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Studies on the Centrally Acting Muscle Relaxants: The Spinal Reflex and the Spinal Gamma-Aminobutyric Acid Level in Young Chickens[1]

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As a part of the research on the mechanisms of action of the centrally acting muscle relaxants, the tentative correlation between the effect on the polysynaptic reflex and gamma-aminobutyric acid (GABA) level in the spinal cord was studied in young chicks. The spinal GABA concentration of chicks was found to be 12.1 mg/100 g wet tissue. The spinal GABA level was not significantly altered by several relaxants and stimulants. It was found that amino-oxyacetic acid (AOAA), an inhibitor of GABA transaminase, markedly increased the spinal GABA level in chicks. The inhibitory effect of mephenesin or exogenous GABA on the crossed extensor reflex in AOAA-pretreated chicks was not significantly different from that in AOAA-untreated ones. Consequently, evidence for the direct correlation between the action of the centrally acting muscle relaxant and the GABA level in the spinal cord could not be obtained.

Introduction

The gamma-aminobutyric acid (GABA) level in the animal brain was reported to increase in the state of a decreased excitability and to decrease in an increased excitability.[3] This finding suggested the correlation between the physiological function and the GABA level in brain. Since then, many authers have reported the inhibitory effect of exogenous GABA on the brain function and the influences of centrally acting drugs on the brain GABA level (*e.g.* Sytinskii, *et al.*[4]).

As to the spinal cord, it was reported that topical application of high doses of GABA reversibly depressed the spinal reflexes.[5–7] The strong effect of GABA was shown to inhibit the activity of spinal neurons when applied iontophoretically,[8] although the depressant action resembling inhibitory transmitter in the mammalian spinal cord had been neglected by the same authers.[9] Although glycine has recently been shown to be associated with spinal neurons that release a transmitter mediating inhibition in motoneurons,[10] GABA does not fail to be an inhibitory transmitter candidate in the spinal cord.

While researching on the mechanisms of action of the centrally acting muscle relaxants,[11] we were interested in the tentative correlation between the effects on the polysynaptic reflex

1) Preliminary report of this work was presented at the 27th International Congress of Pharmaceutical Sciences, Montpellier (France), September, 1967.
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4) I.A. Sytinskii and T.N. Priyatkina, *Biochem. Pharmacol.*, **15**, 49 (1966).
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9) D.R. Curtis, J.W. Phillis and J.C. Watkins, *J. Physiol.*, **146**, 185 (1959).
10) M.H. Aprison and R. Werman, "Neurosciences Research," Vol. 1, ed. by S. Ehrenpreis and O.C. Solnitzky, Academic Press, New York and London, 1968, pp. 143—174.
11) H. Fukuda, K. Watanabe, Y. Kudo and T. Ohshima, *Yakugaku Zasshi*, **88**, 1338 (1968).

and the GABA level in the spinal cord. The alterations in the concentration of spinal GABA caused by relaxants and stimulants were measured, and the influence of these drugs on the crossed extensor reflexes was investigated in both untreated chicks and amino-oxyacetic acid (AOAA)-pretreated ones. The purpose of this work was to investigate whether GABA was concerned in the mechanism of action of the centrally acting muscle relaxants.

Methods and Materials

Male chicks, Golden neck strain ranging in age from 2 to 17 days after hatching, were used.

1) **Measurement of GABA in the Spinal Cord and the Brain**——The measurement was made according to the methods described by Sytinsky, *et al.*[12] with some alterations. After decapitation, the brain and the spinal cord of chicks were removed and put into salted ice immediately. After 1 min, the brain was separated into cerebrum, brain stem and cerebellum, and then the spinal cord and the parts of the brain were weighed. The procedures up to this time were done within 10 min. Each tissue was homogenized with glass homogenizer in about seven volumes of 75 per cent ethanol and allowed to stay overnight at 0° for extraction. The homogenate was centrifuged for 10 min at 2000 *g* and the supernatant liquid was collected. The residue was washed with 75 per cent ethanol and the combined supernatant liquid was evaporated to dryness in a boiling-water bath. This dried material was added with water to 5 ml and centrifuged to remove insoluble materials, and the supernatant was evaporated again to dryness. This final extract was added with water to 0.5 ml for each brain tissue and 0.25 ml for spinal cord. Ascending paper chromatography was carried out by spotting the specimens on filter paper (Toyo Roshi No. 51) for separation of GABA. In each experiment three doses from 1 γ to 10 γ of GABA were used for the reference standards. Samples and standard solutions were developed by solvent: *n*-butanol-acetic acid-water (4:1:1 v/v) for 15—18 hr at room temperature. After evaporating the solvent the sheets were sprayed on both sides with 0.5 per cent solution of ninhydrin in butanol and were heated for 30 min at 80° for complete coloring. A spot was detected at *Rf* 0.53, corresponding to the standard sample of GABA, and this portion was cut off and eluted with 5 ml of water. The color was read at 570 mμ in the spectrophotometer and the GABA concentration was calculated from the standard curve obtained simultaneously.

2) **Polysynaptic Reflex of the Spinal Chicks**——Polysynaptic reflexes were recorded by the method introduced by Bowman, *et al.*[13] and modified by Fukuda, *et al.*[11] The chicks were anesthetized with chloralose (50—70 mg/kg) injected intraperitoneally. The trachea was cannulated and artificial respiration was employed. In order to make the spinal chick preparations, the spinal cord was doubly tied at the level of C 10 and cut between two ligatures under additional anesthesia with ether. Crossed extensor reflexes of the leg were elicited by the stimulation of the contralateral n. fibularis. The reflexes were written on the kymograph. Drug effect was expressed as percentage of the mean of maximal responses for 3 min to the mean of responses for 3 min before injection. Drug solution was injected into jugular vein *via* a polyethylene tube.

In some experiments, arterial blood pressure was simultaneously measured from the carotid artery by means of a Nihonkohden transducer (MP-4T) and displayed on an ink writing oscillograph (WI-180), operating at a proper speed of 15 mm/min.

3) **Sciatic Nerve-gastrocnemius Muscle Preparation *in Situ***——The twitches of gastrocnemius were recorded in response to the stimulation of the peripheral stump of severed sciatic nerve in chicks under chloralose anesthesia (50—70 mg/kg, *i.p.*). Drugs were injected into jugular vein.

4) **Drugs**——Drugs used were chloralose (Tokyo-kasei), mephenesin (Myanol, Chugai), diazepam (supplied by Takeda Chem. Industries), picrotoxin (B.H.D.), strychnine nitrate (Shin-teikoku Seiyaku), gamma-aminobutyric acid (GABA) (Tokyo-kasei), amino-oxyacetic acid (AOAA) (Aldrich Chem.) and thiosemicarbazide dihydrochloride (TSC-2HCl) (Tokyo-kasei). Diazepam was used as the solution in 0.5% Tween 80, and other drugs were dissolved in 0.9% saline.

Results

GABA Level in Spinal Cord and Brain of Chicks

The GABA concentrations were measured in cerebrum, brain stem, cerebellum and spinal cord of chicks. As shown in Table I, the individual variations of the GABA level in each region were small. The spinal GABA level (12.1±1.6 mg/100 g) was lower than that in each region of the brain.

12) I.A. Sytinsky and N.T. Thinh, *J. Neurochem.*, **11**, 551 (1964).
13) W.C. Bowman, B.A. Callingham and G. Osuide, *J. Pharm. Pharmacol.*, **16**, 505 (1964).

Table I. GABA Level in Spinal Cord and Brain of Chicks

Region	Concentration (mg/100 g)
Cerebrum	28.3±1.6 (10)
Brain stem	41.5±0.5 (14)
Cerebellum	27.1±1.4 (5)
Spinal cord	12.1±0.8 (6)

Results are given as mean ±S.E., and are based on wet tissue weight.
The number of animals is shown in parentheses.

GABA Level in Spinal Cord of the Chicks dosed with Stimulants and Depressants

As shown in Table II, any significant alteration in the spinal GABA level was not induced by the introduction of each effective dose of chloralose, mephenesin, diazepam, picrotoxin and strychnine. Exogenous GABA, injected even in a high dose of 500 mg/kg, did not change the spinal GABA level.

Table II. Alterations of the Spinal GABA Level by Several Stimulants and Depressants in Chicks

Treatment	Dose (mg/kg)	Time[a] (min)	Concentration[b] (mg/100 g)
Control	—	—	12.1±0.8 (6)
Chloralose	60 *i.p.*	30	11.4±0.6 (10)
Mephenesin	150 *i.p.*	10	13.5±0.3 (4)
	200 *i.p.*	10	10.6±0.4 (6)
Diazepam	6 *i.p.*	10	12.6±0.7 (5)
Picrotoxin	10 *i.p.*	15	10.3±2.3 (5)
Strychnine nitrate	1 *s.c.*	20	13.7±0.8 (6)
GABA	500 *i.p.*	10	13.1±0.6 (6)

a) time between drug administration and decapitation
b) Results given as mean±S.E., and based on wet tissues. Number of animals shown in parentheses.

Effects of Mephenesin and Exogenous GABA on the Spinal GABA Level of AOAA-pretreated Chicks

As has already been shown in Table II, mephenesin did not significantly alter the spinal GABA level in chicks. However, there remains the possibility that centrally acting muscle relaxants such as mephenesin may influence the spinal GABA level under the other conditions (*e.g.* pretreatment of AOAA or thiosemicarbazide dihydrochloride (TSC–2HCl)).

Highly significant increase in spinal GABA level was observed after the subcutaneous injection of AOAA, an inhibitor of GABA transaminase.[14] The time course for GABA level after AOAA treatment is shown in Fig. 1.

Three hours after the injection of 10 mg/kg of AOAA, for instance, the GABA level was about five times as much as the control level. It was examined whether or not mephenesin might influence the GABA level elevated by AOAA. As seen in Fig. 1, mephenesin (100 mg/kg) did not alter the elevated spinal GABA level in AOAA-pretreated chicks as well as in the case of AOAA-untreated ones. A high dose of GABA (500 mg/kg) did not have any effect on the elevated GABA level.

Following the intraperitoneal injection of TSC–2HCl to chicks, the spinal GABA level was not significantly altered contrary to the expectation (13.0±0.93 mg/100 g for six preparations, 60 min after injection), whereas the level in cerebellum was markedly decreased (16.2±

14) D.P. Wallach, *Biochem. Pharmacol.*, **5**, 323 (1961).

1.71 mg/100 g). So, the performance of the experiment in TSC–2HCl-treated animals was given up.

Effects of Mephenesin and GABA on the Crossed Extensor Reflex in AOAA or TSC-2HCl-treated Chicks

It was shown in the previous report[11] that mephenesin depressed the crossed extensor reflex of the leg in spinal chicks. In the present study, high doses of GABA (150, 300 mg/kg) were found to depress the reflex slightly (Table III), and elicited twitches of gastrocnemius muscle were not influenced by the same or higher dose of GABA, excluding the possibility that the inhibitory action of GABA depends upon the effects at neuro–muscular junction or muscle.

Then, it was examined whether or not the pretreatment by AOAA or TSC–2HCl may give some influence upon the depressive action of drugs on the spinal reflex, in the purpose of elucidating the tentative correlation between the muscle relaxative activity of drugs and the spinal GABA level.

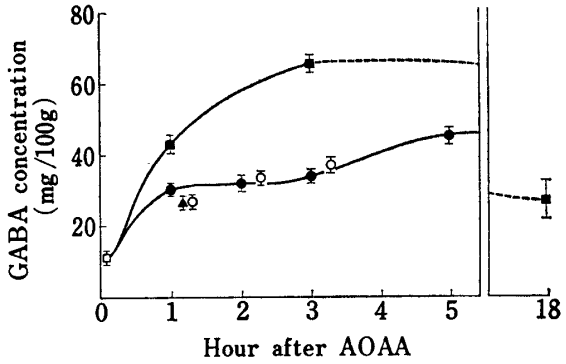

Fig. 1. The Increase of the Spinal GABA Level in AOAA-pretreated Chicks and the Effects of Mephenesin and Exogenous GABA on the Increased GABA Level

□: GABA concentration in the spinal cord of untreated chicks (control)
●: GABA concentration 1, 2, 3 and 5 hr after the injection of AOAA (5 mg/kg, *s.c.*)
■: GABA concentration 1,3 and 18 hr after the injection of AOAA (10 mg/kg,*s.c.*)
○: Mephenesin (100 mg/kg) was injected intraperitoneally each 1, 2 and 3 hr after AOAA (5 mg/kg, *s.c.*), and 10 min later GABA concentration was measured.
▲: GABA (500 mg/kg) was injected intraperitoneally 1 hr after AOAA (5 mg/kg, *s.c.*), and 10 min later GABA concentration was measured.
Each point represents the mean of the GABA concentration in six preparations, with the S.E. indicated.

Table III. Effects of Mephenesin and GABA on the Crossed Extensor Reflex in AOAA or TSC-Pretreated Spinal Chicks

Treatment	Dose (mg/kg, *i.v.*)	Extensor reflex[a]			
		Control	Treated with AOAA[b] before 3 hr	Treated with AOAA[b] before 5 hr	Treated with TSC–2HCl[c] before 3 hr
Mephenesin	10	77.0± 6.0 (5)	74.8± 2.7 (5)[d]	57.3± 9.2 (5)	67.3±4.7 (5)
	20	53.1±12.4 (5)	56.3±10.5 (5)	49.5±15.7 (5)	53.2±7.8 (6)
GABA	100	88.6± 6.6 (7)	—	—	87.8±6.1 (9)
	150	83.6±11.0 (5)	80.0± 6.2 (5)	89.2± 9.4 (5)	—
	300	73.5± 5.8 (5)	89.9±10.0 (5)	90.1± 4.7 (5)	79.0±5.6 (11)

a) Drug effect is expressed as percentage of the mean±S.E. of maximal responses for 3 min to the mean of responses for 3 min before injection. Number of animals is shown in parentheses.
b) amino-oxyacetic acid, 5 mg/kg, subcutaneously
c) Thiosemicarbazide dihydrochloride, 20 mg/kg, intraperitoneally
d) Reflex in AOAA or TSC-treated chicks is not significantly different from that in each untreated control ($p=0.05$).

AOAA given intravenously in a dose of 5 mg/kg slightly depressed the extensor reflex, and a dose of 10 mg/kg caused a gradual decrease. With subcutaneous injection of AOAA (5 mg/kg), however, significant change in the spinal reflex was not observed for five hours.

Either mephenesin (10, 20 mg/kg) or GABA (150, 300 mg/kg) was injected intravenously 3 and 5 hr after the administration of AOAA (5 mg/kg) respectively. The preparation for spinal reflex had been made at least 30 min before administration of the drugs. In 3 or 5 hr after the subcutaneous injection of AOAA (5 mg/kg), the spinal reflex was not changed, whereas GABA level in the spinal cord was in a state of marked elevation (Fig. 1). The result is shown in Table III. There could not be found the significant influence of AOAA-pretreatment upon

the inhibitory effect of mephenesin or GABA on the crossed extensor reflex in spinal chicks. Beside, as seen in Table III, the results in TSC-2HCl-pretreated chicks were found to be the same as in AOAA-pretreated ones. In some experiments, arterial blood pressure was simultaneously measured. Direct relationship between the effect on the polysynaptic reflex and the change of blood pressure (*e.g.* a rise of pressure by GABA) could not be recognized, however.

Discussion

In the previous study,[11] it was shown that the central depressants such as mephenesin and diazepam depressed the crossed extensor reflex and the stimulants increased it in young chicks. It was an important point in this work to see whether or not these drugs alter the GABA level in spinal cord. As to brain, Sytinskii, *et al.*[4] reported that pharmacological substances such as caffeine and meprobamate resulted in an alteration in the brain GABA level. Sisken, *et al.*[15] reported that intraperitoneal injection of GABA (4.9—10.0 g/kg) into young chicks resulted in elevation of GABA in the optic lobe and hemispheres. Contrary to the expectation, the spinal GABA level in chicks was not significantly altered by the introduction of each effective dose of chloralose, mephenesin, diazepam, picrotoxin, and also GABA (Table II). Then, following possibilities will be considered with reference to centrally acting muscle relaxants: First, there might not be a correlation between the mechanism of spinal reflex and the GABA level in the spinal cord. Second, even though endogenous GABA is important for the reflex mechanism, GABA might not participate the mechanism of reflex inhibition of these drugs. Third, although GABA level is important for the reflex and also for the mechanism of action of these drugs, the essential change could not be caught by the present method. These possibilities remain to be solved in future.

AOAA was reported to inhibit the transaminase in the brains of several species of animals, causing marked elevations in the brain concentrations of GABA.[14] Also in the present study, GABA level in the spinal cord of chicks was markedly elevated by the administration of AOAA (Fig. 1). AOAA (5 mg/kg, *i.v.*) slightly depressed the crossed extensor reflex in chicks. However, the increase in spinal GABA level could not be considered a cause of the inhibition of the reflex, because the reflex was not changed even in a state of marked elevation of GABA after subcutaneous injection of AOAA (5 mg/kg) (Fig. 1).

Van Gelder[16] reported that administration of GABA to animals which had received AOAA either enhanced or caused severe impairment of motor function, although no evidence could be obtained that exogenous GABA penetrated into the central nervous system under those conditions. In the present study, however, intraperitoneal injection of GABA (500 mg/kg) or mephenesin (100 mg/kg) did not have any influence on the high concentrations of spinal GABA elevated by the administration of AOAA (Fig. 1). Furthermore, the ratio of inhibition by mephenesin and GABA in spinal chicks pretreated with AOAA or TSC–2HCl was not significantly different from that in untreated spinal ones (Table III). Consequently, evidence for the direct correlation between the action of mephenesin or exogenous GABA and the GABA level in the spinal cord could not be found.

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16) N.M. van Gelder, *Biochem. Pharmacol.*, **15**, 533 (1966).