.net/)

3.3 Hippocampus and learning

3.3.1 Role in general memory

Hippocampus play importance role in the formation of new memories about general experience (episodic memory) but does not affect on specific experience (semantic memory) (O'Kane, G., Kensinger, E. A., and Corkin, S. 2004). These memories often go on in a lifetime and stop to play importance role to holding the memories after the memory consolidation (figure 3.4) (Joseph R. Manns, *et al*, 2003, Larry R. Squire, *et al*. 2004). Dysfunction of the hippocampus usually shows the deficit of forming new information (anterograde amnesia) and

normally also impact on process to recall memory before hippocampal dysfunction (retrograde amnesia).

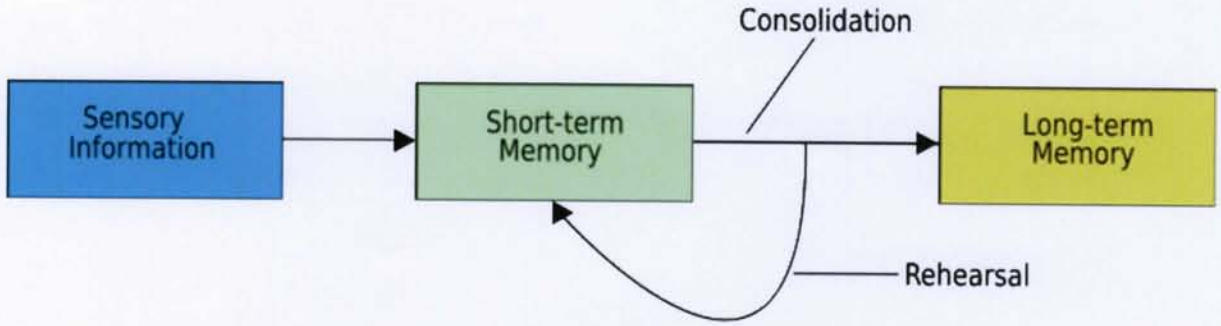


Figure 3.4 Diagram illustrating consolidation of short-term memories into long-term memories. (Atkinson, R. C., & Shiffrin, R. M. 1968)

3.3.2 Role on spatial memory

Many previous studies suggest that hippocampus play major role in storing and processing spatial information (Morris, R. G. M., *et al.* 1982). In animal models have shown that neurons in the hippocampus have spatial firing fields which called place cells. Some cells fire when the animal finds itself in a particular location by irrespectively in direction of travel, while most are at least partially sensitive to head direction and direction of travel. In rats, some cells which called context-dependent cells, may depend on the animal's past (retrospective) or expected future (prospective). It's possible to tell where the animal is by looking at the firing of cell because different cells fire at different locations. In human, place cells involve in finding their way around in a virtual reality town.

Figure 3.5 Spatial firing patterns of place cells which recorded from the same location in the dorsal CA1 layer of a rat (Skaggs, W. E., *et al.* 1996).

3.3.3 Ethanol and Hippocampus

Ethanol can produce detectable impairments in memory after only a few drinks and, as the amount of ethanol increases, so does the degree of impairment. Long-term ethanol treatment can induce structural changes in central nervous system (Pfefferbaum, A., *et al.* 1997, Clive Harper and Izuru Matsumoto, 2003, Wright *et al.*, 2003, Cohen, N. J. & Eichenbaum, H, 1993). Many studies focus on the changes in the hippocampus that special relevance in memory processes (Beracochea *et al.*, 1986; Victor, 1994). Hippocampal formation was reported that was the region which more sensitive to the long-term ethanol treated (Garcı´a-Moreno, L. M. *et al.*, 2002). The long-term ethanol intake induces loss of hippocampal neurons that worsens during withdrawal (Paula-Barbosa *et al.*, 1993). The hippocampal dysfunction show in an impaired ability to successful performance to solve tasks that depend on spatial search strategies (J.W. Wright *et al*, 2003).

CHAPTER 4

MATHERIALS AND METHODS

4.1 Subjects

Male ICR mice (National Laboratory Animal Centre, Mahidol University, Thailand), were used in this experiment. The animals were 8 weeks old and weighted between 30 - 35 g at the beginning of the experiment.

Throughout the experiments, the animals are house in five per one standard laboratory cage with bedding. All mice are allowed to access to food and tap water *ad libitum* and maintained on t emperature and humidity controlled (12 hr light/dark cycle) throughout the experiment. Behavior testing will be done in the dark from 7.00 p.m. to 1.00 a.m. which is the active phase of the animals.

All animals were dyed for swimming in the Morris Water Maze to make the contrast between the water color (creamy white) and the mice color (white). The purpose of coloring provides better version of black and white under video recording and tracking.

4.2 Methods

4.2.1 Ethanol treatment

The animals were divided into 3 major groups: ethanol treated group, vehicle control group and the control group, 10 animals per group. The major groups were divided into two groups, spatial learning group and non-spatial learning group.

The Mice were treated in chronic condition via the intraperitoneal (*i.p.*) route with 4.4 g/kg/day of ethanol (20% v/v diluted from 90% ethanol in an isotonic sterile 0.9% saline solution) (n=10) or saline treatment (n=10) for 21 days (10.00 a.m.) (Isabel *et al.*, 2005). In contrast, the control group (n=10) were reared under the same condition without any injection. After 21 days,

the control, the vehicle control and ethanol treated group were housed for 1 day before starting the behavioral experiment protocols (Adapted from Quadros, I.M.H., *et al.*, 2005.).

4.2.2 Assessment of ethanol withdrawal-induced hyperexcitability

Physical signs of hyperexcitability will be measured suddenly after injection on days 1, 7, 14, and 21. The ethanol dependence will be rated by scale shown in Table 1 (Umathe, S.N., *et al.*, 2008). Each mouse will be lifted gently by the tail, spun gently through a 180° arc and held 30 cm away under an angle-poised lamp (60 W) for 3 s. (Adapted from Goldstein, D.B. & Pal, N., 1971., Watson, W.P., *et al.*, 1997., Umathe, S.N., *et al.*, 2008.)

Sr. no.	Withdrawal signs	Score
1	Vocalization on handling	1
2	Urination on handling	1
3	Defecation on handling	1
4	Caudal posture (0–3)	
	Limp or normal tail	0
	Stiff, curls around fingers	1
	Stiff, curls around finger, stays elevated after release	2
	Spontaneous abnormal posture of tail such as lift above back, stiff, curls around fingers and stays elevated after released	3
5	Tremor (0–3)	
	No tremors	0
	Mild tremor in one portion of body (i.e., face)	1
	Occasional generalized tremor	2
	Constant generalized tremor	3
6	Startle (0–3)	
	None	0
	Twitch	1
	Jump or freeze	2
	Exaggerated jump or freeze	3
7	Convulsions on handling (0–3)	
	None	0
	Short duration clonic	1
	Multiple clonic	2
	Tonic-clonic	3
8	Death	10

Table 4.1 Show rating score for ethanol withdrawal sign (Umathe, S.N., *et al.*, 2008.).

4.2.3 The Morris water maze test

Spatial learning

After the end of 21 days period, the animals were tested in a circular pool filled to a depth of 40 cm with the nontoxic white-colored paint at 23 ± 2 °C. The pool was divided into 4 quadrants (Figure 4.1). A platform (10 cm square) was submerged 1 cm below the surface of water in a place with multiple cues on all sides (Figure 4.2). Each mouse was tested during four dairy trials on successive 4 days. The position of the platform was kept in the same place, in the center of the third quadrant, throughout the experiment. During the four trials, each mouse was started once from four start position and allowed to search for the platform. Mouse was released in quadrant 1 to quadrant 4. The trial ended either when the animal climbed onto the platform or when the maximum of 60 sec elapsed. After each successful trial the animal will have to remain on the platform for a short amount of 15 sec and rest between each trial for 15 sec. If the mouse had not found the platform at the end of the trial, it was gently led to it and allowed to rest for 15 sec. After 4 days training, probe trial test was done in day 5. During the test mice would be released in the first quadrant and allowed to swim for 45 seconds.

Figure 4.1 The circular pool was divided into 4 quadrants (Q1, Q2, Q3 and Q4). The red points are represents releasing point.

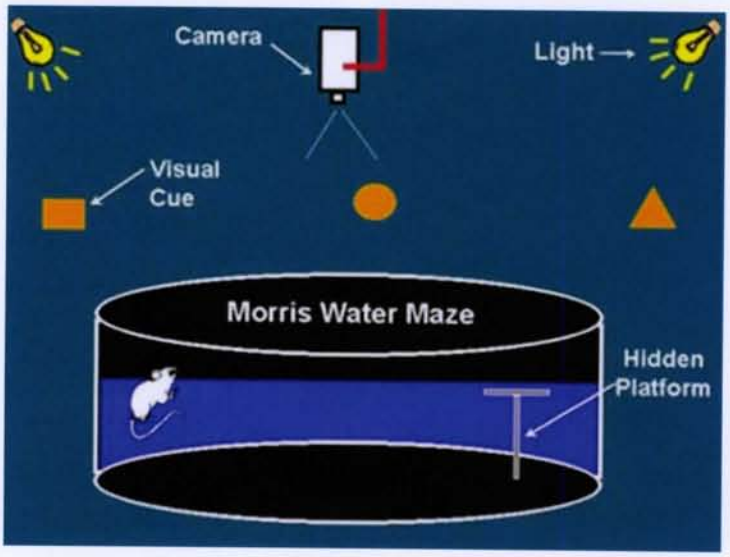


Figure 4.2 spatial learning tasks.

4.2.4 Visual Cued Task (non-spatial learning) Experiment

The non-spatial ability will be measured in Morris water maze, the hidden platform will be replaced with a platform that will be marked with a pole that above the water surface. There will be four trials in each day. Each trial will be similar to described for the hidden platform task, except that animal will see the location of the escape platform. (Adapted from Vorhees, C.V., & Williams, M.T., 2006)

4.2.5 Testing Parameters

Performance in the Morris water maze will tested one day after the last ethanol treatment. For behavioral testing we will measure, *escape latency* (time that animal use in the pool and when it locate the platform). Escape latency time for each animal spent in the pool will be analyzed in training day; the significantly decrease escape latency time indicated that the animal had learned the hidden platform task by using extra-maze spatial cues as a strategy to locate the platform.

4.2.6 Statistical Analysis

3x4 two-way mixed method analysis of variance (two-way ANOVA) with treatment (saline or ethanol or control) and day of training (day 1, day2, day 3 and day 4) as dependent variables. The level of significance was set at $p = 0.05$.

4.2.7 Sacrifice and brain perfusion

The experiment mice were deeply anesthetized with 60 mg/kg sodium pentobarbital by intraperitoneal injection (*i.p.*). Level of anesthesia was checked by an absence of corneal reflexes and a flexor withdrawal response to the noxious paw pinch. After deeply anesthetized, mice were placed on back in a dissection tray and made a surgical cut along chest midline to expose the ribs. Then they were made lateral cuts bisecting ribs on both sides. When the heart was exposed, a small canulae were inserted into the aorta by passing the left ventricle. The mice were perfused by 250 ml of 0.1 M phosphate buffer solution (PBS; pH 7.4) and the follow with 250 ml of 4 percent of paraformaldehyde in 0.1 M PBS. The brain were separated from the skull and placed into a fixative, 4 percent of paraformaldehyde, at least 1 day. The brain tissues were moved/submerged into a 30 percent of sucrose solution in PBS at 4°C until the tissues have sunk.

4.2.8 Gelatinized glass slides preparation.

The glass slides were coated with 0.5% gelatin solution (gelatin 1.5g; chromium potassium sulphate 0.15 mg; distilled water 300 ml) and dried at the room temperature for 20 minutes at least 2 times and then dried at 55-60 for at least 3 hours before use.

4.2.9 Serial sectioning with Cryostat

The whole brain was embedded in cryomatrix and freezed in the cryostat. The brain was cut in the sagittal section at 30 micrometers under frozen condition, temperature about -13°C.

The section in the cutting media was serially placed on the gelatin coated slide and air dried in room temperature about 27°C at least three days before staining. After these steps, the brain sections were stained with cresol violet.

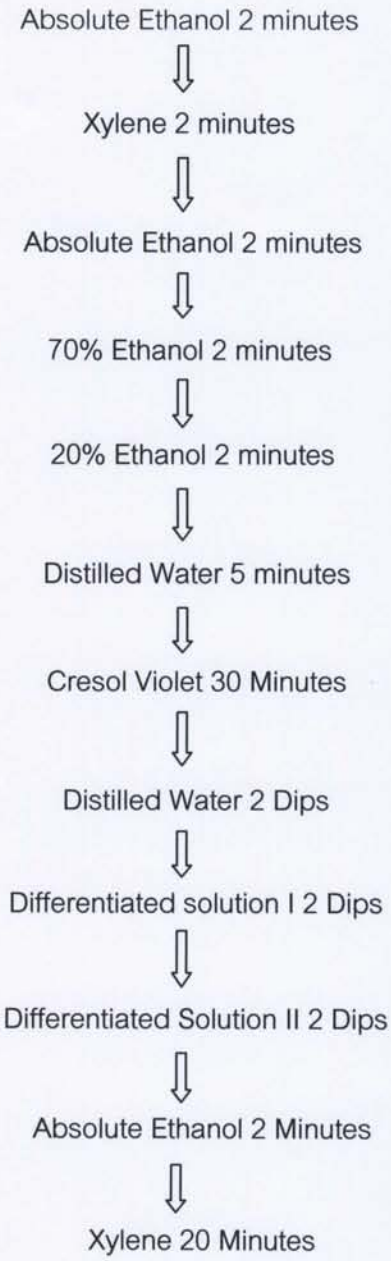


Figure 4.3 Diagram shows the protocol for stain with Cresol Violet.

CHAPTER 5

RESULTS

In this study, we investigated the role of chronic ethanol on spatial and non-spatial learning in mice. Study of spatial and non-spatial learning was performed by Morris water maze test. Mice were divided into 3 major groups; group 1 is ethanol treated group, group 2 is vehicle control group and group 4 is control group, 10 animals per group. The major groups were divided into two groups, spatial learning group and non-spatial learning group. Mice were treated with chronic condition of ethanol (20% v/v diluted from 90% ethanol in an isotonic sterile 0.9% saline solution) or saline treatment for 21 days then spatial and non-spatial study was performed. Behavior data was analyzed by video tracking system, Ethrovision (Noldus Information Technology, Netherland).

5.1 Morris water maze test results

5.1.1 Spatial learning

The results show ethanol-treated group was not significantly different in escape latency time. In contrast, control group and vehicle control group were significantly decreased escape latency time.

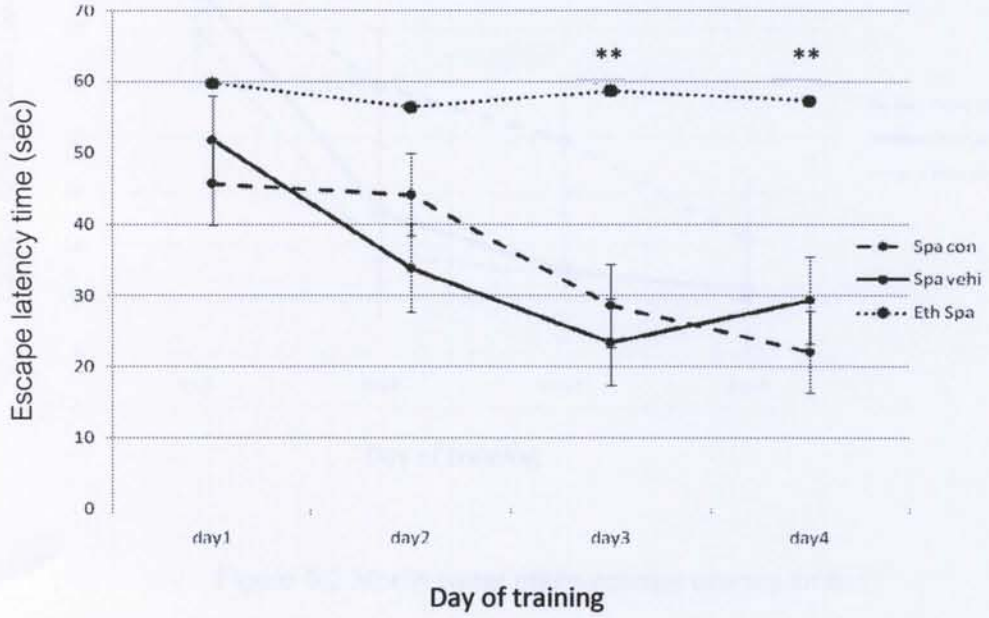


Figure 5.1 Morris water maze-escape latency time.

The figure showed the mean escape latency time that mice spend for found hidden platform. The results showed that escape latency time different in day 3 and 4. At the day 3, escape latency time in ethanol treated group was significantly different when compared with control group and vehicle control group (**: $p < 0.05$).

5.1.2 Non-spatial learning

The results show both ethanol treated group, vehicle treated group and ethanol treated group were not significantly different in escape latency time.

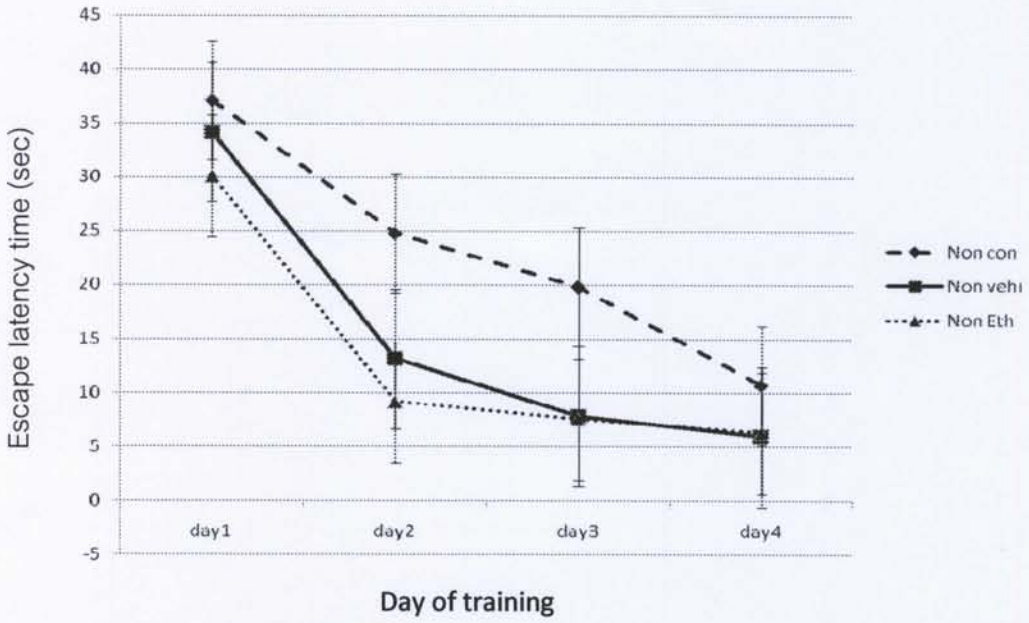
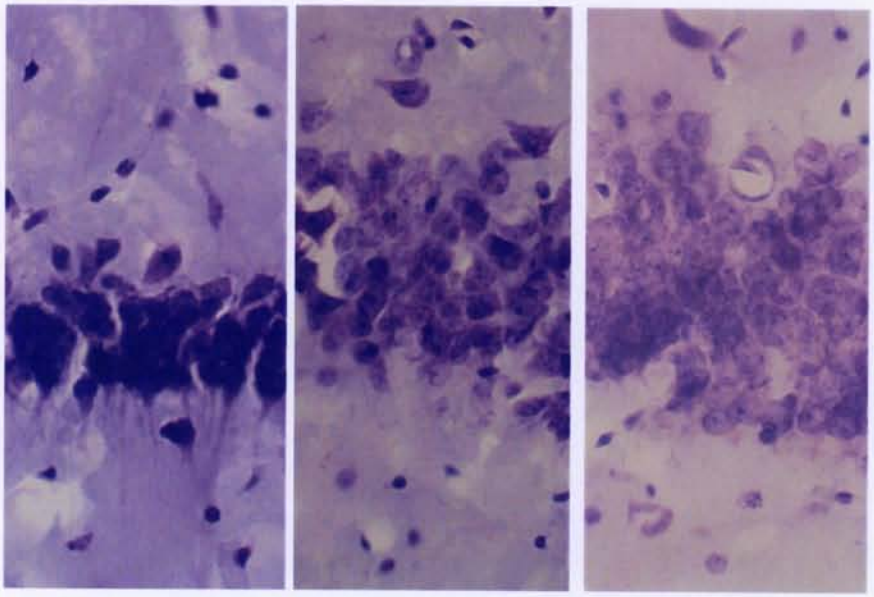


Figure 5.2 Morris water maze-escape latency time.

The figure showed the mean escape latency time that mice spend for found visible platform. The results showed the escape latency time was not significantly different when compared with control group and vehicle control group.

5.2 Hippocampus serial section

The brain samples of mice were slided into serial section and stained with cresol violet (figure 5.2).



Control Group Vehicle Control Group Ethanol-treated Group

Figure 5.2 Different level of Hippocampus (40X) in spatial learning task.

The figures present the region of hippocampus in the serial section, from the first slide following from group (control group, vehicle control group and ethanol treated group). These figures were strained with cresol violet (violet staining), which color shows the neuron region.

CHAPTER 6

DISCUSSION

The experimental designs were divided to study the behavioral. The experimental were performed in laboratory animals because of the chronic ethanol treated study, is limited in humans. According to C57BL mice are always use to study the spatial learning (Vorhees and Williams, 2002). Although, ICR mice are able to utilize for spatial learning study (Adam *et al.*, 2002). Moreover ICR mice are cheap, inquisitive, easy to take care, and wildly used as the subject of the experiment. ICR mice were obtained from National Laboratory Animal Centre, Mahidol University, Thailand (NLAC). The experiment used the mice that were about 8 weeks old (56 days) which were age of puberty (young adult mice). At this age the mice brain was developed completely (from NLAC).

To study the effect of chronic ethanol treated, ethanol was injected via the intraperitoneal (*i.p.*) route with 4.4 g/kg/day of ethanol (20% v/v diluted from 90% ethanol in an isotonic sterile 0.9% saline solution) or saline treatment (Adapted from Isabel *et al.*, 2005). And we desired to control doses which mice were treated by inject ethanol via intraperitoneal (*i.p.*).

In control group, mice were housed under the same condition with vehicle treated group and ethanol treated group except mice were not injected. Treatment of ethanol and normal saline were performed for 21 days, which is effective period to study (Isabel *et al.*, 2005,). However, Barbara Malinowska *et al.*, 1999 used less time than 21 days. Despite, that experiment was done in different source of ethanol by mice were given liquid diet containing 7% ethanol.

There were principle findings in this study. First, the chronic treating of ethanol (4.4. g/kg/day) eliminated spatial learning in the Morris water maze, dependent of training day. This effect was observed in any effect on the escape latency time of mice during training in tasks. However, the chronic treating of ethanol doesn't attenuate acquisition of non-spatial learning.

6.1 Effect of ethanol on spatial and non-spatial learning

This spatial performance in Morris water maze test in ethanol treated group was compare to control group and vehicle control group. The result showed the ethanol treated mice had apparent worse in locating the hidden platform in the Morris water maze as evidenced by significantly different in escape latency time than control group and vehicle control group. Many evidences were represented that physiological effect of ethanol such as disrupt the acquisition of memory (clive harper & izuru matsumoto, 2005). It has been reported that ethanol produce a significant disrupt in performance of working memory, and impaired reaction times for both memory and attention (Jones & jones, 1980, Nilsson *et al.,* 1989, Shawn Echison *et al.,* 2001).

In 2001, it has been reported that acute effects of ethanol (2.5 g/kg) disrupt the acquisition of spatial learning. Nevertheless, low dose of ethanol (0.5 g/kg) improve spatial learning task which was independent of age (Shawn Echison *et al.,* 2001). And in 2005, it has been reported in same way that ethanol-treated rat (2 g/kg *i.p.*) has significantly escape latency time higher than saline-treated control rats (Sircar & Sircar, 2005).

In non-spatial learning study, ethanol-treated mice reveal a learning performance comparable to control group. They were not significantly different time to found visible platform. Likewise, this result show in the same way with the previous study (Shawn Echison *et al.,* 2001, Sircar & Sircar, 2005).

In conclusion, the results of this study demonstrated that ethanol have the effect on spatial learning and memory which is hippocampus-dependent memory. In contrast, ethanol have no effect on non-spatial learning and memory.

CHAPTER 7

CONCLUSIONS

From the experiment, results can be concluded that

1. Ethanol treated group is significantly different in escape latency time when compared with control group in spatial learning task. This may assume that ethanol impair the spatial learning ability in Morris water maze.
2. Ethanol treated group is significantly different in escape latency time when compared with vehicle control group in spatial learning task. This proposes that ethanol impair the spatial learning ability in the Morris water maze but saline solution doesn't impact on the spatial learning ability in the Morris water maze.
3. Vehicle control group isn't significantly different in escape latency time when compare with control group in spatial learning task. This suggest that saline solution doesn't impact on the spatial learning ability in the Morris water maze.
4. Ethanol treated group isn't significantly different in escape latency time when compare with control group in non-spatial learning task. This propose that ethanol doesn't impact on the non-spatial learning ability in the Morris water maze.
5. Ethanol treated group isn't significantly different in escape latency time when compare with vehicle control group in non-spatial learning task. This assume that ethanol doesn't impact on the non-spatial learning ability in the Morris water maze.
6. Vehicle control group isn't significantly different in escape latency time when compare with control group in non-spatial learning task. This suggest that saline solution doesn't impact on the non-spatial learning ability in the Morris water maze.

Conclusively, ethanol impairs spatial learning, whereas saline solution doesn't impair. In contrast, ethanol and saline solution doesn't enhance non-spatial learning. However, the underline mechanism is still illusinated and could be further study.

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APPENDIX

Appendix A: Stains and Reagent list

1) Ethyl alcohol 25%

- Absoluted (or 95%) Ethyl alcohol	250 ml
- Saline solution	750 ml

2) Buffer Solution

2.1) 0.1 M 4X Phosphate Buffer (pH 7.4) – 4.5 L

- Sodium Di-hydrogen Phosphate Monohydrate	49.50 g
- Di-sodium Hydrogen Phosphate Anhydrous	204.48 g
- Distilled water	4,500 ml

3) Fixative

3.1) 4% Paraformadehyde in 0.1 M Phosphate Buffer (pH 7.4) – 5 L

- Paraformadehyde	200 g
- 0.1 M Phosphate Buffer	5,000 ml

4) Gelatin

4.1) 0.5% Gelatin solution for Coated Glass Slide

- Gelatin Powder	1.5 g
- Chromium Potasium Sulphate	0.15 g
- Distilled water	300 ml

5) Stain and Differentiators

5.1.) 0.1% Cresyl Violet – 1 L

- 0.1 % Cresyl Violet in DH_2O

5.2.) Differentiator I for Cresyl Violet Staining – 900 mL

- 70% Ethyl alcohol	810 ml
- Acetic acid (conc.)	9 ml
- Distilled Water	81 ml

5.3.) Differentiator II for Cresyl Viololet Staining – 900 mL

- Absoluted (or 95%) Ethyl alcohol	810 ml
- Acetic acid (conc.)	9 ml
- Distilled Water	81 ml

6) Miscellaneous

6.1) 30% Sucrose solution for staining brain – 100 ml

- Sucrose	30 ml
- 0.1 M Phosephate Buffer	100 ml

6.2) Acid alcohol for Glass Slide cleaning – 100 ml

- 70% Ethyl alcohol	100 ml
- Glacial Hydrochloric acid	4 drops

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