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Effect of Calmodulin Antagonists on Calcium and Ethanol-Induced Sleeping Time in Mice

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SUTOO, D., K. AKIYAMA AND K. IIMURA. *Effect of calmodulin antagonists on calcium and ethanol-induced sleeping time in mice.* PHARMACOL BIOCHEM BEHAV **23**(4) 627–631, 1985.—This investigation was carried out to determine if calcium prolongation of ethanol-induced sleep is mediated by calmodulin and a calmodulin-dependent protein kinase. The duration of ethanol-induced sleeping time in ddY male mice was measured following the administration of $CaCl_2$ (20, 40, 80 and 200 μmol/kg, intraperitoneally (IP)) both with and without the calmodulin antagonists, W-7: [N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide] (4.2 μg/mouse, intraventricular (IVT)) or trifluoperazine (TFP; 1.8 μg/mouse, IVT). When $CaCl_2$ was administered in a dose dependent manner the duration of ethanol-induced sleep was prolonged. The prolongation was antagonized by W-7 and TFP. When mice were treated with W-7 or TFP together with serotonin (5-HT; 15 nmol/mouse, IVT), dopamine (DA; 30 nmol/mouse, IVT) or norepinephrine (NE; 30 nmol/mouse, IVT), the sleeping time induced by ethanol and calcium was enhanced. This finding suggests that W-7 and TFP selectively inhibit the synthesis of 5-HT, DA and NE, but they do not affect other neuronal functions of these biogenic amines. The results would suggest a probable mechanism in which Ca^{++} prolongs ethanol-induced sleeping time by activating tyrosine hydroxylase and tryptophan hydroxylase through intracerebral calmodulin and calmodulin-dependent protein kinase, which subsequently raise the levels of 5-HT, DA and NE.

Sleeping time Ethanol Calcium Calmodulin W-7: [N-(6-Aminohexyl)-5-chloro-1-naphthalene-sulfonamide] Trifluoperazine Serotonin Dopamine Norepinephrine

A number of workers have supported the view that biogenic amines may be involved in the action of ethanol derivatives [10,11]. Furthermore Rosenfeld [18,19] reported an increase in ethanol-induced sleep in mice pretreated with either serotonin (5-HT) or dopamine (DA). Additionally, the duration of ethanol-induced sleep in mice has been shown to increase by the administration of norepinephrine (NE) [7] or L-3,4-dihydroxyphenylalanine (L-DOPA) [2]. Moreover, Petersen *et al.* [17] has described an enhancement in ethanol-induced sleep after raising the levels of biogenic amines in the brain by monoamine oxidase inhibitors.

In addition to these findings, workers have observed that the duration of ethanol-induced sleep in mice and rats is prolonged by the administration of divalent cations such as Ca^{++}, Mn^{++}, Zn^{++} and Cd^{++} [8, 9, 13]. This ability of divalent cations to prolong ethanol-induced sleeping time was markedly increased by Ca^{++} ionophores. But this effect is reduced to the original duration by the intraventricular (IVT) injection of a chelating agent [13,21]. Earlier we reported that prolongation of ethanol-induced sleep by calcium ions was antagonized by the administration of the inhibitor of the 5-HT synthesizing enzyme, p-chlorophenylalanine (PCPA), and catecholamine synthesizing enzyme blockers, α-methyltyrosine (αMPT) and diethyldithiocarbamate (DDC). Therefore, we suggest that the increase in ethanol-induced sleeping time due to calcium results from the increase in biogenic amines in the brain [20].

Recently brain slice tests have suggested that tyrosine hydroxylase (T-OH) [5] and tryptophan hydroxylase (TRP-OH) [3, 4, 12] activities may be regulated by a calmodulin-dependent protein kinase through calcium ions. Based on these findings it seems possible that the prolongation of the ethanol-induced sleeping time induced by calcium ions results from an increase in T-OH or TRP-OH activities in the brain through the action of a calcium-calmodulin-dependent protein kinase.

The present study was carried out in order to clarify how calcium is able to prolong ethanol-induced sleeping time. For this purpose the calmodulin antagonists, W-7: [N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide] and trifluoperazine (TFP), were administered to mice in order to inhibit the T-OH and TRP-OH activities in the brain; the sleeping time was then measured following the administration of ethanol, both with and without $CaCl_2$.

METHOD

Male mice of the ddY strain were obtained from Doken (Ibaraki, Japan). They were housed in groups of 8–10 in stainless steel cages at room temperature (22 ± 2°C) for more than one week before use in the experiments and were kept on a 12/12 hour light/dark cycle. The mice weighed 20–25 g (approximately 35 days old) at the start of the experiment and all animals were used only once.

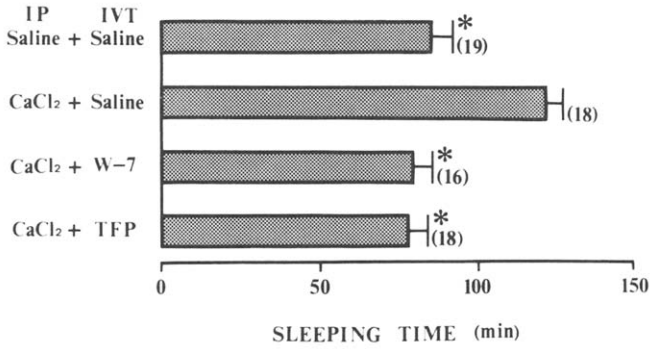

FIG. 1. Effect of W-7 and TFP on calcium and ethanol-induced sleeping time in mice. Saline or $CaCl_2$ (40 μmol/kg) was injected IP one hr before the injection of ethanol (4.5 g/kg, IP), and saline, W-7 (4.2 μg/mouse) or TFP (1.8 μg/mouse) was injected IVT 15 min before the injection of ethanol. Data shown as means ± S.E. (N). *Significantly different from $CaCl_2$ plus saline ($p<0.01$; Dunnett's t-test).

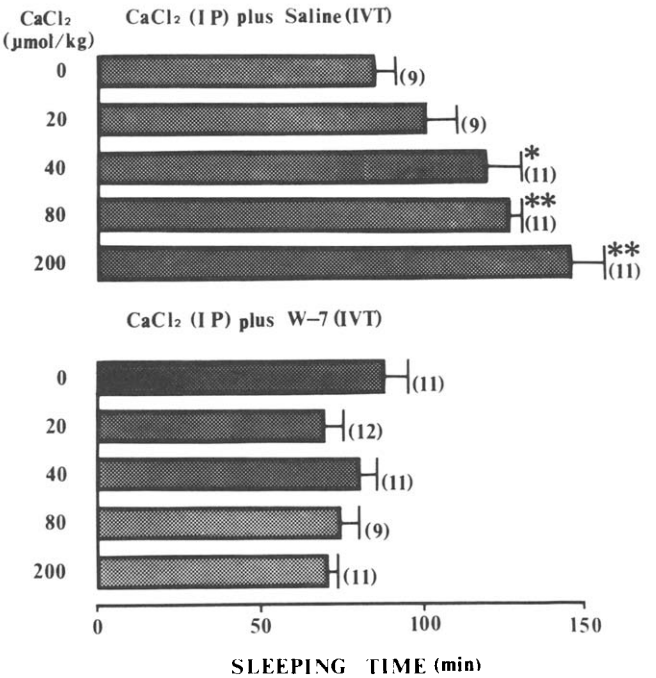

FIG. 2. Effect of W-7 on the prolongation of ethanol-induced sleeping time by each dose of $CaCl_2$. Mice were injected with $CaCl_2$ (IP) and W-7 (4.2 μg/mouse, IVT) 1 hr and 15 min, respectively, before the injection of ethanol (4.5 g/kg, IP). Data shown as means ± S.E. (N). *$p<0.05$; **$p<0.01$ when compared with saline (IP) plus saline (IVT) by Dunnett's t-test.

The drug and chemical sources were as follows: ethanol (99.5%, v/v), calcium chloride, L-DOPA, DA hydrochloride, NE, Nakarai Chemicals, Ltd. (Kyoto, Japan); 5-HT creatinine sulfate, Merck and Co., Inc. (West Point, PA); W-7, Seikagaku Kogyo Co. Ltd. (Tokyo, Japan); TFP dihydrochloride, Sigma Chemical Co. (St. Louis, MO). All drugs and chemicals were dissolved in physiological saline.

Intraventricular injections were performed in conscious mice as described by Clark *et al.* [6] using an injection volume of 5 μl/mouse. A dose of 4.2 μg/mouse of W-7 or 1.8 μg/mouse of TFP was injected IVT. These doses were chosen because the concentration of W-7 required to displace 50% of the labeled W-7 from calmodulin or to produce 50% inhibition of phosphodiesterase activation has been shown to be 31 μM and 28 μM respectively for W-7 [15]. The brain volume of the mice in this study was measured to be about 0.37 ml. The concentration of W-7 attained was approximately 30 μM, when diffused uniformly throughout the brain. The dose of TFP was calculated from the concentration required to produce a 50% inhibition of phosphodiesterase activity (10 μM) [16] when taking into consideration the volume of the mouse brain. Fifteen nmol/mouse of 5-HT, DA or NE, and 30 nmol/mouse of L-DOPA, DA or NE was injected IVT. These dosages were based on studies by Cott *et al.* [7] and Yamamoto *et al.* [21]. Ethanol (30%, v/v) was injected at a dose of 4.5 g/kg together with 20, 40, 80 or 200 μmol/kg $CaCl_2$ by the intraperitoneally (IP) route. In our previous study, $CaCl_2$ was administered intravenous (IV) in contrast to the IP injection used in the present study. This change in the route of $CaCl_2$ administration was intended to reduce the excessive loading of the heart with a high concentration of $CaCl_2$. In a pilot test, a markedly higher effect was obtained by IV injection of $CaCl_2$ at the same dose.

Sleeping time duration is defined as the time from the loss of the righting reflex to the time when the righting reflex occurs twice within 30 sec; room temperature, extraneous sounds, food intake, and ethanol concentrations were carefully controlled.

The effects of the various drugs on ethanol-induced sleeping time were compared between groups of mice pretreated with the test substances, which were given either IP or IVT prior to the injection of the ethanol. Injections made by the IP route were made 1 hr prior to ethanol injection and those given IVT received the injection 15 min before the ethanol. The groups were as follows: (a) after saline was injected both IP and IVT; (b) after administration of $CaCl_2$ (40 μmol/kg, IP) and saline (IVT); (c) after administration of $CaCl_2$ (40 μmol/kg, IP) and W-7 (IVT); and (d) after the administration of $CaCl_2$ (40 μmol/kg, IP) and TFP (IVT).

Also, ethanol-induced sleeping time was measured following the administration of 20, 40, 80 or 200 μmol/kg of $CaCl_2$ both with and without W-7. Mice were given $CaCl_2$ (IP) and saline (IVT), or $CaCl_2$ (IP) and W-7 (IVT) for 1 hr and 15 min, respectively, prior to the administration of ethanol.

Additionally, the duration of ethanol-induced sleeping time was compared between groups of mice pretreated with 40 μmol/kg $CaCl_2$, a calmodulin antagonist and biogenic amine using the injection schedule stated above. Groups of mice were given: (a) saline (IP and IVT); (b) $CaCl_2$ (IP) and saline (IVT); (c) saline (IP) and 5-HT (15 nmol/mouse, IVT); (d) saline (IP) and L-DOPA (30 nmol/mouse, IVT); (e) saline (IP) and DA (15 nmol/mouse, IVT); (f) saline (IP) and DA (30 nmol/mouse, IVT); (g) saline (IP) and NE (15 nmol/mouse, IVT); (h) saline (IP) and NE (30 nmol/mouse, IVT); (i) $CaCl_2$ (IP) and W-7 (IVT); (j) $CaCl_2$ (IP) and a solution consisting of W-7 and 15 nmol/mouse 5-HT (IVT); (k) $CaCl_2$ (IP) and mixture solution consisting of W-7 and 30 nmol/mouse DA (IVT); (l) $CaCl_2$ (IP) and mixture solution consisting of W-7 and 30 nmol/mouse NE (IVT); (m) $CaCl_2$ (IP) and TFP (IVT); (n) $CaCl_2$ (IP) and a solution consisting of TFP and 15 nmol/mouse 5-HT (IVT); (o) $CaCl_2$

TABLE 1

EFFECT OF CALMODULIN ANTAGONISTS AND BIOGENIC AMINES ON ETHANOL-INDUCED SLEEPING TIME IN MICE

Treatment	Dose	N	Sleeping Time (min ± S.E.)	$p^{\S}$
IP, IVT				
Saline, Saline		11	88.36 ± 7.24	
$CaCl_2$, Saline	40 μmol/kg	11	131.27 ± 3.58	<0.01*
Saline, 5-HT	15 nmol/mouse	11	125.51 ± 8.36	<0.01*
Saline, L-DOPA	30 nmol/mouse	11	103.26 ± 7.28	NS*
Saline, DA	15 nmol/mouse	11	91.35 ± 8.29	NS*
	30 nmol/mouse	11	123.47 ± 6.45	<0.01*
Saline, NE	15 nmol/mouse	11	103.79 ± 8.92	NS*
	30 nmol/mouse	11	120.90 ± 7.60	<0.05*
$CaCl_2$, W-7	40 μmol/kg, 4.2 μg/mouse	11	82.48 ± 7.38	
$CaCl_2$, W-7 + 5-HT	40 μmol/kg, 4.2 μg/mouse + 15 nmol/mouse	12	111.79 ± 7.36	<0.05†
$CaCl_2$, W-7 + DA	40 μmol/kg, 4.2 μg/mouse + 30 nmol/mouse	11	105.69 ± 6.51	<0.05†
$CaCl_2$, W-7 + NE	40 μmol/kg, 4.2 μg/mouse + 30 nmol/mouse	10	130.80 ± 9.17	<0.01†
$CaCl_2$, TFP	40 μmol/kg, 1.8 μg/mouse	9	80.22 ± 5.50	
$CaCl_2$, TFP + 5-HT	40 μmol/kg, 1.8 μg/mouse + 15 nmol/mouse	12	104.58 ± 6.76	<0.05‡
$CaCl_2$, TFP + DA	40 μmol/kg, 1.8 μg/mouse + 30 nmol/mouse	11	102.28 ± 5.92	<0.05‡
$CaCl_2$, TFP + NE	40 μmol/kg, 1.8 μg/mouse + 30 nmol/mouse	12	123.37 ± 5.00	<0.01‡

Mice were injected IP and IVT 1 hr and 15 min, respectively, before the injection of ethanol (4.5 g/kg, IP).

* Compared to saline plus saline.

† Compared to $CaCl_2$ plus W-7.

‡ Compared to $CaCl_2$ plus TFP.

§ By Dunnett's *t*-test.

NS = not significant ($p>0.05$).

(IP) and a solution consisting of TFP and 30 nmol/mouse DA (IVT); and (p) $CaCl_2$ (IP) and a solution consisting of TFP and 30 nmol/mouse NE (IVT).

One-way analysis of variance was performed for each experiment as follows: (1) effect of saline plus saline, $CaCl_2$ plus saline, $CaCl_2$ plus W-7 and $CaCl_2$ plus TFP on ethanol-induced sleeping time (Fig. 1); (2) effect of various dosages of $CaCl_2$ on ethanol-induced sleeping time (top in Fig. 2); (3) effect of W-7 on the prolongation of ethanol-induced sleeping time by each dose of $CaCl_2$ (bottom in Fig. 2); (4) effect of saline plus saline, $CaCl_2$ plus saline and saline plus biogenic amine on the ethanol-induced sleeping time (top in Table 1); (5) effect of $CaCl_2$ plus W-7 and $CaCl_2$ plus W-7 plus biogenic amine on the ethanol-induced sleeping time (middle in Table 1); and (6) effect of $CaCl_2$ plus TFP and $CaCl_2$ plus TFP plus biogenic amine on the ethanol-induced sleeping time (bottom in Table 1). As F values of (1), (2), (4), (5) and (6) indicated significance, data from these animals were further analyzed using Dunnett's *t*-test for multiple comparisons. A probability of 0.05 or less was considered significant.

RESULTS

As can be seen in Fig. 1, IP injection of $CaCl_2$ at a dose of 40 μmol/kg increased ethanol-induced sleeping time by approximately 40% when compared to the saline group in mice. This finding is in agreement with the previous observations that IVT or IV administration of $CaCl_2$ enhances ethanol-induced sleep [8,20]. In contrast to this, when W-7 or TFP was IVT injected after IP administration of 40 μmol/kg $CaCl_2$, the enhancing effect of $CaCl_2$ on ethanol-induced sleep was not enhanced.

In Fig. 2, the effects of various concentrations of Ca^{++} on the ethanol-induced sleeping time in the presence and in the absence of W-7 are compared. Pretreatment of mice with $CaCl_2$ increased, in a dose-dependent manner, the duration of sleeping time. A dose of 20 μmol/kg, 40 μmol/kg, 80 μmol/kg or 200 μmol/kg prolonged sleep by approximately 20%, 40% ($p<0.05$), 50% ($p<0.01$) or 70% ($p<0.01$), respectively, when compared to saline. This result agrees with a report where mice were treated IVT with $CaCl_2$ [8]. On the other hand, ethanol-induced sleep was not prolonged at any dose of $CaCl_2$ when IP injection of $CaCl_2$ was followed by IVT administration of W-7.

As can be seen in Table 1, ethanol-induced sleeping time of mice treated IVT with 5-HT (15 nmol/mouse), DA (30 nmol/mouse) or with NE (30 nmol/mouse) was significantly enhanced (40% longer than controls). However, the sleeping

time induced by ethanol was not affected by the IVT injection of L-DOPA (30 nmol/mouse), the injection of DA (15 nmol/mouse), or 15 nmol/mouse of NE. Furthermore, when mice were treated IVT with both W-7 and 5-HT (15 nmol/mouse), W-7 and DA (30 nmol/mouse) or W-7 and NE (30 nmol/mouse), the sleeping time induced by ethanol and calcium was enhanced by 35% ($p<0.05$), 30% ($p<0.05$) or 60% ($p<0.01$), respectively, over those given only W-7. Also, ethanol and calcium induced sleeping time following IVT injection of TFP and 5-HT, TFP and DA, or TFP and NE were enhanced significantly by 30% ($p<0.05$), 30% ($p<0.05$) or 55% ($p<0.01$), respectively, when compared to mice treated with TFP alone.

DISCUSSION

The interrelation between ethanol-induced sleep and the levels of biogenic amines in the brain has been extensively investigated. These studies have shown that the administration of monoamine oxidase inhibitors will increase the levels of 5-HT, DA and NE, and will also prolong ethanol-induced sleep [17]. Also, it has been reported that the administration of 5-HT, DA or NE to mice will increase ethanol-induced sleep [7, 18, 19]. Moreover, the administration of L-DOPA to mice has been said to markedly increase ethanol-induced sleep and to raise the DA level in the brain [2].

As for the effects of divalent cations, Ca^{++}, Mn^{++}, Zn^{++} or Cd^{++} have been demonstrated to increase ethanol-induced sleep if the ion is injected IVT, IP or IV [8, 9, 13, 20]. We have been investigating the mechanism by which divalent cations enhance ethanol-induced sleep in relation to the biogenic amine level in the brain. On the basis of our findings in a previous study [20], we have suggested that Ca^{++} facilitates the synthesis of 5-HT, DA and NE together with which increases their biogenic amine release, and ethanol-induced sleep is consequently enhanced. The present study has been carried out to examine whether the ethanol-induced sleep which can be enhanced by Ca^{++} is mediated by calmodulin and a calmodulin-dependent protein kinase.

In the present study, W-7 and TFP were used as calmodulin antagonists. Although more than several dozen drugs have been reported as calmodulin antagonists, W-7 is the most suitable compound because of its very high affinity for calmodulin and small toxicity to other cellular constituents [1]. TFP is a well-known inhibitor which has long been used in pharmacological studies. However this compound is known to affect not only calmodulin but also other cell membranes. The IC_{50} volume for the phosphodiesterase activation by W-7 has been reported to be 28 μM, while that for the displacement of ^3H W-7 from calmodulin is 31 μM [15]. In the present experiments, since the brain volume of the mice used was about 0.37 ml, 4.2 μg of W-7 was IVT injected into each mouse. This volume of the W-7 solution should produce a concentration of about 30 μM when uniformly diffused throughout the brain. Since the IC_{50} volume for the inhibitory action of TFP on phosphodiesterase is 10 μM [16], 1.8 μg of TFP was IVT injected into each mouse based also on the brain volume observed.

In this present study, ethanol-induced sleep was increased by $CaCl_2$; however when mice were given IVT W-7 or TFP, sleep was not enhanced by administration of $CaCl_2$. This result indicates that Ca^{++}-sensitive ethanol-induced sleep is being blocked by calmodulin antagonists. Combining the above results with our previous findings [20] that the administration of PCPA, an inhibitor of the 5-HT synthesizing enzyme, or αMPT and DDC, inhibitors of catecholamine synthesizing enzymes such as those for DA, NE, etc., abolished the enhancement by Ca^{++} of ethanol-induced sleep, we suggest the following mechanism. Calcium ions activate T-OH and TRP-OH via cerebral calmodulin and calmodulin-dependent protein kinase; this then causes a rise in the levels of 5-HT, NE and DA. As a result, ethanol-induced sleep is enhanced by Ca^{++}.

This model is supported by reports that show T-OH and TRP-OH can be activated by Ca^{++}. Experiments with brain slices have shown that the activity of TRP-OH is increased when neuronal cells are depolarized by a high $[K^+]$ in the presence of Ca^{++} [3] or when the intracellular Ca^{++} concentration was raised by a Ca^{++} ionophor [12]. Since this activation of TRP-OH by depolarization was blocked by TFP, it seemed reasonable to conclude that a calmodulin-dependent protein kinase participates in this activation [4]. It is also known that the activity of T-OH in incubated brain slices can be enhanced by applying a high $[K^+]$ to cause neuronal depolarization in the presence of external Ca^{++}. When Ca^{++} was omitted from the incubation medium, the addition of high $[K^+]$ failed to induce the T-OH activation. Therefore it has been suggested that Ca^{++} is involved in the activation of T-OH [5]. In keeping with the line of evidence we have also reported that IV injection of $CaCl_2$ (20 μmol/kg) raised the cerebral level of DA by 25% [20]. However, our mechanism must include combination with monoamine release increase.

In this present study, when $CaCl_2$ was injected alone, ethanol-induced sleep was prolonged in a nearly dose-dependent manner, however, pretreatment of mice with both $CaCl_2$ and W-7 did not produce an increase in sleeping time at any of the doses used. This finding agrees with a reported property of calmodulin. Namely, the blocking action of a calmodulin antagonist can not be eliminated by raising the Ca^{++} concentration but can be eliminated by adding excessive amounts of calmodulin [14]. It may also be suggested from these results that calmodulin and calmodulin-dependent protein kinase participate in the ethanol-induced sleep which can be prolonged by Ca^{++}. Incidentally, a similar modification to the action of ethanol and Zn^{++}, Cd^{++} or Hg^{++} was found when pretreated with W-7, PCPA or αMPT (Sutoo, Akiyama and Iimura, submitted for publication).

The duration of ethanol-induced sleeping time, observed after the administration of $CaCl_2$ followed by the simultaneous injection of a calmodulin antagonist and a biogenic amine, is shown in Table 1. When 5-HT, DA or NE were IVT administered into mice without injecting $CaCl_2$, 5-HT (15 nmol/mouse), DA (30 nmol/mouse) and NE (30 nmol/mouse) were found to prolong significantly ethanol-induced sleeping time, however, at a lower dose DA (15 nmol/mouse) and NE (15 nmol/mouse) did not show a significant prolongation. This finding is in agreement with reported results [7,21]. However, L-DOPA (30 nmol/mouse) did not extend sleeping time when compared to control mice. But Blum *et al.* [2] injected 400 mg/kg of L-DOPA IP and observed marked prolongation of ethanol-induced sleeping time and raised DA levels in the whole brain as well. At the L-DOPA dose used in the present study, however, no significant change was observed. On the other hand, when W-7 or TFP was IVT administered together with 5-HT, DA or NE after $CaCl_2$ injection, ethanol-induced sleeping time was significantly prolonged compared to mice receiving W-7 or TFP

alone after $CaCl_2$ injection. This finding suggests that the prolongation of ethanol-induced sleeping time by Ca^{++} was at first blocked by W-7 or TFP and was subsequently increased by 5-HT, DA or NE. It is also suggested that W-7 and TFP selectively inhibit the synthesis of 5-HT, DA and NE, but they do not affect other neuronal functions of these biogenic amines.

The results and the above discussion of the present study would suggest a probable mechanism in which Ca^{++} prolongs ethanol-induced sleeping time by activating T-OH and TRP-OH through intracerebral calmodulin and calmodulin-dependent protein kinase which subsequently raise the levels of 5-HT, DA and NE.

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