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EFFECTS OF ACUTE AND CONTINUOUS PENTOBARBITAL ADMINISTRATION ON THE GAMMA-AMINOBUTYRIC ACID SYSTEM

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Abstract—Effects of acute anesthetic doses and chronic administration of pentobarbital on γ-aminobutyric acid (GABA) and glutamic acid levels in mouse brain have been investigated. Acute administration of pentobarbital caused an increase in the brain level of GABA which was associated with pentobarbital-induced narcosis. This was further substantiated by the finding that pentobarbital-induced sleeping time was prolonged when brain GABA level was elevated by the administration of either amino-oxyacetic acid (AOAA), an inhibitor of GABA-2-oxoglutarate aminotransferase (GABA-T), or glutamate, the precursor of GABA. In addition, the activity of L-glutamate-1-carboxylase (GAD) measured during pentobarbital-induced narcosis was higher than that of the control group. On the other hand, chronic administration of pentobarbital resulted in a decrease of both GABA and glutamate levels. There was a concomitant 30 per cent decrease in the activity of GAD. This was confirmed by the finding that the rate of brain GABA accumulation induced by AOAA administration in tolerant mice was slower than that of the non-tolerant animals. Brain GABA remained at significantly lower levels after an abrupt withdrawal from pentobarbital; however, brain glutamate levels showed no significant difference as compared to the control group. It appears that the GABA system in the central nervous system may be involved in barbiturate narcosis and further linked with the development of tolerance to barbiturate.

Ample evidence has been presented to suggest that γ-aminobutyric acid (GABA) is an important inhibitory synaptic transmitter in the central nervous system of vertebrates [1–9]. It has been shown that chronic administration of pentobarbital decreases the turnover of the metabolic pool of GABA [10]. However, contradictory data exist concerning the acute and chronic effects of barbiturates on brain GABA levels in rodents. Some investigators reported that brain GABA concentrations were increased [11], while some observed no change [10, 12, 13] and others reported decreased concentrations [14].

Pharmacological manipulations of the GABA system had suggested indirectly that GABA may be involved in barbiturate narcosis. The administration of amino-oxyacetic acid (AOAA), a potent inhibitor of GABA-2-oxoglutarate aminotransferase (GABA-T), potentiated the pentobarbital hypnosis [15]. Also, it was reported that both desipramine and pargyline elevated the brain concentration of GABA and prolonged barbital-induced narcosis [16].

In view of the inconsistent findings, we have investigated the effects of acute and chronic administration of pentobarbital on brain L-glutamate-1-carboxylase (GAD) activities and brain levels of GABA and glutamate. The results obtained on the effects of AOAA and glutamate on pentobarbital-induced narcosis and brain levels of GABA and glutamate also further substantiated the finding that the GABA system may be involved in barbiturate-induced narcosis.

METHODS AND MATERIALS

Male ICR mice weighing 24 ± 2 g (Charles River, Wilmington, MA) were used in the various experiments. Animals were maintained on standard laboratory chow and tap water and were housed in a room lighted artificially for 12 hr of the day. The chemicals and their suppliers were as follows: GABase (a partially purified cell-free preparation from *Pseudomonous fluorescens* containing GABA-T and succinic semialdehyde dehydrogenase), sodium pentobarbital and pyridoxal-5-phosphate from Sigma Chemical Co., St. Louis, MO; nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine nucleotide (NAD^+), reduced glutathione (GSH), AOAA, GABA, bovine serum albumin and L-glutamic acid from Cal-Biochem, La Jolla, CA; Aquasol and [U-^{14}C]glutamic acid from New England Nuclear, Boston, MA; and L-glutamate dehydrogenase from Boehringer Mannheim Corp., San Francisco, CA.

Determination of GABA and glutamate concentrations. Preparation of brain extracts and the fluorometric determination of GABA and glutamate levels were as described by Jakoby and Scott [17] and Graham and Aprison [18] with only minor modifications. Animals were decapitated and brains were removed and frozen immediately in crushed dry ice. After weighing each brain, it was homogenized in precooled 75% ethanol with a Polytron homogenizer. After centrifugation at 20,000 *g* for 30 min, the supernatants were dried under an air stream and then resuspended in water. The slightly cloudy suspension was recentrifuged in a model L5-65 ultracentrifuge at 100,000 *g* for 30 min. Aliquots (100 μl) of the supernatant were used for GABA and glutamate assays.

The levels of GABA were determined by the enzymatic fluorometric method [17, 18]. Briefly, the NADPH, formed in the presence of GABA and

α-ketoglutarate by GABase, is directly measured. The glutamate levels also were determined fluorometrically by measurement of NADH formation from L-glutamate and NAD^+ using a purified preparation of beef liver glutamate dehydrogenase.

Measurement of GAD activity. The activity of GAD (EC 4.1.1.15) was determined by measuring the $^{14}CO_2$ formation from [U-^{14}C]L-glutamic acid according to the method described by Roberts and Simonsen [19] with minor modifications. Each brain was homogenized with a glass homogenizer immediately in 5 ml of ice-cold 0.1 M potassium buffer, pH 6.5, containing 0.03% GSH at 4°. Each assay mixture (total volume 1.0 ml) in a 18 × 150 mm test tube contained 100 μmoles of potassium phosphate buffer, pH 6.5, 100 μmoles L-glutamate (0.9 μCi), 4 μmoles GSH and 1 μmole pyridoxl-5-phosphate. Two-tenth ml of 1 M hyamine hydroxide solution in methanol was placed in a plastic vial hung on the rubber stopper in the test tube. The reaction was started by injecting 2.5 mg of brain homogenate protein at 37°. After 30 min, 0.2 ml of 4 N H_2SO_4 was injected to stop the reaction and to release CO_2. After shaking another 90 min, the contents of the plastic vials were transferred to a liquid-scintillation-counting vial which contained 10 ml Aquasol. The enzyme activity in μmoles glutamate decarboxylated/30 min/100 mg of protein of brain homogenate was calculated from the $^{14}CO_2$ liberated from [U-^{14}C]L-glutamate. The protein content of brain homogenate was determined by the method of Lowry *et al.* [20] with crystalline bovine serum albumin as a standard.

Effect of acute treatment of pentobarbital on brain levels of GABA and glutamate and GAD activity. Mice were injected with sodium pentobarbital at either 37.5 or 75 mg/kg, i.p. Controls received saline. The sleeping time induced by pentobarbital was recorded. The duration of sleeping time was taken as the time between loss of the righting reflex and the time at which the animal righted itself. The brain levels of GABA and glutamate and GAD activity were determined at 0, 10, 30, 60 and 120 min after the administration of the drug.

Effect of AOAA on pentobarbital sleeping time and brain levels of GABA and glutamate. Mice were divided into three groups of 20 mice in each group. Two groups of mice received either 20 or 40 mg/kg (i.p.) of AOAA and the other group received saline as control. At the end of 2 hr, six mice from each group were sacrificed and the supernatant of the brain homogenate was prepared for GABA and glutamate assays. The rest of the animals from each group received sodium pentobarbital, 60 mg/kg, i.p. The sleeping time induced by pentobarbital was recorded.

Effect of glutamate on pentobarbital sleeping time and brain levels of GABA and glutamate. Sleeping time was measured after the i.p. injection of pentobarbital, 60 mg/kg, given 5 min after pretreatment with saline, 300 or 600 mg/kg of L-glutamic acid (i.p.), which was adjusted to pH 7.0 with 2 N sodium hydroxide. The brain levels of GABA and glutamate were determined at 0, 10, 30 and 60 min after the administration of glutamate or vehicle.

Chronic administration of pentobarbital by pellet implantation. Specially formulated pentobarbital pellets containing 75 mg of free acid were implanted for 3 days in mice as reported previously [21, 22]. The control animals were implanted with placebo pellets for the same period of time. This treatment conveys about 6-fold tolerance to the loss of righting reflex response to pentobarbital.

Effect of AOAA on brain GABA and glutamate levels in tolerant animals. Brain levels of GABA and glutamate were estimated at 0, 10, 30 and 60 min after the administration of AOAA, 20 mg/kg, i.p. Comparisons were carried out in mice implanted with pentobarbital and placebo pellets for a period of 72 hr.

Statistical tests for significance. Statistical analysis for significance was checked by the one-tail *t*-test and the P values are shown in the tables and figures.

RESULTS

Effect of acute pentobarbital administration on sleeping time, brain levels of GABA and glutamate, and the GAD activity in the mouse. In the animals given Na-pentobarbital, 37.5 or 75 mg/kg, i.p., the sleeping time of mice was 12.5 ± 2.1 or 59.8 ± 4.2 min respectively.

Acute pentobarbital administration increased brain GABA levels and reduced the levels of glutamate (Fig. 1). The brain GABA levels in the animals receiving sodium pentobarbital, 75 mg/kg, i.p., was increased significantly at 10 and 30 min but returned to control levels by 60 min, the same time that mice regain their righting reflex. On the other hand, the glutamate levels in mice receiving the same dose of pentobarbital were significantly lower in comparison with those of the control group at 10, 30 and 60 min after administration but returned to the control levels at 120 min. At 37.5 mg/kg of sodium pentobarbital administration, there was a significant increase in GABA levels

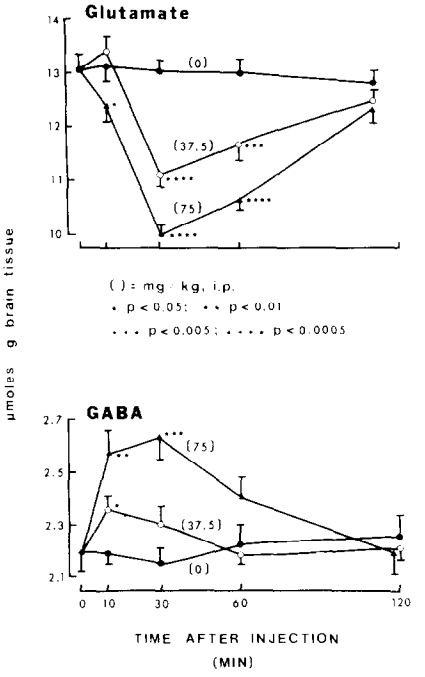

Fig. 1. Effect of acute pentobarbital administration on brain GABA and glutamate levels. Sodium pentobarbital was dissolved in saline. The animals given saline alone were used as the control. Values shown in the figure are means from four mice ±S.E.

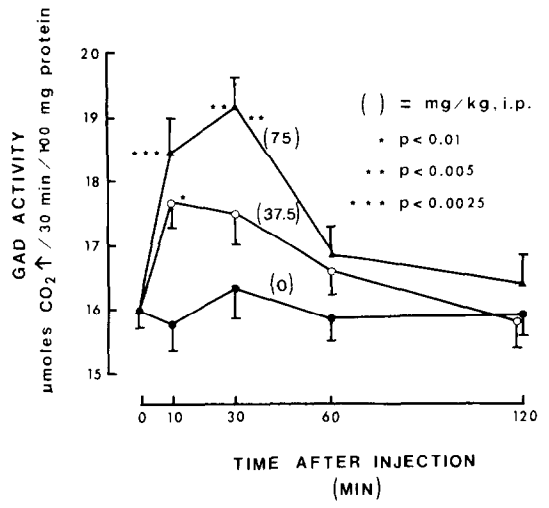

Fig. 2. Effect of acute pentobarbital administration on GAD activity. Values shown in the figure are means from four mice ±S. E.

at 10 min with a return to control by 30 min. However, the glutamate levels were decreased significantly at 10, 30 and 60 min in comparison with the saline control group. The values returned to the control level at 120 min. Measurement of GAD activities at the same time intervals after the administration of sodium pentobarbital showed that the increase of GAD activity corresponded to the increase of GABA levels (Fig. 2).

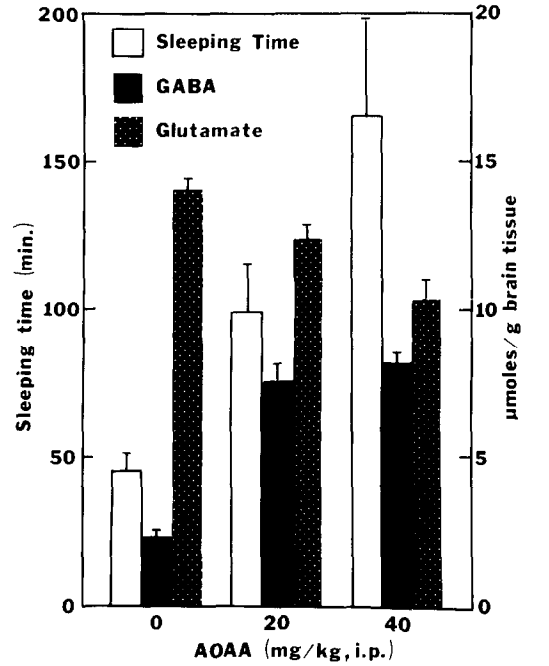

Fig. 3. Effect of AOAA on pentobarbital-induced narcosis and brain levels of GABA and glutamate. AOAA, 0, 20 or 40 mg/kg, i.p., was given 2 hr before a challenge dose of sodium pentobarbital, 60 mg/kg, i.p. Sleeping times shown in the figure are means from fourteen mice ± S. E.; brain levels of GABA and glutamate are from six mice ±S. E.

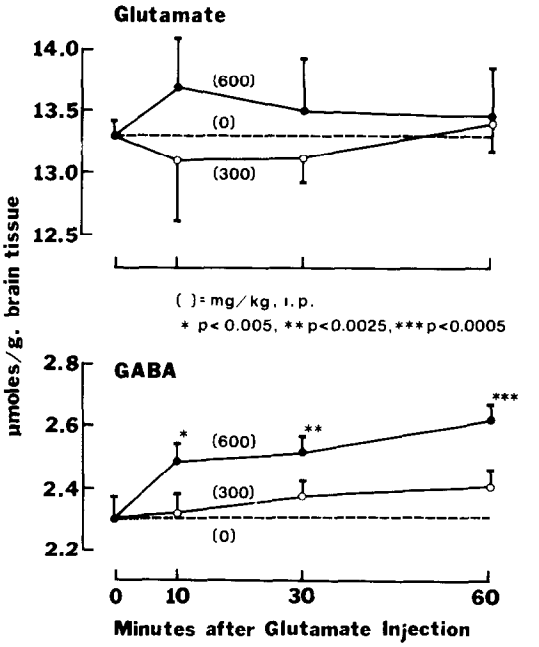

Fig. 4. Effect of glutamate administration on brain GABA and glutamate levels. Values shown in the figure are means of four mice ±S. D. The animals treated with saline were used as the control.

Effect of AOAA on pentobarbital-induced narcosis and brain levels of GABA and glutamate. AOAA, an inhibitor of GABA-T, prolonged the pentobarbital sleeping time (Fig. 3) at both doses tested when administered 2 hr prior to the pentobarbital challenge. Brain GABA levels increased (227 and 250 per cent) and glutamate levels decreased (12 and 26 per cent) after AOAA pretreatment.

Effect of glutamate administration on pentobarbital narcosis and brain GABA and glutamate levels. The data in Table 1 demonstrate that the larger dose of glutamate (600 mg/kg) given 5 min before the challenge of pentobarbital significantly increased sleeping time by 75 per cent. The lower dose of glutamate had no significant effect.

Acute administration of glutamate, 600 mg/kg, i.p., increased the level of brain GABA, as shown in Fig. 4. A significant increase was evidenced as early as 10 min. The level of GABA remained elevated at 1 hr after administration of glutamate. At a glutamate

Table 1. Effect of glutamate administration on pentobarbital-induced narcosis*

Glutamate (mg/kg, i.p.)	No. of animals	Sleeping time (min ± S. E.)	Significance (P)
0	9	41.7 ± 3.9 (29–60)	
300	10	49.1 ± 4.0 (29–79)	>0.05
600	10	73.0 ± 5.3 (50–104)	<0.0005

* Five min after glutamate administration, the animals were challenged with 60 mg/kg, i.p., of sodium pentobarbital. Numbers in parentheses indicate the range of values.

Table 2. Effect of chronic pentobarbital administration on brain levels of GABA and glutamate and the GAD activity in the mouse

Experimental group	GABA (μmoles/g brain tissue ± S.E.; N = 4)	Glutamate (μmoles/g brain tissue ± S.E.; N = 4)	GAD activity (μmoles CO_2 30 min 100 mg protein ± S.E.; N = 6)
Pentobarbital	1.95 ± 0.05	12.44 ± 0.22	9.95 ± 0.77
Placebo	2.10 ± 0.03	13.24 ± 0.24	13.69 ± 1.35
	P < 0.025	P < 0.025	P < 0.025

dose level of 300 mg/kg, the brain GABA level was not significantly elevated. Brain glutamate levels were not altered significantly by either pretreatment.

Effect of chronic pentobarbital administration on brain levels of GABA and glutamate and the GAD activity in the mouse. As shown in Table 2, in mice receiving pentobarbital pellet implantation for 3 days, the brain GABA and glutamate levels were slightly (but significantly) lower than that of the placebo control group. The GAD activity in the brains of pentobarbital-pellet-implanted animals was decreased significantly to 72.5 per cent of the control level.

Effect of AOAA on GABA and glutamate in tolerant and non-tolerant animals. After injection of AOAA, 20 mg/kg, i.p., the expected increase in brain GABA content occurred in both tolerant and non-tolerant mice. However, as shown in Fig. 5, the rate of GABA accumulation in tolerant mice was 30 per cent slower than in the controls. On the other hand, AOAA had no effect on glutamate levels at 10 and 60 min after AOAA administration.

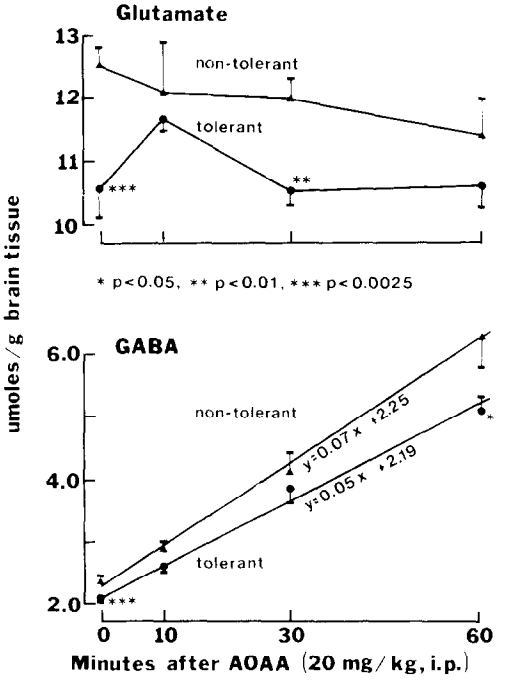

Fig. 5. Effect of AOAA on brain GABA and glutamate levels in tolerant mice. A dose of 20 mg/kg, i.p., of AOAA was given to each mouse which had been implanted with a pentobarbital pellet for 72 hr. The animals receiving placebo implantation were used as the control. Values shown in the figure are means of four mice ± S.E.

Effect of abrupt withdrawal of pentobarbital on brain GABA and glutamate levels in the mouse. Measurement of GABA and glutamate of the brain after the withdrawal of pentobarbital pellets revealed that there was a significant decrease of GABA which was not accompanied by a change in glutamate. As shown in Table 3, after removal of the pentobarbital pellet, the brain GABA level was decreased about 15 per cent at 3 hr and 8 per cent by 23 hr. The glutamate level immediately before pentobarbital pellet removal was significantly lower than that of the control group. However, the glutamate levels were not significantly different at 3 and 23 hr after the removal of pentobarbital pellets as compared to the control group.

DISCUSSION

The data from the present studies indicate that an increase in brain levels of GABA is associated with pentobarbital-induced narcosis in mice. When the loss of righting reflex in the mouse was induced by a lower dose of sodium pentobarbital, 37.5 mg/kg, i.p., the duration of sleeping time was short and the period of elevation in GABA level also was short. On the other hand, in mice receiving a higher dose of sodium pentobarbital (75 mg/kg), the sleeping time of the animals was prolonged. With this treatment, the brain level of GABA was significantly increased and persisted for a longer period of time. Our results in the mouse substantiate the prior evidence provided on rats, indicating that the acute administration of an anesthetic dose of sodium pentobarbital caused a small but significant increase in the GABA concentration within a short time (1 hr) after the administration of the drug [10]. The time course studies of GAD activity in the brain after administration of two different doses of sodium pentobarbital also substantiate the findings on brain GABA level. We also found that when brain GABA levels were increased with the administration of AOAA, an inhibitor of GABA-T, or glutamate, the precursor of GABA, the pentobarbital-induced sleeping time also was prolonged. Thus, it appears that GABA is associated with pentobarbital-induced narcosis.

Although the acute administration of sodium pentobarbital and AOAA significantly decreased the brain level of glutamate, there was no correlation with pentobarbital-induced narcosis. The administration of glutamate prolonged the pentobarbital sleeping time. Under these conditions the brain level of glutamate was not elevated, but brain GABA levels were significantly elevated at 10, 30 and 60 min after the administration of glutamate, indicating a correlation between

Table 3. Effect of abrupt withdrawal from pentobarbital on brain GABA and glutamate levels in the mouse*

Amount (μmoles/g brain tissue)	Time after withdrawal (hr)	Withdrawal	Control	Significance
GABA	0	1.98 ± 0.05	2.13 ± 0.04	$P < 0.025$
	3	1.82 ± 0.02	2.14 ± 0.04	$P < 0.0005$
	23	1.91 ± 0.03	2.08 ± 0.04	$P < 0.0005$
Glutamate	0	12.08 ± 0.11	12.79 ± 0.12	$P < 0.0025$
	3	11.96 ± 0.19	12.16 ± 0.35	NS
	23	12.27 ± 0.07	11.99 ± 0.20	NS

* Values shown are means of four mice ± S. E. NS = not significant.

GABA levels but not glutamate levels, and pentobarbital-induced narcosis.

The current study also demonstrated that chronic pentobarbital administration by pentobarbital pellet implantation caused significant decreases in both GABA level and GAD activity. In addition, the GABA levels in the brains were significantly decreased for at least 23 hr in mice after withdrawal of the pentobarbital pellet. Although evidence with rats indicates that the chronic administration of barbiturate did not cause marked changes in the brain concentration of GABA or in the activity of GAD or GABA-T [10], we obtained contrary evidence in the mouse. The discrepancy can be explained by the differences in experimental conditions, ours being more drastic. In the rat experiments, barbiturate was given in the drinking water as a chronic administration of barbiturate; therefore, the degree of tolerance development probably was not sufficient to affect the GABA systems. With our recent development of pentobarbital pellet implantation [21, 22], we were able to induce a much greater degree of tolerance to the hypnotic effect of pentobarbital in a shorter time period than that produced by the conventional parenteral or oral administration techniques. Under such conditions we have demonstrated that the chronic administration of pentobarbital caused marked changes in the concentration of GABA in mouse brain and in the GAD activity.

Decreased brain levels of GABA have been casually related to the occurrence of convulsions (Killam and Bain [23] and Killam [24]). Eidelberg *et al.* [25] and Wallach [26] also have shown that GABA might have anticonvulsant properties. Recently, Wood and Peesker [27] re-evaluated the anticonvulsant action of GABA-elevating agents and concluded that all agents which elevate the content of GABA in the brain are potential anticonvulsant agents. Although no neurochemical basis for barbiturate abstinence properties has been established, Essig [28] showed that AOAA could serve as an anticonvulsant against barbiturate abstinence seizures in dogs. Direct measurement of brain GABA during barbiturate intoxication and withdrawal in current studies supports the findings in the withdrawal dogs [28].

The precise mecchanism by which the GABA system is involved in pentobarbital narcosis, tolerance and physical dependence remains to be elucidated. We are in the process of seeking answers to this question by studying regional distributions of glutamate and GABA levels and of GAD and GABA-T in discrete areas of the brain after acute or chronic administration of pentobarbital.

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