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Influence of Alkylamides of Glutamic Acid and Related Compounds on the Central Nervous System. I. Central Depressant Effect of Theanine

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Theanine (N-ethylglutamine), a flavourous constituent of tea leaves, was studied in respect of the influence on the central nervous system in mice.

Theanine was shown to inhibit the convulsive action of caffeine, but was ineffective against pentetrazole, picrotoxin, strychnine, pipradrol and bemegride as well as L-glutamine. Neither theanine nor L-glutamine effected on the prolongation of the sleeping time after the injection of hexobarbital sodium in mice. Using ^{14}C-labeled theanine, it was found that the intraperitoneally administered theanine was taken up by brain tissue without undergoing the metabolic changes in a 30 minutes. The intracerebral level of γ-aminobutyric acid was shown to increase significantly at 30 minutes following the intraperitoneal administration of theanine in mice.

In 1949, Sakato[2] isolated an amino acid, theanine (L-GluNHEt), from Japanese tea leaves and identified it as N-ethylglutamine amide. The presence of L-GluNHEt in other varieties of tea was confirmed by Cartwright, *et al.*[3] And L-GluNHEt has been thought to be a flavourous constituent of tea leaves.

On the other hand, caffeine, a central stimulant, has been known to be a pharmacological active constituent of tea. Hayashi,[4] however, reported that the magnitude of stimulant effect produced by the water extract of tea is less marked than that seen following administration of caffeine in a dose comparable to its content in the extract of tea leaves.

This report concerned with the studies on the pharmacological action of L-GluNHEt on the central nervous system comparing with other alkylamides of glutamic acid (Glu).

Material and Method

Drugs——L-Glutamine (L-GluNH_2) used in the present study was a commercial product manufactured by Wako Pure Chemical Industries. L-GluNHEt and N-ethylglutamiamine (L-GluNHMt) were synthesized by us according to the method described by Furuyama, *et al.*[5] Caffeine (Toyo Seiyaku-Kasei Co.), pentetrazole (Cardiazol, Sankyo Co.), picrotoxin (Wako Pure Chemical Industries), strychnine (Stchinin, Fuso Pharmaceutical Industries), bemegride (Antibarbi, Tanabe & Co.), pipradrol (Meratran, Shionogi & Co.) and Hexobarbital sodium (Oltopan-Sodium, Dainippon Pharmaceutical Co.) were purchased.

Laboratory Animal——Male ddY-mice, weighing 18 to 20 g, were used aftrer starvation for more than 16 hours.

Methods

1) **Effect on Tonic Convulsion**——Fifteen minutes after intraperitoneal administration of L-GluNH_2 or L-GluNHEt in water solution, the mice were given with the central stimulants intraperitoneally or subcutaneously and the incidence of convulsion was determined. For the estimation of the interval to onset tonic convulsion by caffeine, the stimulant and amides were administered intraperitoneally at the same time. To the control mice, saline instead of the amides was administered.

2) **Effect on the Sleeping Time Induced by Hexobarbital Sodium**——Fifteen minutes after administration of L-GluNH_2 or L-GluNHEt, the mice were given intraperitoneally hexobarbital sodium (60 mg/kg).

1) Location: *2-1, Oshika-2-chome, Shizuoka.*
2) Y. Sakato, *Nippon Nogeikagaku Kaishi*, **23**, 262 (1949).
3) R.A. Cartwright, E.A.H. Roberts and D.J. Wood, *J. Sci. Food Agr.*, **5**, 597 (1954).
4) E. Hayashi, "Collection of Scientific Papers Commemorating the 10th Anniversary of the Foundation of the Shizuoka College of Pharmacy," 1963, p. 118.
5) T. Furuyama, T. Yamashita and S. Senoh, *Bull. Chem. Soc. Japan*, **37**, 1078 (1964).

The interval from evident disappearance of righting reflex to recovery from it was determined as the sleeping time.

3) **Assay for the Uptake of L-GluNHEt by Brain and Liver**——L-GluNHEt-1,2,3,4,5-^{14}C was prepared from L-Glu-U-^{14}C according to Furuyama, *et al.*[5] The specific radioactivity of the preparation was 13.4μCi/m mole. At 15 or 30 minutes after administration of the radioactive L-GluNHEt, the mice were rapidly frozen in liquid nitrogen, then brain and liver were separated. The tissues thus obtained were processed by the method of Tsukada, *et al.*[6] and used for amino acid analysis by two dimensional paper chromatography on Toyo-Roshi No. 51, 40×40 cm. The solvent systems used were pyridine–water (4: 1) for the first dimension and phenol–water (4: 1) for the second dimension. The *Rf* values for GluNHEt, γ-aminobutyric acid (GABA), $GluNH_2$, Glu and asparitic acid (Asp) by using those solvents are as shown in Table I.

Table I. *Rf* Values of Amino Acids

Compound	Pyridine–water	Phenol–water
L-GluNHEt	0.73	0.87
GABA	0.45	0.78
$GluNH_2$	0.39	0.58
Glu	0.42	0.27
Asp	0.33	0.15

Radioactive spots were identified by radioautography and cut out from the paper. The papers were immersed in toluene scintillator (consisting of 2,5-diphenyloxazole, 4 g; 1,4-bis-2-(5-phenyl-oxazolyl)benzene, 0.1 g and 1000 ml of toluene) and measured for the radioactivity by the liquid scintillation counter (Packard, Model Tricarb 314F). All radioactivities obtained were corrected for background.

4) **Effect on the Intracerebral Free Amino Acid Pattern**——Mouse brain was separated as described above, and amino acids were determined by the amino acid analyzer (Hitachi, Model KLA-3B).

Result

1) Inhibition of Tonic Convulsion by Central Stimulants

Such central stimulants as caffeine, pentetrazole, picrotoxin, strychnine, bemegride and pipradrol were used at their minimum lethal doses.

The number of mouse which showed tonic convulsion within 3 hours after administration of the stimulants are shown in Table II.

Table II. Effects of L-$GluNH_2$ and L-GluNHEt on the Convulsion Induced by Central Nervous System Stimulants in Mice

Stimulant	Dose		Response A/B		
	Stimulant mg/kg	Amide m moles/kg	Control	L-$GluNH_2$	L-GluNHEt
Caffeine	270 *i.p.*	2.5	10/10	9/10	7/10
		5.0		6/10	4/10
		10.0		2/10	1/10
Pentetrazole	119 *s.c.*	10.0	9/10	9/10	10/10
Picrotoxin	6.5 *s.c.*	10.0	10/10	6/ 6	6/ 6
Strychnine	0.95 *s.c.*	10.0	9/10	6/ 6	6/ 6
Bemegride	48 *s.c.*	10.0	8/10	6/10	6/10
Pipradrol	140 *i.p.*	10.0	10/10	6/ 6	6/ 6

The stimulant was injected 15 min after intraperitoneal injection of the amides or saline.
A: The number of animal which showed tonic convulsion.
B: The number of animal used.

6) Y. Tsukada and Y. Nagata, *Seikagaku*, 33, 51 (1961).

It seemed that the both amides specifically inhibited against the action of caffeine. And L-GluNHEt delayed significantly the onset of tonic convulsion as shown in Table III.

TABLE III. Effects of L-GluNH$_2$ and L–GluNHEt on Caffeine–Induced Convulsion in Mice

Compound	No. of mouse	Dose m moles/kg	Tonic convulsion onset time ± S.D.	No. of survival
Control	14	—	16′17″ ± 10′20″	0
L-GluNH$_2$	14	9.0	19′53″ ± 9′15″	0
L-GluNHEt	14	9.0	32′01″ ± 14′40″ ($10^{a)}$)$^{b)}$	4

a) No. of the dead
b) $P<0.01$
Caffeine (300 mg/kg) was injected intraperitoneally and the drugs were administered intraperitoneally at the same time.

2) Effect on Hexobarbital Sodium-Induced Sleeping Time

Neither L-GluNHEt nor L-GluNH$_2$ in a dose of 5.0 or 10.0 m moles per kg affected the sleeping time of mice induced by hexobarbital sodium (60 mg/kg) as shown in Table IV.

TABLE IV. Effects of L-GluNH$_2$ and L-GluNHEt on Hexobarbital Sodium-Induced Sleeping Time in Mice

Compound	Dose m moles/kg	Sleeping time ± S.D. min
Control	—	28 ± 14 (10)
L-GluNH$_2$	5.0	23 ± 14 (10)
	10.0	29 ± 12 (10)
Control	—	19 ± 8 (10)
L-GluNHEt	5.0	23 ± 9 (10)
	10.0	21 ± 10 (10)

Figures in parentheses are No. of animals.

3) Uptake of L-GluNHEt by Mouse Brain and Liver

After intraperitoneal administration of 7.7 m moles per kg (1.18×10^8 cpm/kg) of L-GluNHEt, unchanged L-GluNHEt was found in brain and liver tissue as shown in Table V.

In brain tissue, only L-GluNHEt was detectable on the radioautograms.

TABLE V. The Uptake of L-GluNHEt-1,2,3,4,5-^{14}C by Brain and Liver *in Vivo*

Time (min)	Brain		Liver	
	cpm $\times 10^2$/g	μ moles/g	cpm $\times 10^2$/g	μ moles/g
15	33.3	0.21	682.7	4.40
15	35.7	0.23	512.4	3.31
30	60.9	0.39	319.2	2.06

4) Effects on the Intracerebral Free Amino Acids

Effects of L-GluNH$_2$, L-GluNHMt and L-GluNHEt on the intracerebral levels of amino acids were measured every 15 min after intraperitoneal administration of the amides. The results are shown in Table VI.

TABLE VI. Intracerebral Levels of Free Amino Acids Following Administration of Amides

Amino acid	Amide	Control	Amino acid level (μ moles/g wet weight)[a] 15	30	45
GABA	L-GluNH$_2$	2.67±0.26 (7)	2.64±0.33 (4)	2.86±0.40 (4)	2.64±0.51 (4)
	L-GluNHMt	2.66±0.05 (5)	2.45±0.21 (3)	2.56±0.41 (3)	2.29±0.28 (3)
	L-GluNHEt	2.67±0.26 (7)	2.87±0.37 (6)	3.20±0.33 (5)[b]	2.65±0.14 (4)
Glu	L-GluNH$_2$	10.40±0.88 (7)	9.38±0.67 (4)	10.07±1.22 (4)	10.37±0.46 (4)
	L-GluNHMt	10.41±1.47 (5)	9.07±1.09 (3)	9.25±0.38 (3)	10.80±0.97 (3)
	L-GluNHEt	10.40±0.88 (7)	10.02±0.82 (6)	10.00±1.01 (5)	10.79±0.82 (4)
GluNH$_2$	L-GluNH$_2$	4.86±0.58 (7)	4.98±1.22 (4)	5.80±0.51 (4)[b]	5.39±0.77 (4)
	L-GluNHMt	5.02±0.13 (5)	5.01±0.42 (3)	5.61±0.42 (3)	5.88±0.33 (3)
	L-GluNHEt	4.86±0.58 (7)	4.30±0.45 (6)	4.29±0.34 (5)	4.48±0.06 (4)
Asp	L-GluNH$_2$	2.78±0.54 (7)	2.78±0.52 (4)	3.05±0.52 (4)	3.04±0.65 (4)
	L-GluNHMt	3.03±0.55 (5)	2.34±0.33 (3)	3.02±0.11 (3)	2.73±0.29 (3)
	L-GluNHEt	2.78±0.54 (7)	2.58±0.72 (6)	2.48±0.41 (5)	2.58±0.13 (4)

Amino acid	Amide	Amino acid level (μ moles/g wet weight)[a] 60	75	90 (min)
GABA	L-GluNH$_2$	2.71±0.21 (4)	2.25±0.35 (4)	2.49±0.28 (4)
	L-GluNHMt	2.43±0.33 (3)	2.33±0.12 (3)[b]	2.23±0.06 (3)[b]
	L-GluNHEt	2.64±0.17 (4)	2.31±0.36 (4)	2.31±0.45 (4)
Glu	L-GluNH$_2$	9.94±1.10 (4)	9.41±0.56 (4)	9.81±0.71 (4)
	L-GluNHMt	10.31±1.02 (3)	9.94±2.02 (3)	10.71±0.90 (3)
	L-GluNHEt	10.08±1.08 (4)	10.57±0.50 (4)	10.43±1.81 (4)
GluNH$_2$	L-GluNH$_2$	5.60±0.62 (4)	5.10±0.69 (4)	4.87±0.30 (4)
	L-GluNHMt	5.81±0.79 (3)	5.82±0.51 (3)	5.26±0.37 (3)
	L-GluNHEt	4.41±0.27 (4)	4.28±0.40 (4)	4.01±0.23 (4)[b]
Asp	L-GluNH$_2$	2.97±0.79 (4)	2.47±0.34 (4)	2.62±0.38 (4)
	L-GluNHMt	2.76±0.27 (3)	2.95±0.36 (3)	2.81±0.51 (3)
	L-GluNHEt	2.99±0.25 (4)	2.73±0.22 (4)	2.16±0.26 (4)

Each amide was administered 7.5 mmoles/kg body weight *i.p.*
Figures in parentheses are No. of experiments.
a) mean±S.D.
b) $P<0.05$

Discussion

In the present study it was shown that L-GluNHEt antagonized the central stimulant action of caffeine in mice.

Tower[7] described his observation on a suppresive effect of L-GluNH$_2$ on the convulsion induced by methionine sulfoximine, but the later work by Swinyard, *et al.*[8] could not confirm the result. In our study, L-GluNH$_2$ did not show any antagonistic action against the onsets of tonic convulsions produced by pentetrazole, picrotoxin, strychnine, bemegride and pipradrol.

A study was made to assess as to whether L-GluNHEt might be capable of potentiating hypnotics. This method is generally used for screening some central depressants and tranquilizers. As is evident from the data presented in Table IV, neither L-GluNH$_2$ nor L-GluNHEt prolonged to any significant extent the disappearence of righting reflex that had been produced by hexobarbital sodium.

7) D.B. Tower, *Neurology*, **5**, 113 (1955).
8) E.A. Swinyard, L. Chin, F.R. Cole and L.S. Goodman, *Proc. Soc. Exptl. Biol. Med.*, **94**, 12 (1957).

An evidence has been shown for the anticonvulsant activity of intracerebrally adminstered GABA.[9] And such compounds generally referred to anticonvulsants as hydroxylamine,[10] L-glutamic acid-5-hydrazide[11] and aminoxyacetic acid[12] were shown to increase the cerebral GABA content, while semicarbazide[10c,13] and thiosemicarbazide[10c,14] decreased the level of GABA in brain and produced convulsive responses. Though the intracerebral level of GABA itself is in no way directly implicable in the genesis of convulsive seizure,[10c,14b] the effects of the amino acid amides on the cerebral levels of GABA and related amino acids were estimated.

Significant increase of GABA level was shown in mice 30 min after the administration of L-GluNHEt. Thirty minutes after the intraperitoneal administration of L-GluNHEt, the amide was incorporated in the mouse brain without any metabolic changes such as hydrolysis in our experiment (Table V). Therefore, the increased cerebral GABA seemed to be originated from endogenous amino acids.

Further studies on the mechanisms antagonistic action by L-GluNHEt against the tonic convulsion produced by caffeine are in progress in our laboratory.

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