# CHAPTER IV

# RESULTS

## 1. Dose-response of THF and GBL as Considered from the Loss of the Righting Reflex and Failure in the Rotarod Test

The first objective of our study was to compare the acute toxicity of THF and GBL. We accomplished this objective by studying the dose-response curves of these agents obtaining from intraperitoneal injection and using the righting reflex test and rotarod test in ICR mice as test models. The $TD_{50}$ values (the median toxic dose) for the loss of righting reflex induced by THF and GBL were 15.18 mmol/kg (95% CI, 11.88–19.39 mmol/kg) and 4.60 mmol/kg (95% CI, 3.25-6.51 mmol/kg), respectively (Table 4-1; Figure 4-1). Similarly the $TD_{50}$ values for the failure at the rotarod test for THF and GBL were 7.00 mmol/kg (95% CI, 5.22-9.40 mmol/kg), and 0.85 mmol/kg (95% CI, 0.52-1.38 mmol/kg), respectively (Table 4-2; Figure 4-2). The effect of THF administered by intracerebroventricular injection was also observed. Mice that received THF by intracerebroventricular injection did not show dose-related loss of righting reflex and failure to perform the rotarod test. Instead, convulsions and death were observed. At doses of THF up to 10 µmol/10 µl, mice did not show any deficits on the righting reflex and rotarod test. Mice that received THF at doses of 20 to 200 µmol/10 µl manifested seizures and death in a dose-related manner. The $LD_{50}$ of intracerebroventricular injection of THF was 79.28 µmol/mice (95% CI, 45.87-137.05 µmol/mice).

## 2. Effects of THF and GBL on Locomotor Activity

The second objective was to investigate effect of THF and GBL on locomotor activity. Since THF solution used in the laboratory was preserved with <0.025% butylated hydroxytoluene (BHT), therefore the effect of BHT was observed by administration of 2 mg/kg BHT in corn oil to mice which were then subjected to locomotor investigation. Experimental results revealed that locomotor activities induced by corn oil and 2 mg/kg BHT did not differ from that induced by saline vehicle for the entire 150-min test period (Figure 4-3).

Dose-response analyses were conducted for locomotor activity in which four doses for each of THF and GBL were tested (1, 3, 5, and 10 mmol/kg;

n=10). Mice (receiving an injection of the test compounds or saline vehicle) were immediately placed in the testing chambers. Locomotor activity was measured continuously and summarized in 10-min intervals for a total of 150-min test session. Effects of THF and GBL on the locomotor activity of mice are presented in Figure 4-4 and 4-5 respectively. Both agents produced a dose-dependent suppression in locomotor activity. There was also a main effect of sampling time for each agent. *Post hoc* analyses of the entire 150-min test period revealed that the activity of THF at doses of 1, 3, 5, and 10 mmol/kg differed significantly from that of saline. In addition, THF at doses of 3, 5, and 10 mmol/kg revealed significant effects on locomotion activity starting from 20 min to 150 min of the testing period.

A similar analysis for GBL revealed that locomotor activity at 1, 3, 5, and 10 mmol/kg differed significantly from that of saline. Depressant effects in all groups of mice treated with GBL were observed during the first 10 min. For GBL, doses of 1, 3, and 5 mmol/kg had a strong locomotor activity depression, then followed by modest depressant effect on locomotion. This phenomenon resulted in no significant differences observed among activities at various doses of GBL (1, 3, and 5 mmol/kg) and saline when compared locomotor activity at 40, 60, and 80 min of the testing period, respectively ($p>0.05$). At a GBL dose of 10 mmol/kg, locomotor activity was mostly suppressed and was significantly different from that of saline for the entire 150-min test period.

Due to strong depressant effect of THF and GBL (1-10 mmol/kg), THF and GBL at lower doses (0.1 mmol/kg and 0.3 mmol/kg) were additionally investigated. At 0.1 and 0.3 mmol/kg of THF, locomotor activity was apparently depressed during the first 40 min, then followed by a pronounced locomotor suppression which was significant from 70 min to 150 min. For GBL, a dose of 0.1 mmol/kg had no significant overall effects on locomotor activity except at 120 to 140 min. However, at a dose of 0.3 mmol/kg, GBL significantly reduced locomotor activity during the first 40 min ($p<0.05$), followed by a modest depressant effect on locomotor activity for the next 20 min and then significant reduction of locomotor activity again from 80 through 150 min ($p<0.05$) (Figure 4-6).

### 3. Effects of THF and GBL on Open-Field Activity

Table 4-3 shows mean values of study parameters in the open-field test on groups of mice treated with THF (0.1 and 0.3 mmol/kg), GBL (0.1 and 0.3 mmol/kg), and saline. The parameters included thigmotactic ratio, inner ambulation, outer ambulation, number of rearings, number of groomings, open-field defecation and open-field urination. There were no significant differences among groups in all parameters tested except for the inner ambulation. THF (0.1 mmol/kg) and GBL (0.1 and 0.3 mmol/kg) treated groups decreased inner ambulation in the open-field during the 10-min test period ($p<0.05$).

### 4. Effects of THF and GBL on Elevated Plus Maze

The percentage of open-arm entries and the time spent in open arms were not different among saline-treated, THF-treated (0.1 and 0.3 mmol/kg), and GBL-treated (0.1 and 0.3 mmol/kg) groups. However, DZP-treated group (0.007 mmol/kg) significantly ($p<0.05$) increased the percentage of open arm entries as well as the time spent in open arms (Figure 4-7).

### 5. Effects of THF and GBL on Spontaneous Alternation Behavior

The percentage of spontaneous alternation and total arm entries in the Y-maze test of mice is shown in Figure 4-8 (A) and Figure 4-8 (B), respectively. THF (0.1, 0.3, 1, and 3 mmol/kg) and GBL (0.1 and 0.3 mmol/kg) had no effect on the percentage of spontaneous alternation. THF (0.1, 0.3, and 1 mmol/kg i.p.) and GBL (0.1 and 0.3 mmol/kg i.p.) had no effect on total arm entries. THF at dose of 3 mmol/kg significantly reduced total arm entries. At 1 and 3 mmol/kg of GBL, mice were immobile and the alternation behavior could not be determined.

### 6. Effects of THF and GBL on Spatial Memory

Escape latencies (the time taken to escape onto the hidden platform) in daily training of mice in the water maze task during a 5-day training are shown in Figure 4-9 (A). The daily escape latencies of THF-treated mice (3 mmol/kg) and GBL-treated mice (1 and 3 mmol/kg) were significantly ($p<0.05$) longer than those of control mice.

The percentage of time spent in the platform quadrant (probe trial) was shown in Figure 4-9 (B). The percentage of time spent in the platform quadrant of

THF-treated mice (1 and 3 mmol/kg), and GBL-treated mice (1 and 3 mmol/kg) were significantly shorter than that of control mice ($p<0.05$).

**7. Effects of THF and GBL on Open-Space Swimming Test**

Figure 4-10 presents effects of imipramine (IMI 15 mg/kg, 3 injections per day, i.p.), THF (0.1 and 0.3 mmol/kg, 3 injections per day, i.p.), and GBL (0.1 and 0.3 mmol/kg, 3 injections per day, i.p.), administered during the open-space swimming test on the percentage of mobility time in mice. Mice with imipramine treatment significantly increased percent mobility in Day 2 and Day 3 of testing. All THF- and GBL- treated groups showed no differences in the percentage of mobility time in all 3 days as compared to saline-treated group.

**8. Tolerance of Mice with Chronic THF Treatment on the Loss of Righting Reflex and the Rotarod Test**

Mice receiving saline or THF (5 and 10 mmol/kg, i.p.), once daily for a total period of 14 consecutive days, were then challenged with THF (15 mmol/kg, i.p.) on day 15. The righting reflex and the rotarod test were evaluated until recovery. The percentage of mice receiving a prolonged treatment of THF at a dose of 10 mmol/kg which showed the loss of righting reflex when challenged with THF 15 mmol/kg was decreased significantly ($p<0.05$) up to 165 min as compared to THF naïve group (Figure 4-11).

**9. Effects of THF and GBL on Conditioned Place Preference**

The conditioned place preference of mice was determined after conditioning with morphine (5 mg/kg, s.c.), THF (3 and 5 mmol/kg, i.p.), and GBL (0.5 and 1 mmol/kg, i.p.). Statistical analysis indicated that morphine induced place preference by increasing the place preference score and differences in time spent in the white compartment (between post-conditioning and pre-conditioning sessions). THF- and GBL- treated groups showed no differences in place preference scores or in time spent in white compartment as compared to saline-treated group (Figure 4-12).

## 10. Role of GABA and GHB Receptor Involvement in the Motor Impairment Induced by THF

Mice that received saline pretreatment, followed by THF (15 mmol/kg, i.p.) initially failed in the rotarod test but eventually recovered after 210 min. Full recovery was observed at 360 min (Figure 4-13, 4-14, 4-15, 4-16). Although mice that received CGP-35348 administration (200 mg/kg, i.p.) 15 min prior to THF (15 mmol/kg, i.p.) treatment passed the rotarod test upto 120-150 min, their performance was gradually decreased and did not recover in 360 min. Mice that received NCS-382 administration (250 mg/kg, i.p.) 15 min before THF (15 mmol/kg, i.p.) treatment showed no improvement in the rotarod performance and did not recover in 360-min test session as the control group did (Figure 4-14). Mice that received picrotoxin (2 mg/kg, i.p.), or flumazenil (10 mg/kg, i.p.) administration 15 min before THF (15 mmol/kg, i.p.) treatment showed similar rotarod performance to that of saline pretreatment (Figure 4-15, 4-16, respectively).

**TABLE 4-1 Comparison of $TD_{50}$ Values of THF and GBL as Considered from the Failure in the Righting Reflex Test.**

| Chemical | $TD_{50}$ for failure in the righting reflex test (mmol/kg) | 95 % Confidence Interval (mmol/kg) |
|---|---|---|
| THF | 15.18 | 11.88 -19.39 |
| GBL | 4.60 | 3.25 – 6.51 |

There was a statistically significant difference between the treatment groups ($p<0.05$).

**TABLE 4-2 Comparison of $TD_{50}$ Values of THF and GBL as Considered from the Failure in the Rotarod Test.**

| Drug | $TD_{50}$ for failure in the rotarod test (mmol/kg) | 95 % Confidence Interval (mmol/kg) |
|---|---|---|
| THF | 7.00 | 5.22 – 9.40 |
| GBL | 0.85 | 0.52 – 1.38 |

There was a statistically significant difference between the treatment groups ($p<0.05$).

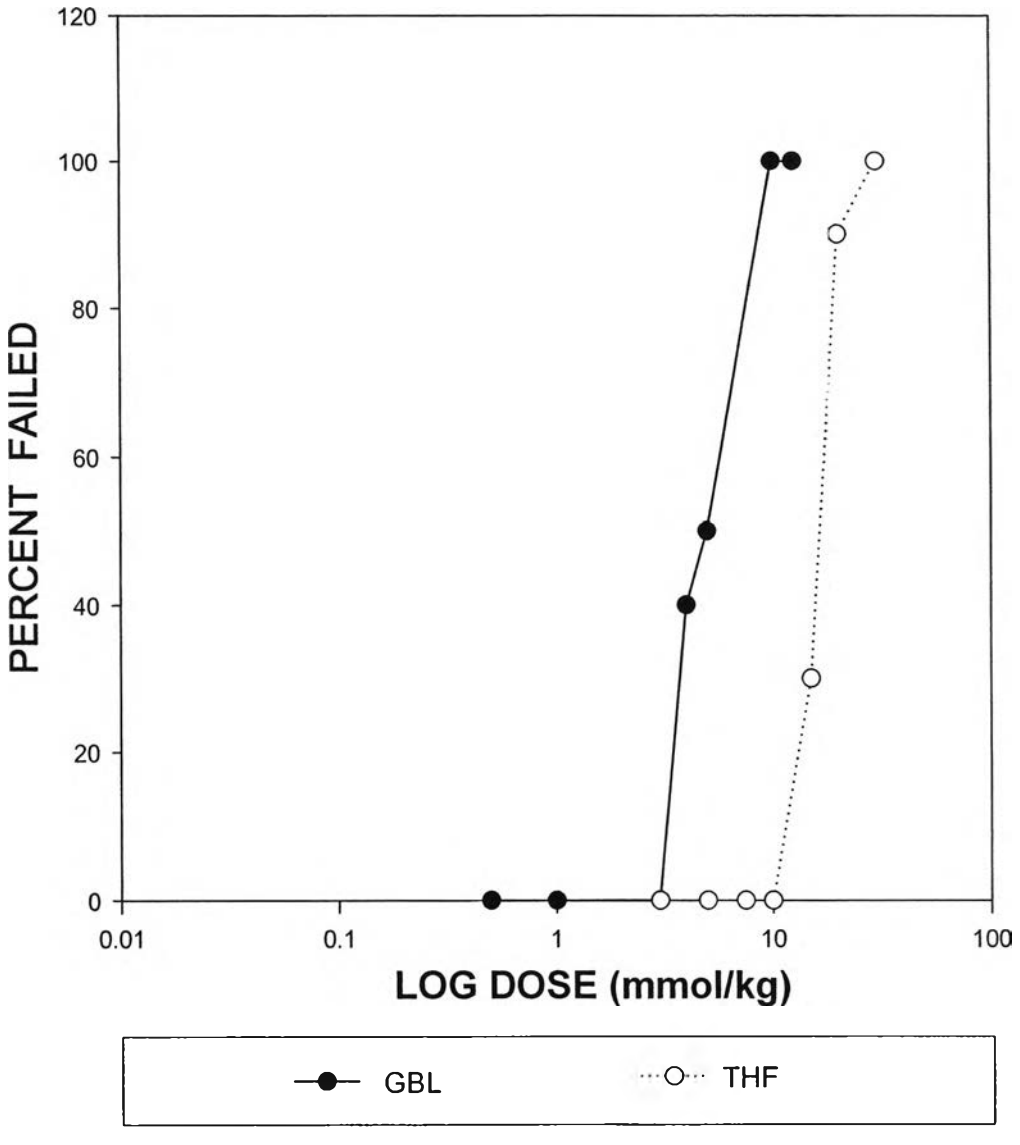


**FIGURE 4-1 Dose-Response Curves of THF and GBL for the Righting Reflex Test.**

Mice (n=10/dose) were administered with incremental doses of either THF (3-30 mmol/kg) or GBL (0.5-12.5 mmol/kg) i.p. and tested for the righting reflex 30 min after the administration.

Values represent the percentage of mice that lost the righting reflex.

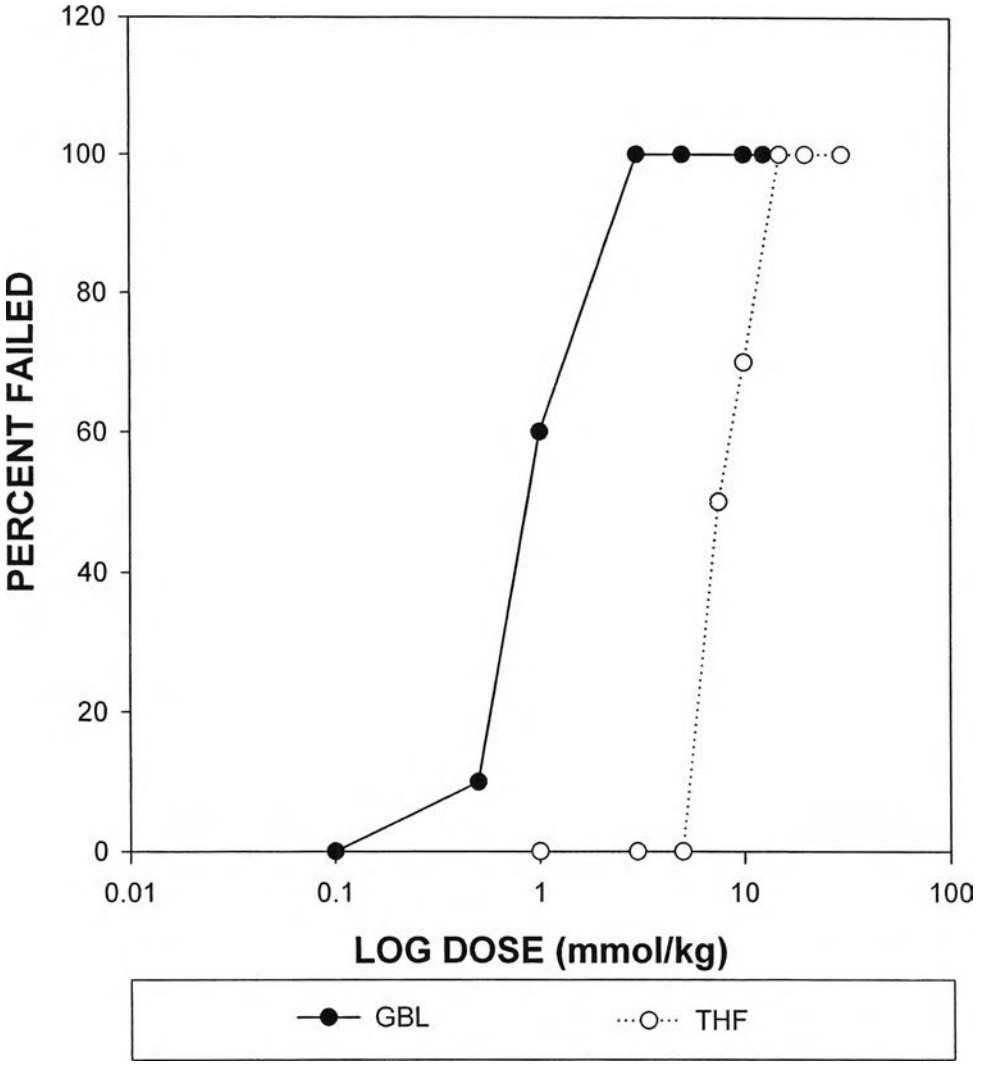


**FIGURE 4-2 Dose-Response Curves of THF and GBL for the Rotarod Test.**

Mice (n=10/dose) were administered with incremental doses of either THF (3-30 mmol/kg) or GBL (0.5-12.5 mmol/kg) i.p. and tested for the rotarod performance 30 min after the administration.

Values represent the percentage of mice that failed to perform the rotarod test.

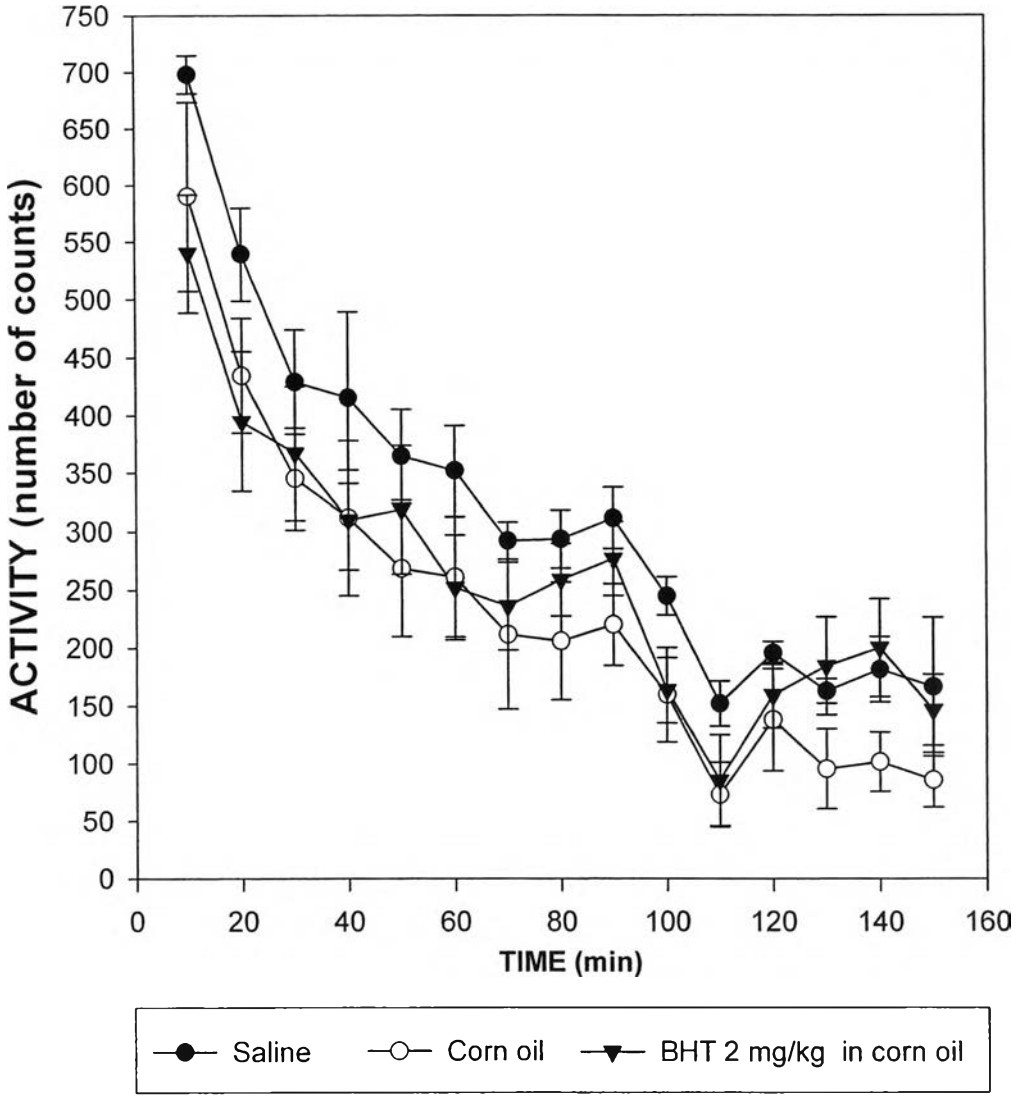


**FIGURE 4-3 Effects of Normal Saline, Corn Oil, and Butylated Hydroxytoluene (BHT) on Locomotor Activity.**

Mice received an intraperitoneal injection of normal saline (10 ml/kg), corn oil (10 ml/kg), or BHT (2 mg/kg), were immediately placed in an activity monitor. Horizontal activity was monitored for 150 min.

Each point represents the mean $\pm$ S.E.M. from a group of 8 mice. No significant differences from the normal saline-treated group were noted.

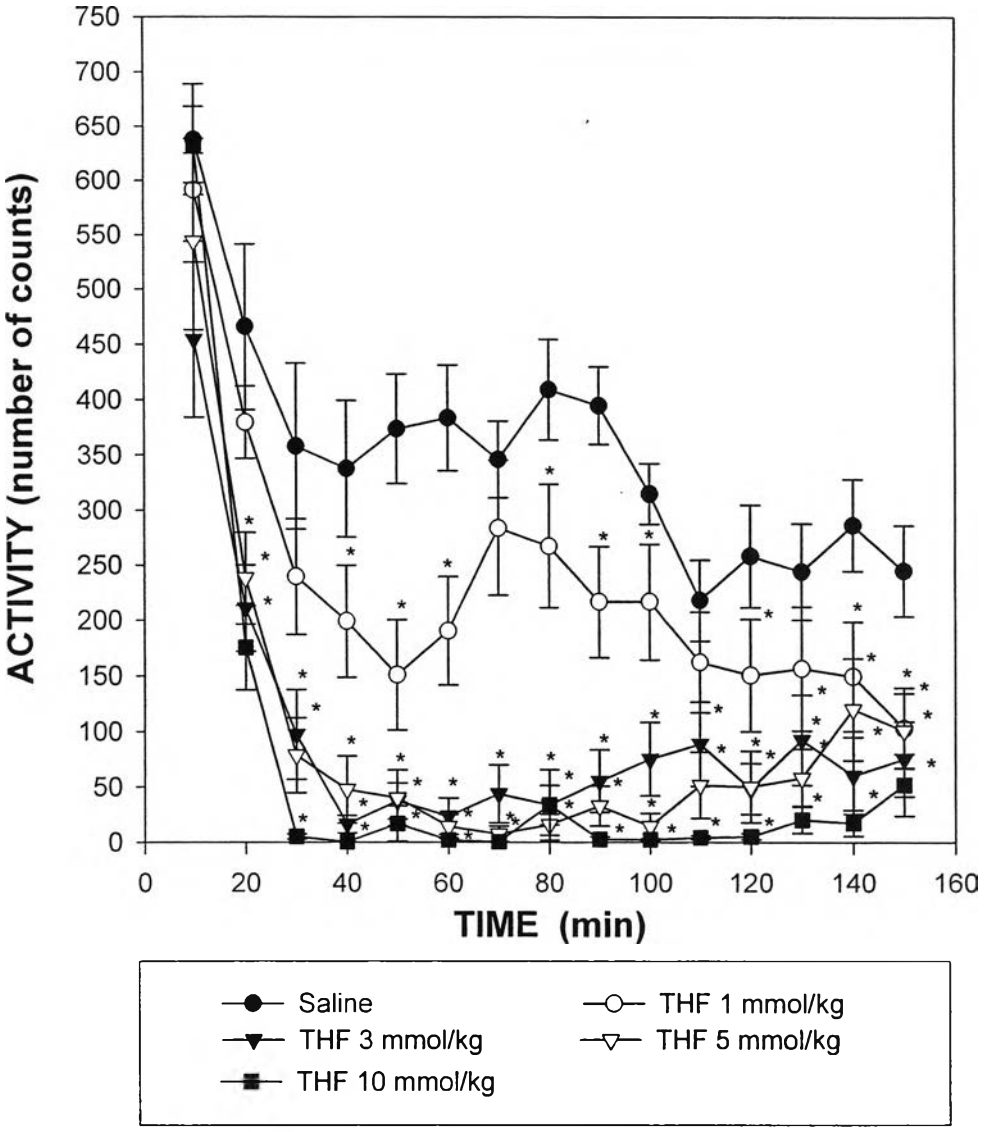


**FIGURE 4-4 Effect of THF on Locomotor Activity.**

Mice received an intraperitoneal injection of normal saline or THF (1, 3, 5, and 10 mmol/kg) were immediately placed in an activity monitor. Horizontal activity was monitored for 150 min.

Each point represents the mean ± S.E.M. from a group of 10 mice.

* denotes significant difference in comparison with the normal saline treated group ($p<0.05$).

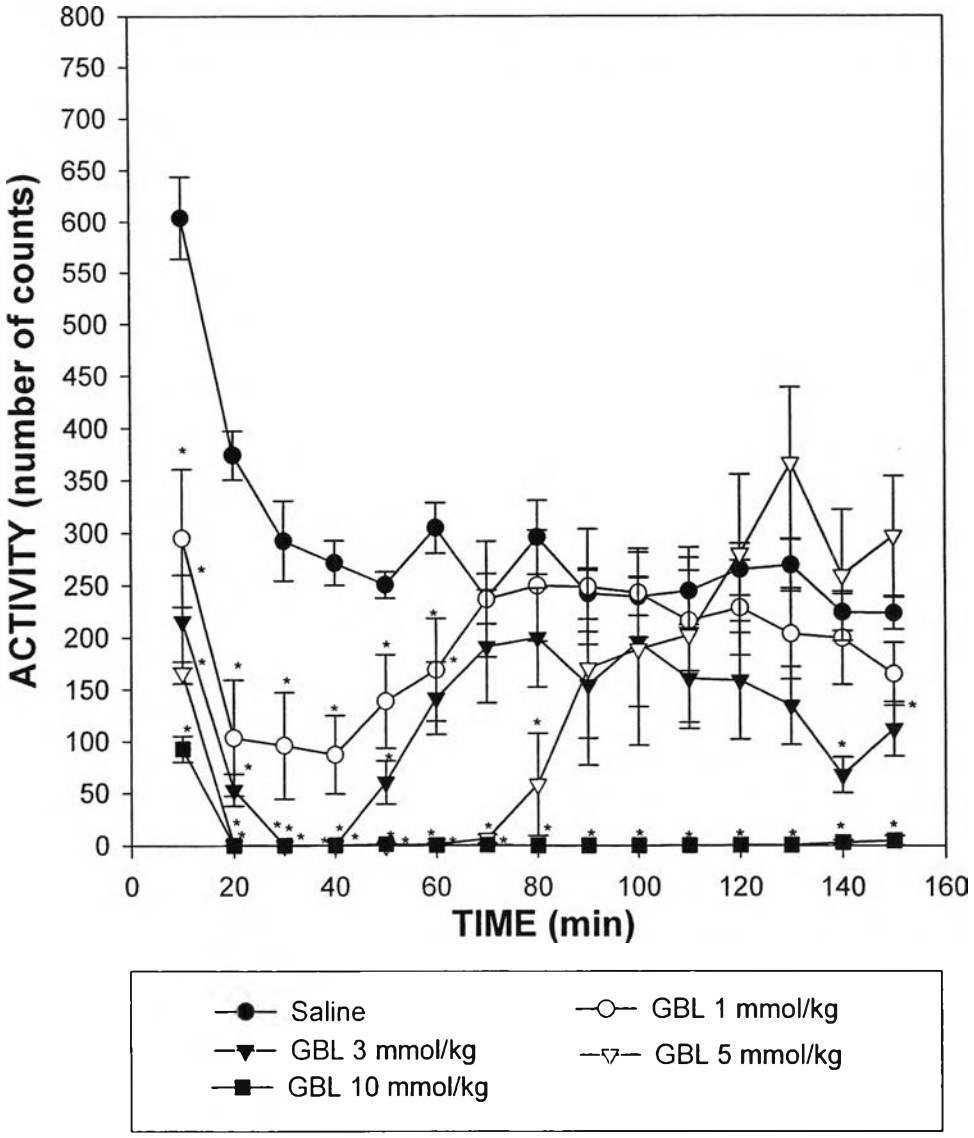


**FIGURE 4-5 Effect of GBL on Locomotor Activity.**

Mice received an intraperitoneal injection of normal saline or GBL (1, 3, 5, and 10 mmol/kg) were immediately placed in an activity monitor. Horizontal activity was monitored for 150 min.

Each point represents the mean ± S.E.M. from a group of 10 mice.

* denotes significant difference in comparison with the normal saline treated group ($p<0.05$).

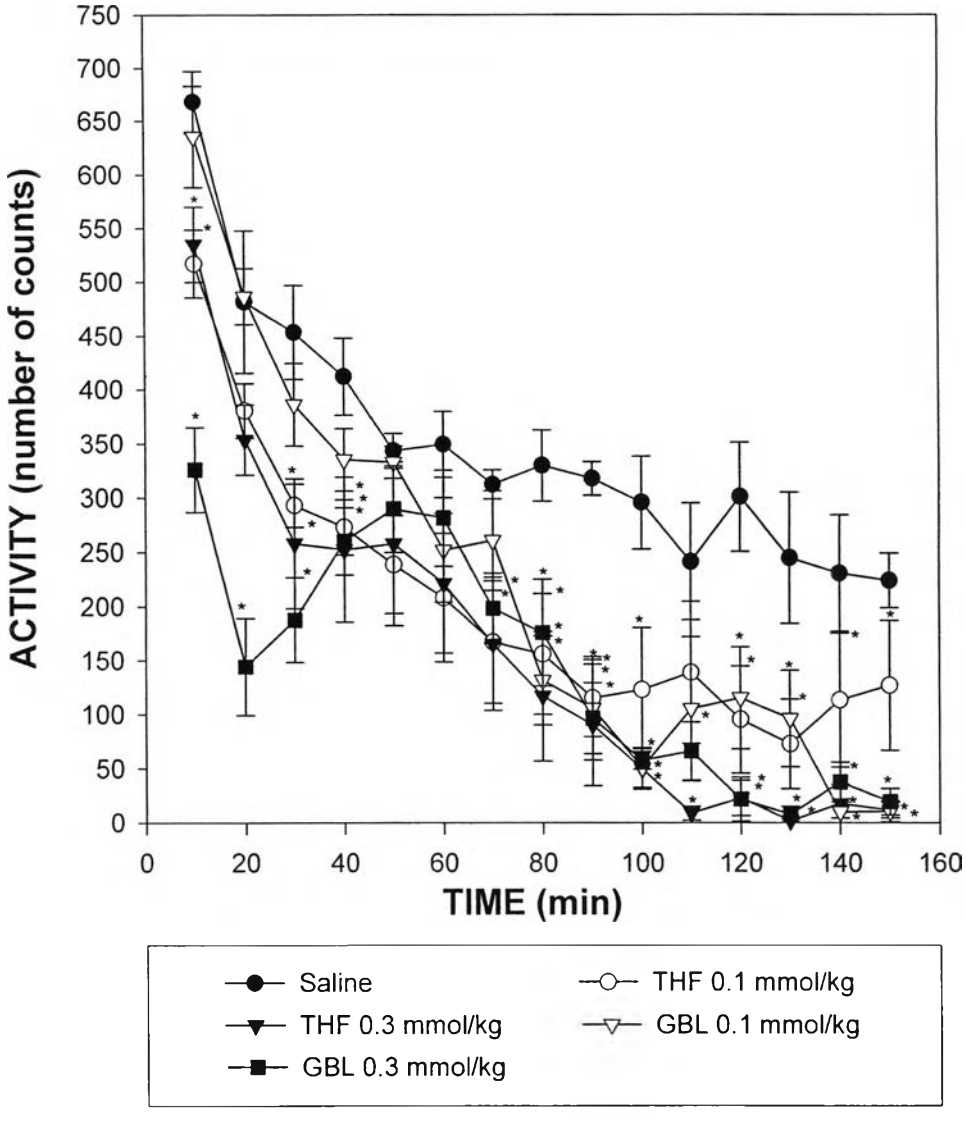


**FIGURE 4-6 Effects of Low Doses of THF and GBL on Locomotor activity.**

Mice received an intraperitoneal injection of normal saline, THF (0.1 and 0.3 mmol/kg), or GBL (0.1 and 0.3 mmol/kg), were immediately placed in an activity monitor. Horizontal activity was monitored for 150 min.

Each point represents the mean ±S.E.M. from a group of 7 mice.

* denotes significant difference in comparison with the normal saline treated group ($p<0.05$).

**TABLE 4-3 Effects of THF and GBL on Open-Field Behavior.**

Thirty minutes after intraperitoneal injection of normal saline, THF (0.1 and 0.3 mmol/kg), or GBL (0.1 and 0.3 mmol/kg), mice were placed in an open-field. Thigmotactic ratio, inner ambulation, outer ambulation, number of rearing, number of grooming, defecation and urination were measured in 10 min.

Each point represents the mean ± S.E.M. from a group of 8 mice.

* denotes significant difference in comparison with the normal saline treated group ($p<0.05$).

| | Saline | THF 0.1 mmol/kg | THF 0.3 mmol/kg | GBL 0.1 mmol/kg | GBL 0.3 mmol/kg |
|---|---|---|---|---|---|
| **Thigmotactic ratio** | 0.33 ± 0.02 | 0.23 ± 0.04 | 0.25 ± 0.04 | 0.21 ± 0.03 | 0.18 ± 0.05 |
| **Inner ambulation** | 111.00 ±21.01 | 55.50± 0.13* | 71.50±0.35 | 48.88± 8.57* | 49.38±17.48* |
| **Outer ambulation** | 217.25 ± 24.52 | 174.88±20.45 | 197.75±16.94 | 181.88±23.61 | 145.13±6.69 |
| **Rearing** | 43.63 ± 5.28 | 37.00 ± 7.60 | 41.75 ± 6.04 | 30.38 ± 9.28 | 26.63 ± 9.50 |
| **Grooming** | 5.75 ± 0.59 | 6.25 ± 1.39 | 3.88 ± 0.97 | 5.00 ± 0.93 | 2.75 ± 0.96 |
| **Defecation** | 0.63 ± 0.18 | 0.25 ± 0.16 | 0.38 ± 0.18 | 0.25 ± 0.16 | 0.38 ± 0.18 |
| **Urination** | 1.25 ± 0.41 | 0.50 ± 0.33 | 1.25 ± 0.53 | 0.38 ± 0.38 | 0.88 ± 0.58 |

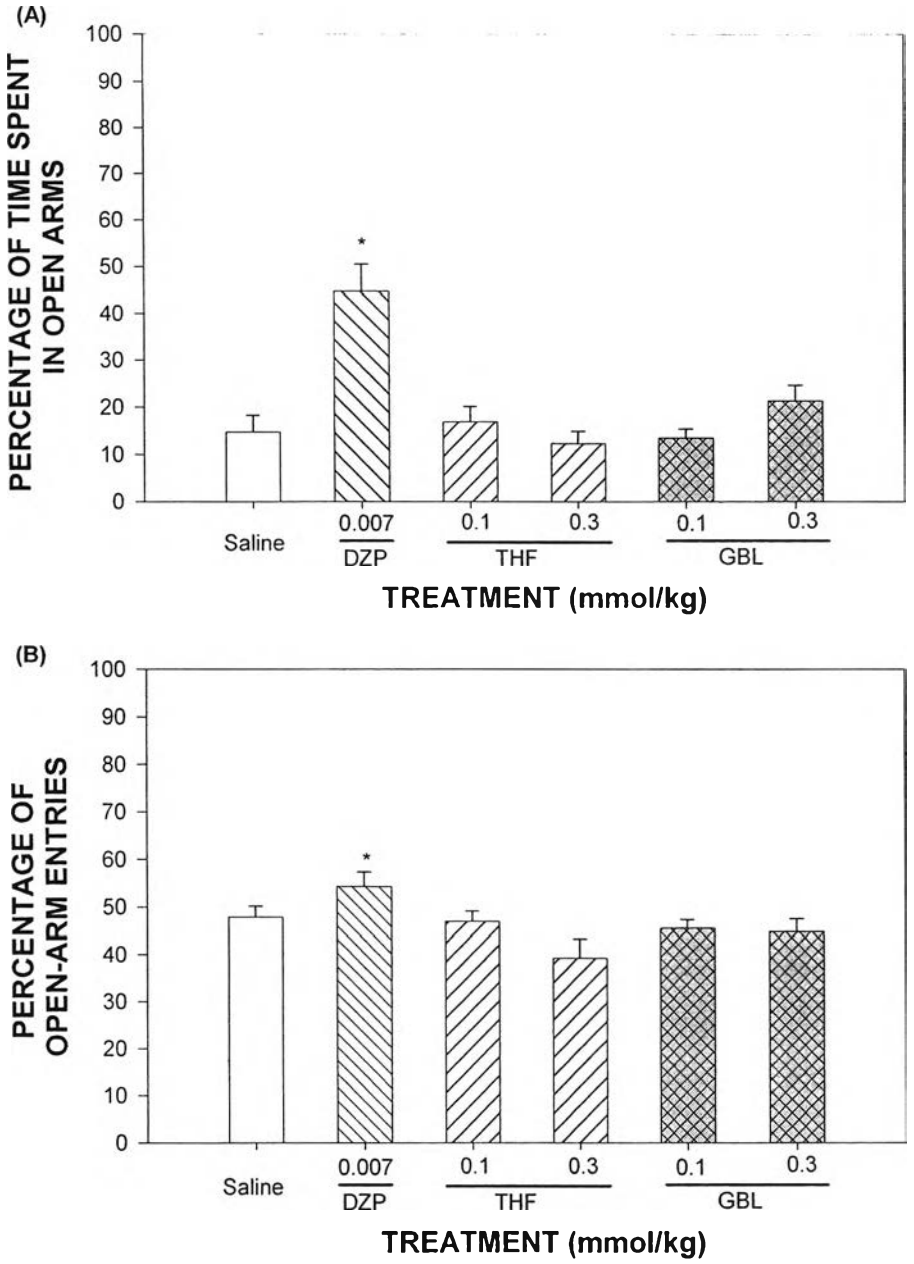


**FIGURE 4-7 Effects of THF and GBL on the Elevated Plus Maze Test.**

(A) Effects of diazepam (DZP, 0.007 mmol/kg), THF (0.1 and 0.3 mmol/kg), and GBL (0.1 and 0.3 mmol/kg), on the percentage of time spent in open arms.

(B) Effects of THF (0.1 and 0.3 mmol/kg) and GBL (0.1 and 0.3 mmol/kg) on the percentage of open-arm entries.

Each point represents the mean ± S.E.M. from a group of 8 mice.

* denotes significant difference in comparison with the normal saline-treated group ($p<0.05$).

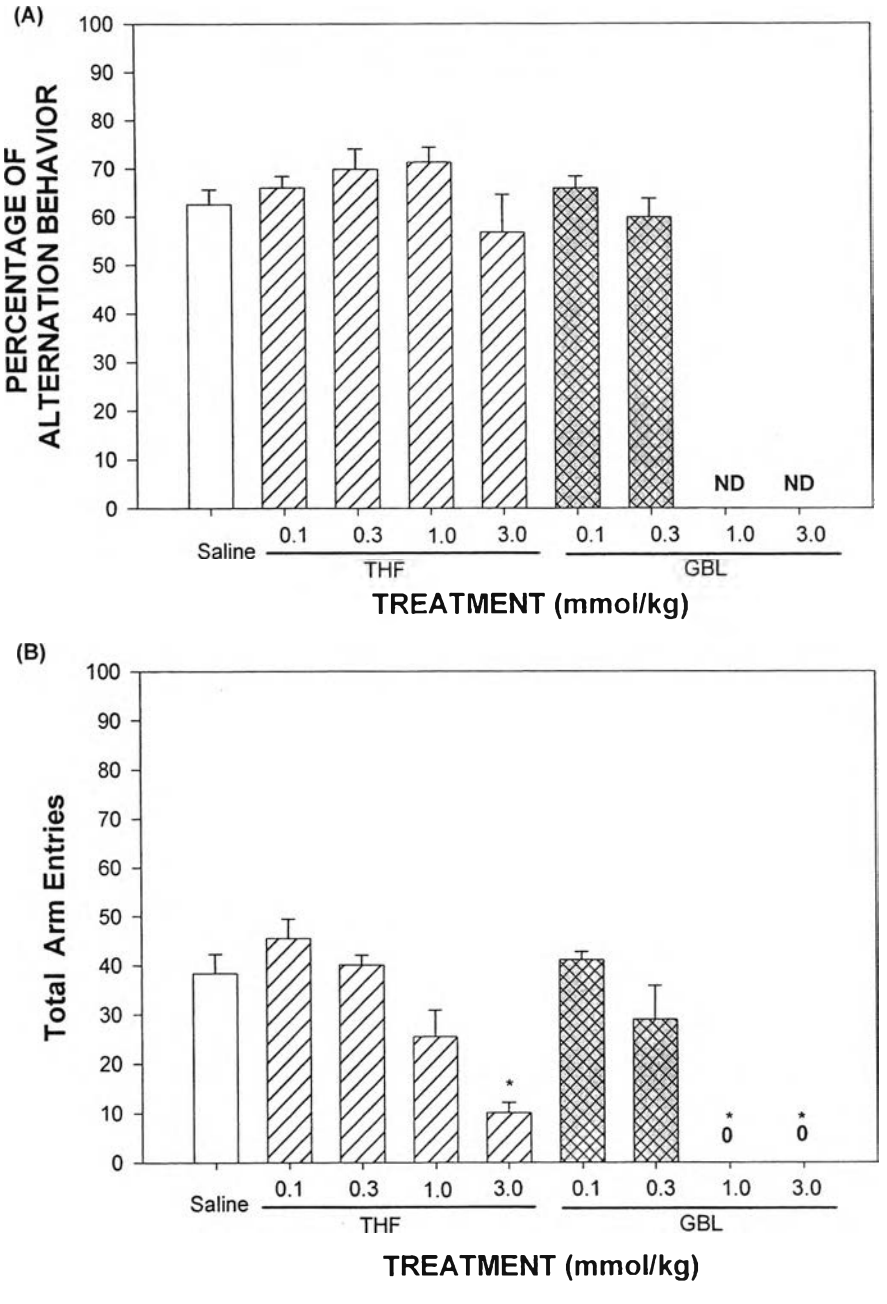


**FIGURE 4-8 Effects of THF and GBL on the Y-Maze Task.**

(A) Effects of THF (0.1 and 0.3 mmol/kg) and GBL (0.1 and 0.3 mmol/kg) on spontaneous alternation behavior.

(B) Effects of THF (0.1 and 0.3 mmol/kg) and GBL (0.1 and 0.3 mmol/kg) on total arm entries.

Each point represents the mean ± S.E.M. from a group of 8 mice.

* denotes significant difference in comparison with the normal saline-treated group ($p<0.05$). ND denotes not determined.

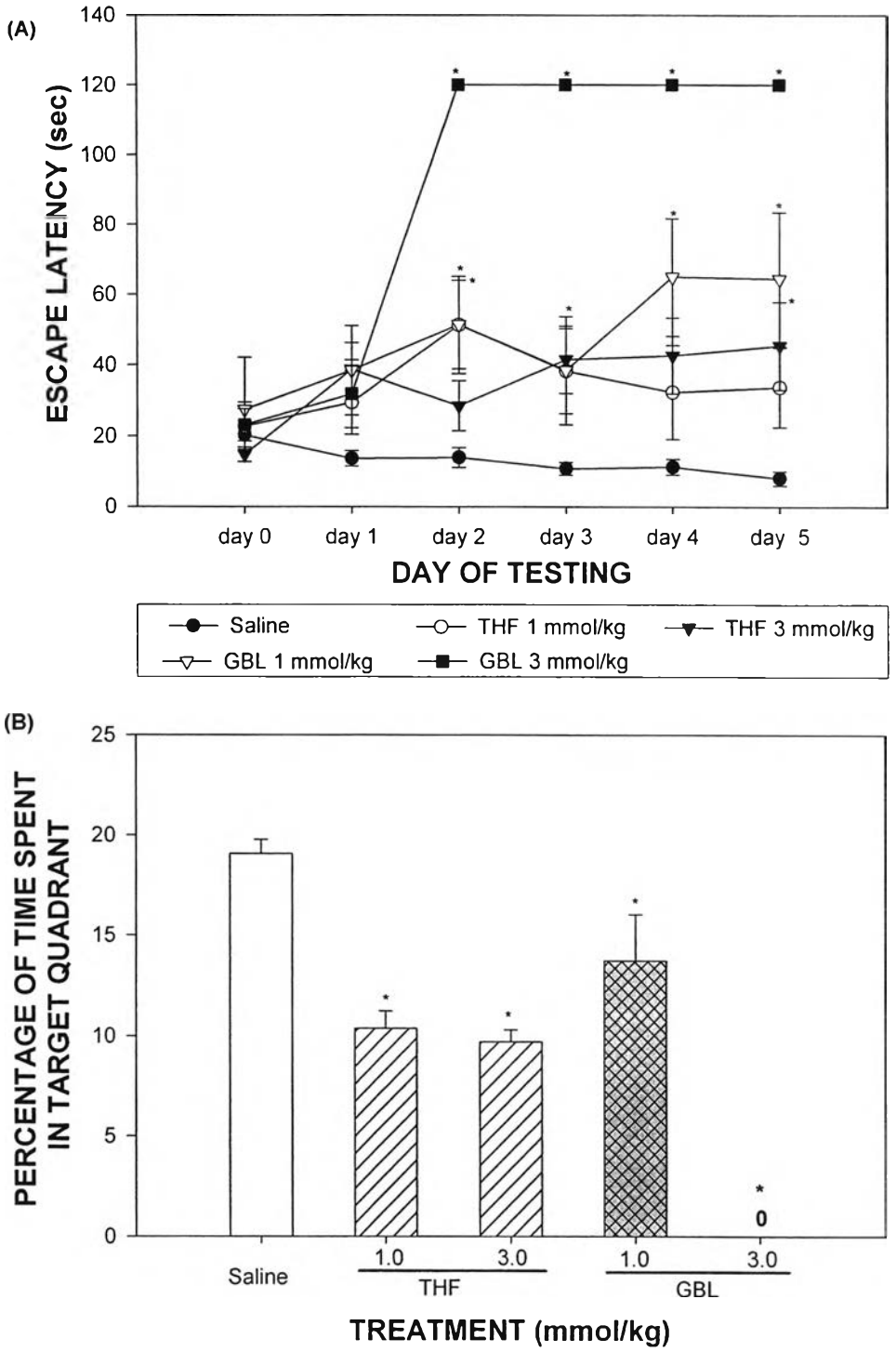


**FIGURE 4-9 Effects of Chronic THF and GBL Treatments on Memory Retrieval as Determined by the Morris Water Maze Test.**

After the learning period (5-day training in a water maze task), mice received an intraperitoneal injection of normal saline, THF (1 and 3 mmol/kg), or GBL (1 and 3 mmol/kg), daily for a period of 6 consecutive days. Thirty minutes after respective i.p. injections, they were subjected to the water maze test, each trial comprising of 120 seconds, everyday for 5 consecutive days. The probe test (90 seconds trial) was performed on day 6.

(A) Comparative effects of chronic THF and GBL treatments on the latency time. Values in day 0 represent baseline escape latencies after the learning period.

(B) Comparative percentage of the time spent in target quadrant.

Each point represents the mean $\pm$ S.E.M. from a group of 8 mice.

* denotes significant difference in comparison with the normal saline-treated group ($p<0.05$).

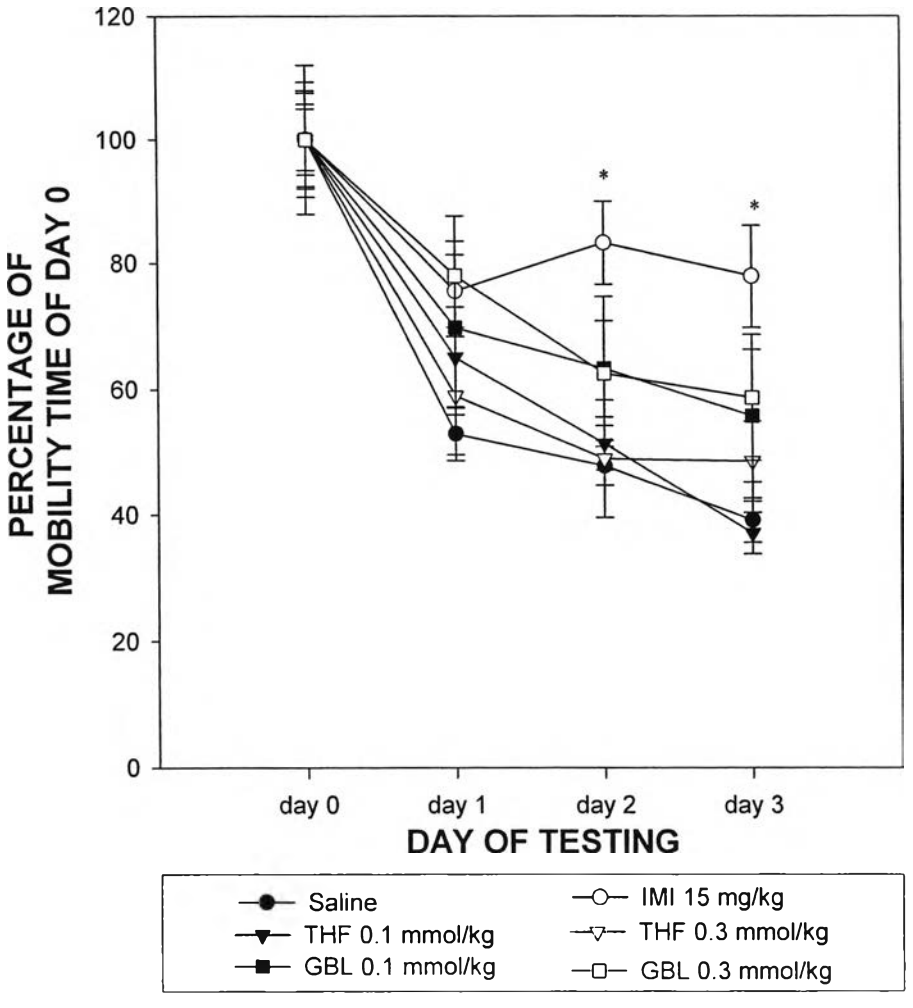


**FIGURE 4-10 Effects of THF and GBL Treatments on the Open-Space Swimming Test.**

Mice were tested on day 0 in the open-space swimming test by placing individually in a circular pool for 15-min session and recording the percentage of mobility time. Thereafter, imipramine (IMI; 15 mg/kg × 3 injections per day, i.p.), THF (0.1 mmol/kg and 0.3 mmol/kg × 3 injections per day, i.p.), or GBL (0.1 mmol/kg and 0.3 mmol/kg × 3 injections per day, i.p.), were administered during which the same procedure of open-space swimming trials was conducted for 3 consecutive days.

Each point represents the mean ± S.E.M. from a group of 8 mice.

* denotes significant difference in comparison with the normal saline-treated group ($p<0.05$).

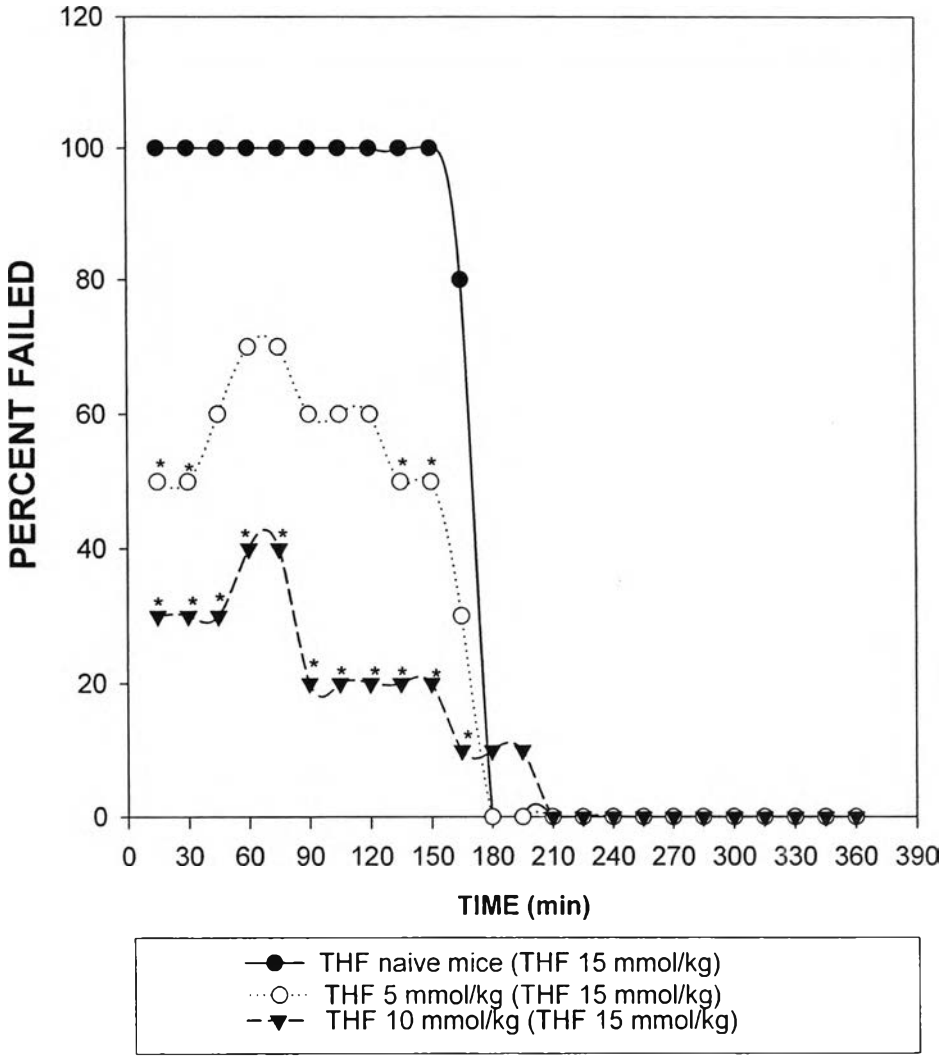


**FIGURE 4-11 Induction of Tolerance to Chronic THF Treatment as Determined by the Righting Reflex Test.**

Groups of 10 mice received THF injections (5 mmol/kg, i.p. and 10 mmol/kg, i.p.) once daily for a total period of 14 consecutive days. Control group (n=10) received normal saline (THF naïve group) for the same period. On day 15, all mice were challenged with THF (15 mmol/kg) and continuously evaluated by the righting reflex test until recovery.

Values represent the percentage of mice that lost the righting reflex.

* denotes significant difference in comparison with the THF naïve group ($p<0.05$) as determined by the Fisher exact two-tailed test.

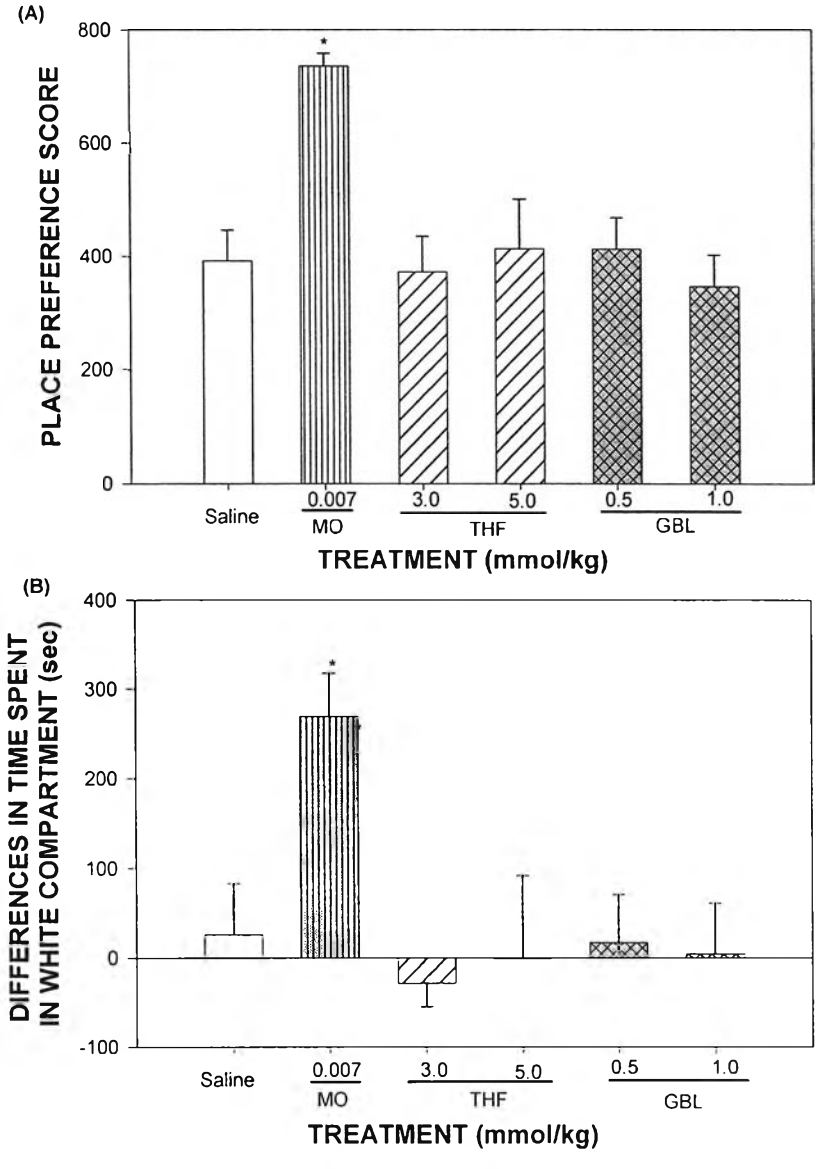


**FIGURE 4-12 Effects of THF and GBL on the Conditioned Place Preference Test.**

(A) Comparative place preference scores after conditioning with morphine (5 mg/kg, s.c.), THF (3 and 5 mmol/kg, i.p.), or GBL (0.5 and 1 mmol/kg, i.p.), in the conditioned place preference test.

(B) Comparative differences in the time spent in the drug-paired compartment after conditioning with morphine (5 mg/kg, s.c.), THF (3 and 5 mmol/kg, i.p.), or GBL (0.5 and 1 mmol/kg, i.p.), in the conditioned place preference test.

Each point represents the mean ± S.E.M. from a group of 6 mice.

* denotes significant difference in comparison with the normal saline-treated group ($p<0.05$).

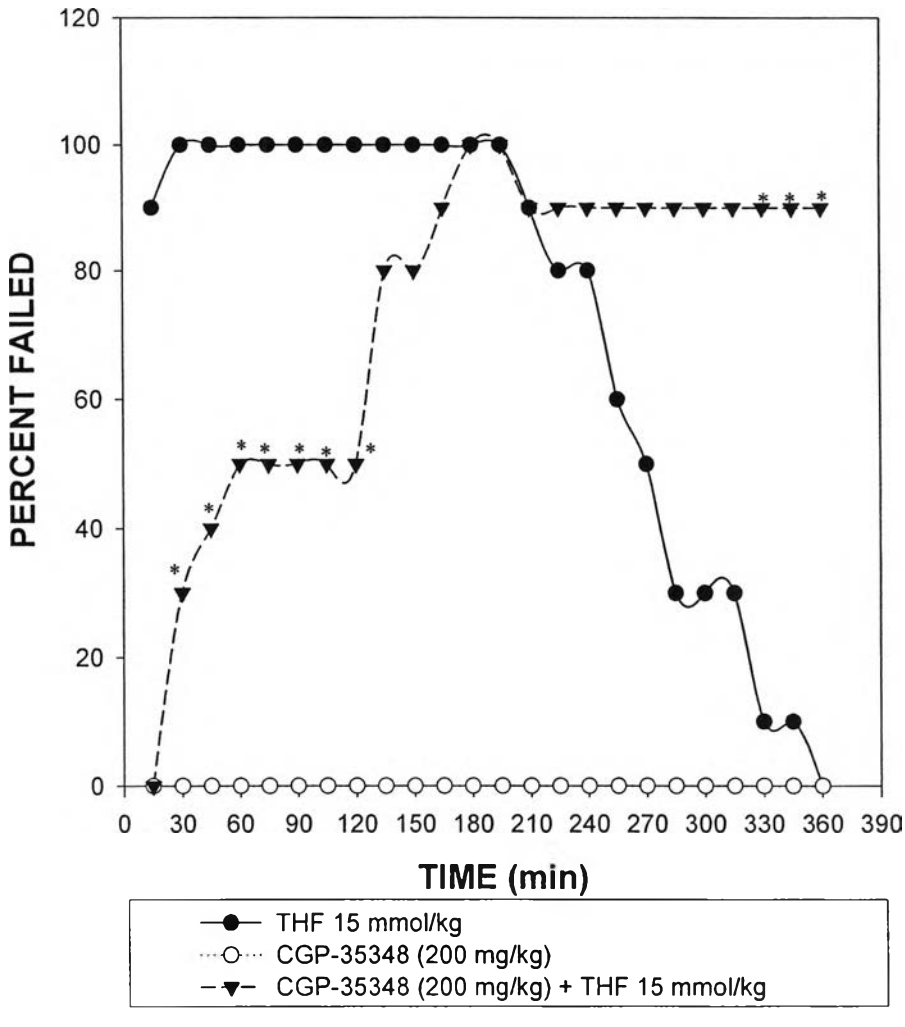


**FIGURE 4-13 Effect of Pretreatment with CGP-35348, a $GABA_B$ Receptor Antagonist, on THF-Induced Impairment of the Rotarod Test.**

Groups of 10 mice received either normal saline i.p. or CGP-35348 (200 mg/kg, i.p.). Fifteen minutes later, they were administered with THF 15 mmol/kg, i.p. (except one group receiving CGP-35348 was followed 15 min later by normal saline, i.p., to serve as a control group). The performance in rotarod test was repeatedly monitored for 6 h.

Values represent the percentage of mice that failed to perform the rotarod test.

* denotes significant difference in comparison with the THF treated group ($p<0.05$) as determined by the Fisher exact two-tailed test.

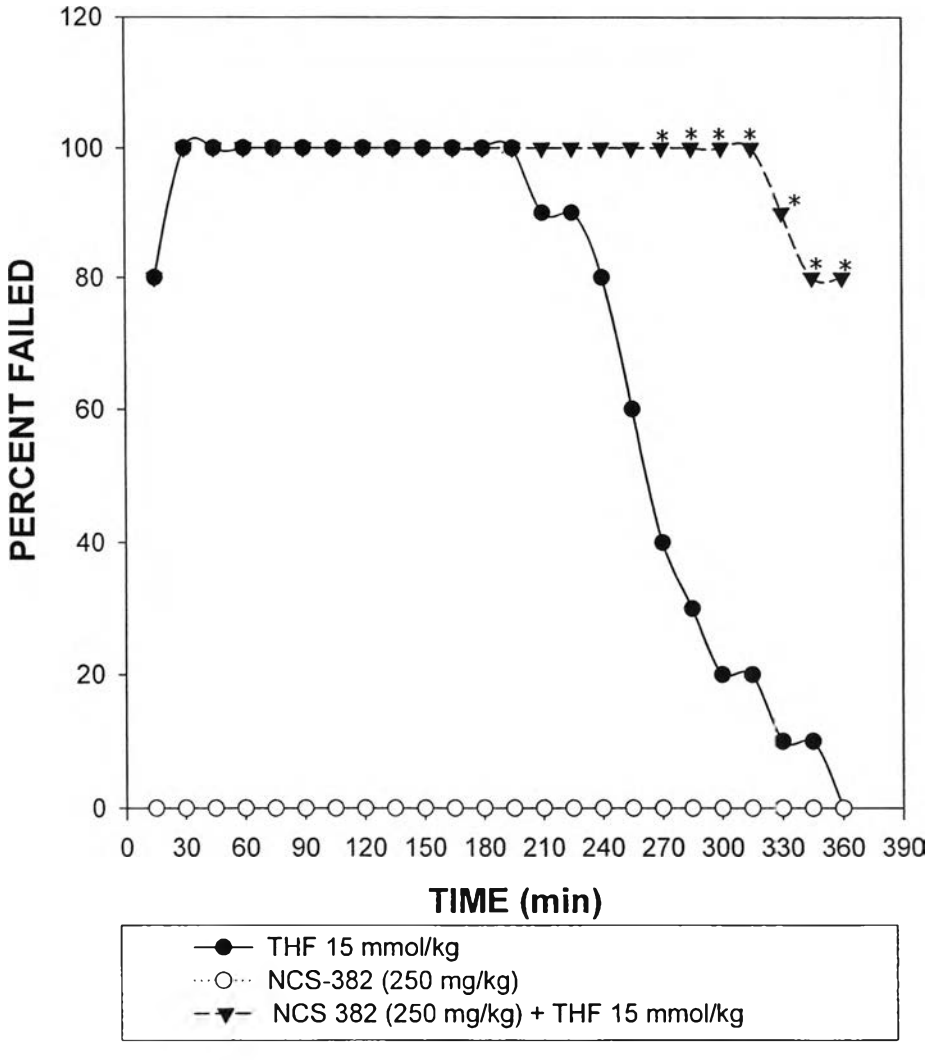


**FIGURE 4-14 Effect of Pretreatment with NCS-382, a GHB Specific Receptor Antagonist, on THF-Induced Impairment of the Rotarod Test.**

Groups of 10 mice received either normal saline i.p. or NCS-382 (250 mg/kg, i.p.). Fifteen minutes later, they were administered with THF 15 mmol/kg, i.p. (except one group receiving NCS-382 was followed 15 min later by normal saline, i.p., to serve as a control group). The performance in rotarod test was repeatedly monitored for 6 h.

Values represent the percentage of mice that failed to perform the rotarod test.

* denotes significant difference in comparison with the THF treated group ($p<0.05$) as determined by the Fisher exact two-tailed test.

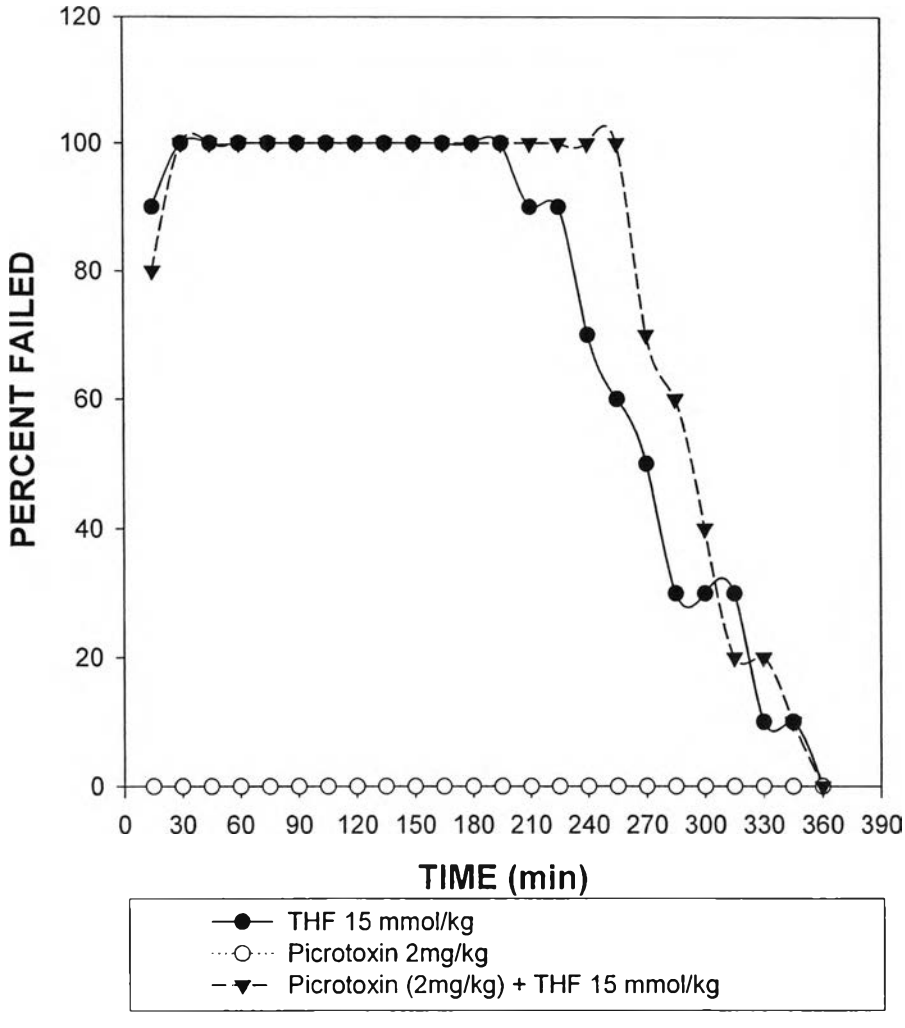


**FIGURE 4-15 Effect of Pretreatment with Picrotoxin, a $GABA_A$ Receptor Antagonist, on THF-Induced Impairment of the Rotarod Test.**

Groups of 10 mice received either normal saline i.p. or picrotoxin (2 mg/kg, i.p.). Fifteen minutes later, they were administered with THF 15 mmol/kg, i.p. (except one group receiving picrotoxin was followed 15 min later by normal saline, i.p., to serve as a control group). The performance in rotarod test was repeatedly monitored for 6 h.

Values represent the percentage of mice that failed to perform the rotarod test.

* denotes significant difference in comparison with the THF treated group ($p<0.05$) as determined by the Fisher exact two-tailed test.

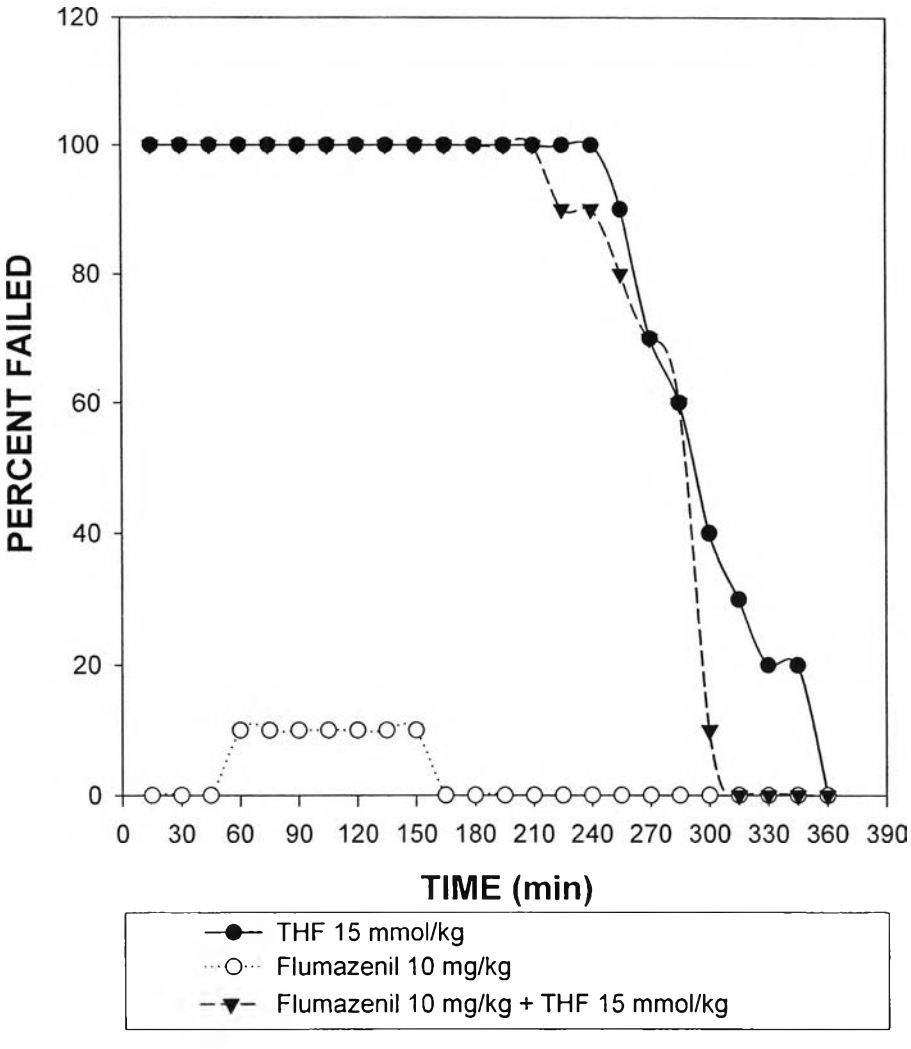


**FIGURE 4-16 Effect of Pretreatment with Flumazenil, a $GABA_A$ Receptor Antagonist, on THF-Induced Impairment of the Rotarod Test.**

Groups of 10 mice received either normal saline i.p. or flumazenil (10 mg/kg, i.p.). Fifteen minutes later, they were administered with THF 15 mmol/kg, i.p. (except one group receiving flumazenil was followed 15 min later by normal saline, i.p., to serve as a control group). The performance in rotarod test was repeatedly monitored for 6 h.

Values represent the percentage of mice that failed to perform the rotarod test.

* denotes significant difference in comparison with the THF treated group ($p<0.05$) as determined by the Fisher exact two-tailed test.