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GAMMA-AMINOBUTYRIC ACID AND RUNNING FITS INDUCED BY GAMMA-MERCAPTOBUTYRIC ACID

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Abstract—The central action of γ-mercaptobutyric acid (GMBA), which has a structure closely resembling γ-aminobutyric acid (GABA), was examined in relation to GABA. A running fit is induced by intraperitoneal injection of 150 mg or 200 mg/kg of GMBA after a latent period of about 4–8 min in the mouse. Determination of the glutamic acid (Glu) and GABA levels and the glutamic acid decarboxylase (GAD) and GABA-α-ketoglutaric acid transaminase (GABAT) activities in the brain fails to reveal any significant alterations.

When GABA is first instilled intracysternally in the rabbit, the GMBA-seizure is prevented so the relationship between GABA and GMBA was studied in further detail.

Aminooxyacetic acid (AOAA) was first administered in the mouse to raise brain GABA level and then GMBA administered. An inverse relationship was found between the brain GABA level and ease of inducing the running fit under a state of lowered GABA level caused by administration of 2-methyl-4-amino-5-hydroxymethyl pyrimidine (OMP), on the other hand, the running fit was more readily induced by GMBA.

IN 1939, Abderhalden showed that a characteristic running fit (RF) was induced in the mouse and rat by administration of 2-methyl-4-amino-5-hydroxymethyl pyrimidine (OMP), the pyrimidine moiety of vitamin B_1, and Makino *et al.*[1,2] revealed that this RF was specifically suppressed by vitamin B_6. It was further suggested by Kodama[3] that the OMP-induced RF originated in a reduction in the brain γ-amino-butyric acid (GABA) level on the basis of the finding that a decline in brain glutamic acid decarboxylase (GAD; EC 4.1.1.15) and γ-aminobutyric acid-α-ketoglutaric acid transaminase (GABAT; EC 2.6.1.19) activities is present, prior to the occurrence of the RF in the OMP administered animal. The pre-condition for this is GABA to have activity as an active substance and Kodama *et al.*[4] made a study of compounds with a structure close to GABA. Experiments with γ-mercaptobutyric acid (GMBA) revealed an antagonistic action between this compound and GABA.

In the present study, the antagonistic action of GMBA and the experimental conditions under which this action occurs were studied in greater detail. Several findings of interest were obtained and are here presented.

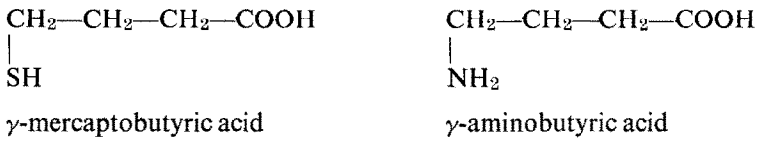
EXPERIMENTAL

Materials

For the intracysternal injection experiments, male rabbits weighing about 2·5 kg were used, while in all the other experiments, male dd mice, ca. 15 g in weight, were used. GMBA (Sankyo Research Laboratory, Japan), aminooxyacetic acid (AOAA), as the hemihydrochloride, (Upjohn Company, U.S.A.) and OMP (Takeda Research

Laboratory, Japan) were given by injection. For intracysternal injection, GABA and GMBA were prepared so that the quantity to be injected was 0·2 ml. The dosage of the agents in the mice experiments was adjusted so as to be 0·01 ml per gram of body weight and was injected i.p. GMBA is a compound in which the amino radical in the γ-position of GABA is replaced by an SH radical. It is a clear, colorless, strongly acidic solution with a putrid odor and a boiling point of 121–124°.

At around pH 5, it readily mixes with water. In the present study, the neutral, aqueous solution was used.



Determination of GABA and glutamic acid

The method of Tsukada *et al.*[6] and Sugawara[7] based on the method of Roberts *et al.*[5] was used as reference and one-dimensional paper chromatography was carried out.

The brain of the mouse killed by decapitation was taken out as soon as possible and frozen in acetone–dry ice. After weighing, 5 ml of 75% ethanol was added and the brain homogenized in a Potter–Elvehjem Type homogenizer while chilling. The homogenate was centrifuged and the supernatant evaporated to dryness. Water was added to the dry residue in a proportion of 1·0 ml per 1 g of wet brain, the residue dissolved and placed in a small test tube. The vessel was washed with 3 ml of water-saturated chloroform and the washing added to the small test tube. After centrifugation, the uppermost layer was taken and used as the test sample. For determination of GABA, 20 μl of the sample was spotted linearly on Toyo Filter Paper No. 51 and for determination of glutamic acid (Glu), 5 μl was spotted. At the same time, standard solutions of GABA and Glu, in strong and weak concentration, were prepared and spotted. The developing solution was water-saturated phenol and the time of development was 15–18 hr. To eliminate the developing solution adequately, the filter paper was exposed to air for 5–8 hr, heated for 15 min at 80° and then washed with ether. The dried paper was placed in 0·5% ninhydrin–acetone solution, heated for 20 min at 80° to colorize. Each spot was cut out and eluted for 20 min in 0·005% $CuSO_4$–75% ethanol solution. A piece of filter paper, corresponding in size and location to the respective spots, was cut out, treated in same manner and taken as the blank. Colorimetric determination was carried out with the Hitachi Type FPW-4 photoelectric photometer at 500 mμ. With the method used, the recovery rate of GABA and Glu was 90–97%.

Determination of GAD activity

The Roberts and Simonsen method[9] based on the method of Baxter and Roberts,[8] wherein the quantity of GABA which increases during incubation of brain homogenate with Glu is determined, was used for reference. Mouse brain, frozen with acetone–dry ice was weighed, homogenized in 0·05 M phosphate buffer (pH 6·3) containing 0·01 M of the reduced form of glutathione(GSH) while chilling with ice and 1·0 ml of homogenate and 0·2 ml of 0·25 M Glu (pH 6·3) combined and incubated at 37°.

A sample in which 0·05 M phosphate buffer was substituted for the Glu was used for the blank. After incubation for 60 min, 3 ml of 75% ethanol was added, the mixture centrifuged, the supernant dried and the GABA content determined according to the previously described method.

Determination of GABAT activity

The Sytinski and Priyatkina method[11] based on the Baxter and Roberts method[10] in which the Glu produced by biosynthesis on incubation of brain homogenate, GABA and α-ketoglutaric acid (KGA) is measured, was used as reference.

Frozen mouse brain was homogenized in 5 ml of 0·1 M borate buffer (pH 8·0), while chilling with ice. To 1·0 ml of the homogenate was added 0·5 ml of 0·05 M GABA (in 0·1 M borate buffer, pH 8·0) and 0·5 ml of 0·05 M KGA (in 0·1 M borate buffer, pH 8·0) and the mixture incubated at 37°. After 60 min, the reaction was stopped by heating for 10 min at 60°. The blank was heated prior to the incubation. To the mixture was added 2·0 ml of water, shaken well, centrifuged and 1·0 ml of the supernant mixed with 3·0 ml of copper–phosphate reagents.[12] From here, the standard Glu was treated in the same manner. To 1·0 ml of the supernant were added 3·0 ml of neocuproine reagent[13,14] and 50 mg of ascorbic acid and after 10 min, colorimetric determination carried out at 470 mμ.

RESULTS

GAD, GABAT Activity and GABA, Glu content of mouse brain after GMBA injection

When GMBA was injected i.p. in the mouse, RF was induced after a latent period of ca. 4–8 min. The relationship between the dose and the incidence of RF is shown in Table 1. It is known that RF, similar in appearance, is induced by OMP and a

TABLE 1. RUNNING FITS OF MICE AFTER ADMINISTRATION OF γ-MERCAPTOBUTYRIC ACID (GMBA) INTRAPERITONEALLY

GMBA (mg/kg)	RF	Death
200	5/5	5/5
150	5/5	3/5
125	4/5	2/5
100	1/5	1/5
75	0/5	0/5

LD_{50}* = 134·5 mg/kg
* by van der Waerden's method (intraperitoneal).

depression of brain GAD, GABAT activity and decrease in GABA was already present prior to the appearance of the RF.[3] The activity of these enzymes in the GMBA induced RF was investigated. It was found that GAD and GABAT activities were unchanged, i.e. 11·06 ± 1·07 μM/g/hr (control 11·41 ± 1·21 μM/g/hr) and 20·74 ± 1·48 μM/g/hr (control 20·45 ± 1·25 μM/g/hr) respectively, and there was no difference in brain GABA and Glu content compared to the control, i.e. 2·34 ± 0·48 μM/g (control 2·44 ± 0·26 μM/g) and 9·38 ± 0·96 μM/g (control 9·77 ± 1·08 μM/g) respectively.

Effect of GABA on GMBA-induced RF of rabbit

The results of the previous experiment seem to indicate that the mechanism of action of GMBA differs from OMP and the RF is induced wholly unrelated to GABA. Since GABA does not cross the blood–brain barrier (BBB),[15] there is no rise in the brain GABA level with oral or i.v. administration in animals. Injection was therefore made directly into the cysterna magna of the rabbit and the relation to the GMBA-induced RF was investigated. The results are shown in Table 2 and it can be seen that

TABLE 2. RUNNING FITS INDUCED BY 10 mg OR 20 mg GMBA IS PREVENTED BY THE PRIOR INTRACYSTERNAL INJECTION OF 20 mg GABA

Controls		Experiments		
Administration method	RF	Administration method	RF	Prevention, %
GMBA 10 mg	7/7	GABA 20 mg; 10 min; GMBA 10 mg	0/7	100
GMBA 20 mg	6/6	GABA 20 mg; 10 min; GMBA 20 mg	0/6	100

the RF induced by GMBA is prevented by the prior intracysternal injection of GABA. This suggests that GABA is related in some way to the GMBA-induced RF.

Prevention of GMBA-induced RF by AOAA

In order to further clarify the relationship between GMBA and GABA, tests to prevent the GMBA-induced RF were conducted with AOAA, which had been shown by Wallach[16] to endogenously increase the GABA level in the brain. Mice were first i.p. given 40 mg/kg of AOAA and 150 mg/kg and 200 mg/kg of GMBA then injected i.p. after set intervals. The rate of appearance of the RF and the GABA level were determined.

Table 3 shows the results and as can be observed, the rate of occurrence of the RF by i.p. injection of GMBA 150 mg/kg is markedly reduced 3–12 hr after injection of AOAA. Even in the animals which showed the RF before this period, a prominent prolongation of the latent period between injection of GMBA and appearance of the RF was noted. The same trend was observed when the dose of GMBA was increased to 200 mg/kg. The change in the brain GABA levels at this time are shown in Fig. 1 and it can be seen that the rate of prevention of the RF rose in parallel with increase in

TABLE 3. EFFECT OF AMINOOXYACETIC ACID (AOAA) ON GMBA-INDUCED RUNNING FITS OF MICE. FIRST 40 mg AOAA/kg WERE INJECTED AFTER SET INTERVALS

	GMBA			
	150 mg/kg i.p.		20 mg/kg i.p.	
	RF	Latent period (min)	RF	Latent period (min)
Control	13/13	4·3	18/18	5·3
	AOAA 40 mg/kg i.p.			
1 hr	6/10	10·2	12/13	12·3
3 hr	4/12	21·3	11/17	13·7
6 hr	3/12	21·5	11/17	16·1
9 hr	1/12	24·0	13/19	15·8
12 hr	3/12	10·0	11/16	13·5
24 hr	11/11	8·7	15/19	9·2

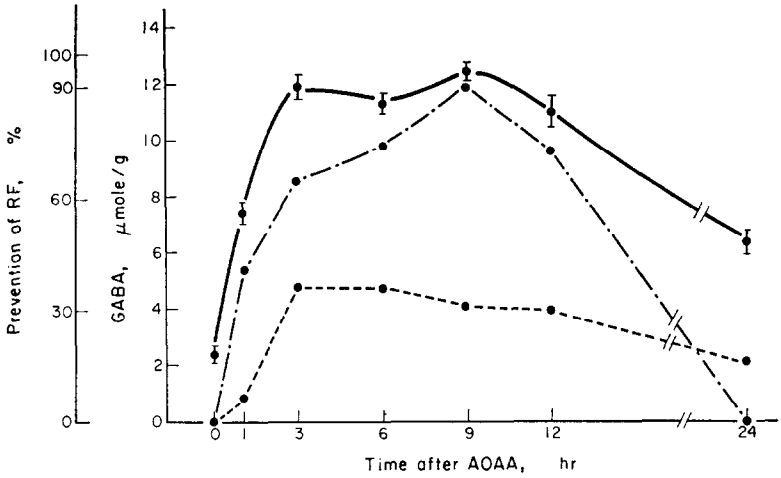


FIG. 1. Effect of 40 mg aminooxyacetic acid (AOAA)/kg on GABA content (———) of brains of mice and on running fits induced by 150 mg (— · — · —) and 200 mg (- - - -) GMBA/kg.

the GABA level, and the GABA curve and the RF-prevention curve closely coincided.

Kuriyama *et al.*[17] have reported that the brain GABA level reaches a plateau 3–4 hr after i.p. injection of 25 mg/kg of AOAA in the male Swiss albino mouse and the plateau is maintained for 4–6 hr after which there is a further rise and a peak is reached after 6 hr. Experiments were therefore, carried out with this dosage. As shown in Fig. 2 a biphasic rise was not found.

Effect of OMP on GMBA-induced RF

In order to ascertain, on the other hand, whether the GMBA-induced RF is increased when GABA is decreased, experiments were conducted with OMP. It was first verified that RF was not induced by i.p. injection of 50 mg/kg of GMBA, 10

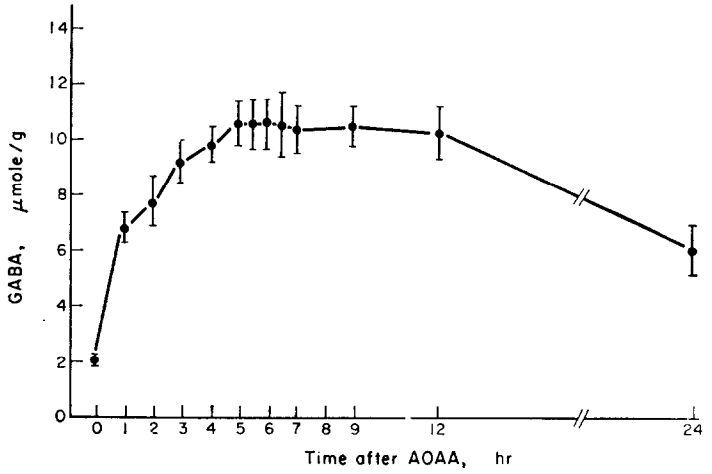


FIG. 2. GABA content of brains of mice after administration of 25 mg AOAA/kg.

min following i.p. injection of 200 mg/kg of OMP in the mouse. The interval between the OMP and GMBA was then extended to 60 and 120 min. Table 4 shows the results. RF became apparent in more than one-half of the animals and the GABA level gradually decreased with time as may be seen in Fig. 3. The same trend was apparent when the GMBA was increased to 100 mg/kg (Table 4).

DISCUSSION

In the analytical investigations on the biological activity of GABA, crustaceans were first used[18] and recently Otsuka *et al.* reported that GABA is released according to stimulation of the inhibitory nerve in the lobster.[35] On the other hand, numerous

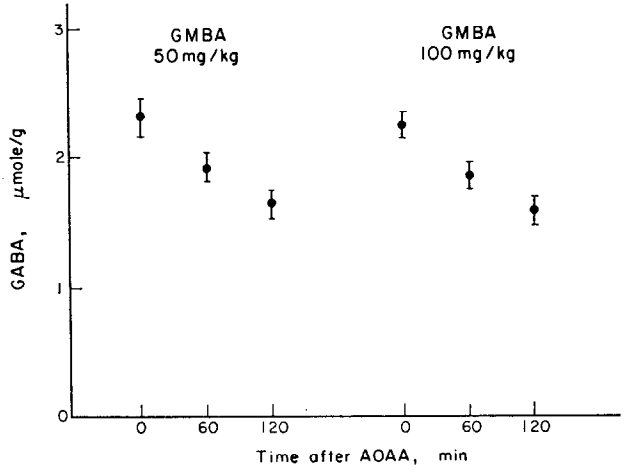


FIG. 3. Effect of 200 mg OMP/kg on GABA content of brains of mice. GABA was administered by methods shown in Table 4.

TABLE 4. EFFECT OF 2-METHYL-4-AMINO-5-HYDROXYMETHYLPYRIMIDINE(OMP) ON GMBA-INDUCED RUNNING FITS OF MICE. 50 mg AND 100 mg GMBA/kg WERE INJECTED INTRAPERITONEALLY AFTER 200 mg OMP/kg

Administration methods	GMBA 50mg/kg	GMBA 100mg/kg
OMP GMBA (10 min)	0/9	5/15
Saline GMBA	0/9	1/13
OMP GMBA (60 min)	5/9	10/12
OMP Saline	0/9	1/12
OMP GMBA (120 min)	6/9	15/15
OMP Saline	1/9	2/14

suggestions have been made regarding the role of GABA in the mammalian CNS; e.g. specific changes occur in the EEG when GABA is applied to the cerebral cortex, the motor neuron of the spinal cord is inhibited by GABA. Pharmacological studies have been conducted on the relation between brain GABA levels and convulsions since it is known that deficiency in vitamin B_6 induces seizures and GAD and GABAT are enzymes which act as a coenzyme for vitamin B_6. Some findings[26–31] indicate a monophasic relationship between seizures and GABA but it has also been proposed that there is no relationship between seizures and GABA.[32–35]

In the RF induced by GMBA, no change in the GABA level was found but in the present study, the GABA level of the whole brain was examined and it may be necessary to observe the changes in specific regions related to seizure activity or in the cellular components of the active site of GABA before a definite conclusion can be made. Though Kamrin *et al.*,[36] and Tews *et al.*,[37] report that no significant change occurred in the GABA level of the whole brain during picrotoxin-induced seizures, Sytinski *et al.*[38] state that on examination of the various parts of the brain in the monkey, a decrease in GABA was found in the gray matter of the occipital and parietal lobes. Elliott[39] has divided GABA into the free form extracted by fresh physiological saline solution and the occult form, which is extracted by alteration of

the pH or using hypotonic saline and states that it is only the occult form which is related to excitation of the brain and seizures are more readily induced by a reduction in occult GABA while seizures may occur even when free GABA is increased.

When a large dose (3 g/kg) of GABA is injected i.v. in the mouse, there is no rise in GABA content of the brain, indicating that, passage of GABA through the BBB is extremely difficult.[15] The attempt was then made to elevate the brain GABA content endogenously by inhibiting GABAT and tests were conducted with inhibitors such as hydroxylamine,[8] AOAA[16] and glutamic acid-γ-hydrazide.[40]

Of these, AOAA has the greatest GABAT inhibiting action and numerous experiments have been conducted using this substance.[8,16,17,41–49] AOAA readily forms the oxime with the aldehydic form of vitamin B_6 *in vitro*[50] and inhibits pyridoxal phosphokinase[48] as well as pyridoxal-dependent enzymes other than GABAT such as GAD[43] and glutamic–pyruvic transaminase.[51] Pyridoxal phosphokinase is more sensitive to hydrazine than GAD[52] and the *in vitro* effect of AOAA is stronger against kinase rather than pyridoxal-dependent enzymes.[48]

Kuriyama *et al.*[17] have examined the GABA content of the brain and the sensitivity to electroshock according to time following injection of AOAA in the mouse and state that the pattern of change in the GABA content with time after injection of 25 mg/kg of AOAA is biphasic and there is a pronounced decrease in the incidence of electro-shock seizure up to 1·5 hr after injection and the seizure sensitivity returns to normal after 6 hr when the brain GABA level reaches a maximum showing that a close interrelationship exists between the GABA content and the incidence of electro-shock seizures.

Da Vanzo *et al.*[48] report that the protective effect against TSC seizure deaths becomes maximal even before the GABA level reaches a maximum at 6 hr and the effect decreases at 6 hr. In present experiment, it was found that the brain GABA level following injection of AOAA did not show a biphasic pattern and furthermore that there was a close relationship between the preventative effect against the GMBA-induced RF and the brain GABA level.

OMP becomes antagonistic to pyridoxal phosphate and apoenzyme only after phosphorylation,[53–55] but the degree of vitamin B_6 enzyme inhibited by OMP depends upon the reciprocal affinity of OMP and pyridoxal phosphate to apoenzyme,[71] and the bond of GAD to pyridoxal phosphate is weaker than GABAT[6,57] so that it is inhibited more strongly by OMP.[3,58,63] The brain GABA level, therefore decreases. When the brain GABA of the mouse is reduced by injection of OMP, the RF is induced by i.p. injection of 50 mg/kg of GMBA, a quantity which will not cause the RF if given alone, and an interrelationship is found between the degree of reduction of GABA and the ease of occurrence of the GMBA-induced RF.

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