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EFFECTS OF ACUTE AND CHRONIC ETHANOL ON THE γ-AMINOBUTYRIC ACID SYSTEM IN RAT BRAIN

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Abstract—The effects of acute and chronic ethanol treatment have been investigated on the cerebral concentration of γ-aminobutyric acid (GABA). In addition, the activities of both L-glutamate 1-carboxylyase (GAD) and 4-aminobutyrate: 2-oxoglutarate aminotransferase (GABA-T) have been measured and also the rate of ^{3}H-GABA disappearance following its intracisternal injection into rat brain. Acute ethanol administration caused no significant change in the GABA concentration. There was, however, an increase in GAD activity, but no change in GABA-T activity or the rate of disappearance of ^{3}H-GABA. Chronic ethanol treatment, on the other hand, caused a 48 per cent increase in GABA concentration. There was no simultaneous change in GAD activity but the GABA-T activity was significantly increased. Again there was no change in the rate of disappearance of ^{3}H-GABA. It is suggested that these effects may be due to an action of ethanol on the metabolic pool of GABA as distinct from the transmitter pool.

ETHANOL is known to be a central nervous system (CNS) depressant but its mode of action is uncertain. It is possible that part of the depression may be mediated by altering the content or metabolism of the putative transmitter, γ-aminobutyric acid (GABA). GABA is present in mammalian brain tissue[1,2] and its likely role as an inhibitory neurotransmitter has been well documented.[3,4] GABA is also known to have a role in intermediary cerebral metabolism[4–7] and there is now much evidence to suggest that GABA exists in at least two pools within the CNS.[8–10]

There is considerable literature on the acute and chronic effects of ethanol on the absolute levels of GABA found in the brain, but the results of these experiments have been mostly inconsistent. Some workers have found an increase in GABA concentration,[11–13] some a decrease[14–16] and others found no change at all.[17] It was therefore interesting to investigate the effects of acute and chronic ethanol administration on the dynamic aspects of the GABA system. This was done by estimating the GABA content, the GABA metabolizing enzyme activities and the rate of disappearance of labelled GABA from rat brain.

METHODS AND MATERIALS

Acute ethanol experiment. Male Wistar rats weighing between 160–200 g were used. Food was withheld for 24 hr before the experiment but drinking water was available *ad lib.* Ethanol was administered intraperitoneally in a dose of 4 g/kg as a 20% solution in saline. Control rats received the same volume of saline. Thirty min after

these injections, groups of experimental and control rats were injected with ^{3}H-γ-aminobutyric acid (^{3}H-GABA) intracisternally and sacrificed at intervals. Parallel groups of rats were sacrificed at the same times after receiving the ethanol for estimation of cerebral GABA and enzyme activities. A limited number of experiments were performed on rats receiving 0·5 g ethanol/kg.

Chronic ethanol experiment. Male Wistar rats weighing 130 g at the start of the experiment were used. The rats were given a normal diet and a 14% solution of ethanol in water as the only available drinking water for 3 weeks. A corresponding control group was given ordinary tap water. Again the rats were starved for 24 hr before receiving an intracisternal injection of ^{3}H-GABA or being sacrificed for estimation of cerebral GABA and enzyme activity.

In each series of experiments, the disappearance of ^{3}H-GABA with time, the GABA content and the activity of both L-glutamate 1-carboxylyase (GAD: EC 4.1.1.15) and 4-aminobutyrate: 2-oxoglutarate aminotransferase (GABA-T: EC 2.6.1. 19) were determined in whole brain. Also, blood alcohol concentrations were determined.

Estimation of the rate of disappearance of ^{3}H-GABA. Rats were injected intracisternally[18] with 10 μCi of [2,3-^{3}H]GABA (N.E.N. Dreieichenhain, W. Germany) in a volume of 20 μl of Merlis solution.[19] In the acute ethanol experiments, the dose of ethanol used was anaesthetic and no further anaesthesia was necessary for the injection procedure. In all control rats and those receiving chronic ethanol pretreatment, light ether anaesthesia was used. The amount of GABA injected was only 0·2% of the endogenous GABA content of brain, so it can be considered a tracer dose. Animals were killed at 0·5, 1, 3 and 5 hr after injection. Within 1 min after death, brains were extracted in 3 vol. of 0·4 N perchloric acid. Amino acids were isolated on columns of Amberlite CG 120, H^+ form, and eluted with 2 N ammonium hydroxide.[20] The eluate was evaporated to dryness and reconstituted in 0·5 ml distilled water. 0·1 ml aliquots were run on Silica gel thin-layer chromatography plates using butanol–acetic acid–water (8:2:2) as solvent, to separate GABA from the other amino acids. The locations of the separated amino acids were visualized by spraying with 0·25% ninhydrin, and the GABA spot scraped off into a counting vial containing 0·4 ml water. In initial experiments, the GABA spot was shown to be free of other amino acids by passage through a Bio-Cal BC 100 amino acid analyser.

The isolated GABA fraction together with 0·1–0·2 ml aliquots of the original crude supernatant and the reconstituted ammonium hydroxide eluate were counted by liquid scintillation spectroscopy using 4 ml of 2-ethoxyethanol plus 10 ml of 0·5% w/v butyl PBD in toluene. The recovery of GABA over the entire procedure was consistently between 56 and 70 per cent. None of the results have been corrected for this recovery.

Determination of GABA concentration. Endogenous GABA was measured using the enzymic method originally described by Jakoby and Scott[21] and modified by Kravitz *et al.*[22] Freeze-dried bacterial extract containing GABA-T and succinic semialdehyde dehydrogenase (EC 1.2.1.) was obtained from Worthington Biochemicals Ltd via Cambrian Chemical Co., Croydon, U.K. This product was claimed by the manufacturers to be free of NADPH oxidase activity, and our own experiments as well as those of Okada *et al.*[23] have shown that any slight oxidase activity present does not affect the GABA assay.

All brain samples used for the assay of endogenous GABA were homogenized in 0·01 N perchloric acid within 1 min of the rat's death and stored frozen. This timing was always strictly followed, and was unlikely to lead to any significant post mortem changes in GABA levels.[24,25]

Determination of GAD activity. The activity of GAD was determined by measuring the production of $^{14}CO_2$ from DL-1-[^{14}C]glutamic acid (Radiochemical Centre, Amersham) according to the method of Roberts and Simonsen.[26] Brains were homogenized in sodium phosphate buffer pH 6·4 containing Triton X-100 (0·5%), β-mercaptoethanol (0·1%) and ethylenediaminetetraacetic acid (0·25%), and stored at 5° overnight. Under these conditions 11 per cent of the activity was lost. Care was taken not to freeze the extracts, since this resulted in a considerable loss of activity. The assays were performed in Warburg flasks, in the presence and absence of added pyridoxal-5′-phosphate.

Determination of GABA-T activity. The activity of GABA-T was determined by the fluorimetric assay of Salvador and Albers.[27] Brains were homogenized in sodium phosphate buffer pH 7·4 and frozen overnight to further break up the mitochondrial membranes to which GABA-T is bound. The presence of added pyridoxal-5′-phosphate was found not to increase the activity of GABA-T and it was also found unnecessary to dialyse the enzyme preparation before doing the assay.

The succinic semialdehyde necessary for the calibration curve was prepared by the method of Bruce *et al.*[28] and its concentration was assayed by the method of Jakoby[29] using preparations of *Pseudomonas fluorescens* as the source of succinic semialdehyde dehydrogenase activity.

Determination of blood alcohol level. Blood samples were taken from animals at the time of death and assayed enzymatically, as described by Bonnichsen.[30]

RESULTS

Acute ethanol experiment. The acute administration of ethanol (4 g/kg) gave blood alcohol levels after 1 hr of 360 ± 14 mg/100 ml blood ($n = 6$), which dropped to 269 ± 13 mg/100 ml blood ($n = 5$) after 5·5 hr. At these blood levels the rats were anaesthetized for the duration of the experiment.

The effect of this dose of ethanol on the endogenous GABA concentration in whole brains is shown in Table 1. It can be seen that there is no significant ($P > 0·05$) increase in the GABA concentration after the acute administration of ethanol.

In the experiments in which ^{3}H-GABA was injected intracisternally, the disappearance from brain of total radioactivity, radioactive amino acids and ^{3}H-GABA was followed. Results from control experiments are shown in Fig. 1. Each tritium-labelled fraction showed an initially fast rate of disappearance which became progressively slower. To facilitate analysis, the early period between 0·5 and 1 hr was treated as one exponential phase and the later period between 3 and 5 hr was treated as a second exponential phase. The fractional rate constants (k) for each phase of the disappearance of ^{3}H-GABA were calculated using the formula $k = 2·303 \times$ slope, and are presented in Table 2. Pretreatment of rats with an acute dose of ethanol did not significantly alter ($P > 0·05$) any of the fractional rate constants.

GAD activity in the whole brain of rats following an acute dose of ethanol is shown in Table 1. When the assay was performed in the presence of exogenous pyridoxal-5′-phosphate, there was a small but significant increase in the GAD activity at 1·5 hr

after the ethanol pretreatment. When no pyridoxal-5'-phosphate was added to the assay mixture, the increase was apparent at 1·5 hr and also at 3·5 and 5·5 hr after the pretreatment. Simultaneous estimates of the activity of GABA-T (Table 1), however,

TABLE 1. EFFECT OF ACUTE AND CHRONIC ETHANOL ADMINISTRATION ON GABA CONCENTRATION AND ENZYME ACTIVITIES

	GABA concn (μmoles/g)	GAD activity (mmoles/kg/hr)		GABA-T activity (mmoels/kg/hr)
			Added pyridoxal-5'-phosphate	
Acute ethanol				
Controls	0·72 ± 0·05 (7)	8·02 ± 0·12 (7)	15·7 ± 0·30 (7)	63·0 ± 3·13 (10)
Time after ethanol administration (hr)				
1	0·74 ± 0·09 (4)	8·43 ± 0·15 (4)	15·9 ± 0·37 (4)	59·9 ± 1·34 (4)
1·5	0·69 ± 0·07 (4)	*8·94 ± 0·16 (4)	*17·3 ± 0·56 (4)	64·2 ± 2·60 (8)
3·5	0·86 ± 0·07 (4)	*8·98 ± 0·33 (4)	16·5 ± 0·28 (4)	60·9 ± 1·35 (4)
5·5	0·80 ± 0·08 (4)	*8·81 ± 0·14 (4)	16·8 ± 0·75 (4)	57·5 ± 1·89 (4)
Chronic ethanol				
Controls	0·98 ± 0·10 (6)	8·70 ± 0·32 (10)	15·3 ± 0·47 (10)	56·8 ± 1·08 (10)
Chronic ethanol group	*1·45 ± 0·09 (6)	8·69 ± 0·35 (10)	15·6 ± 0·38 (10)	*61·7 ± 1·47 (10)

* Values significantly greater than corresponding controls at $P < 0·05$.
Numbers in parentheses represent number of animals.

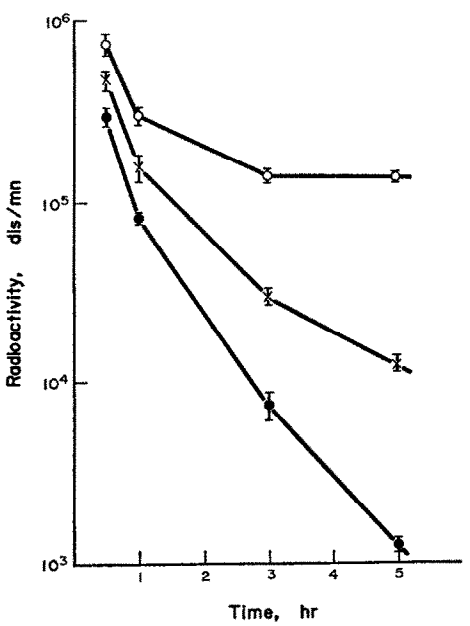

FIG. 1. Rate of disappearance of radioactivity with time in rat whole brain, after the intracisternal injection of 10 μCi of ^{3}H-GABA. Each point is the mean of six experiments ± S.E.M. Total radioactivity (○); radioactive amino acids, (×); and ^{3}H-GABA (●). The fractional rate constants for the various phases are seen in Table 2.

showed that the acute dose of ethanol (either 0·5 or 4 g/kg) had no effect on the activity of this enzyme.

Addition of ethanol in concentrations of 4–40 mg/ml to the incubation mixtures for the assays of both GAD and GABA-T from control rat brains did not significantly affect the enzyme activities. Thus, it is unlikely that the changes in GAD activity in pretreated rats were due to the presence of ethanol in the assay mixture.

TABLE 2. FRACTIONAL RATE CONSTANTS k (sec^{-1}) OF THE DISAPPEARANCE OF RADIOACTIVE GABA AFTER ADMINISTRATION OF ^{3}H-GABA

Control		Acute ethanol	
0·5–1 hr	3–5 hr	0·5–1 hr	3–5 hr
2·44 ± 0·33	0·87 ± 0·09	2·33 ± 0·38	0·94 ± 0·15
Control		**Chronic ethanol**	
0·5–1 hr	3–5 hr	0·5–1 hr	3–5 hr
2·62 ± 0·41	0·48 ± 0·13	2·49 ± 0·39	0·32 ± 0·16

All k values determined from 10–12 points.

Chronic ethanol experiments. Chronic administration of ethanol in the drinking water of rats gave a blood alcohol concentration of 74·7 ± 10·1 mg/100 ml blood ($n = 7$) if the rats were killed at 10 a.m. and 37·8 ± 5·1 mg/100 ml blood ($n = 4$) if the rats were killed at 3 p.m. From Table 3, it can be seen that the brain and body weights of the chronic ethanol group were significantly lower than those of the control group. Also the volume of fluid consumed per day was 2·2-times greater for the control group. There were no apparent differences between experimental and control groups of rats in their gross behaviour.

TABLE 3. EFFECT OF CHRONIC ETHANOL ADMINISTRATION ON GROWTH

	Body wt* (g)	Brain wt (g)	Fluid consumed (ml/day)
Controls	216 ± 3·2 (20)	1·697 ± 0·014 (26)	30 (Water)
Chronic ethanol group	†186 ± 6·2 (20)	†1·624 ± 0·012 (26)	‡14 (Ethanol solution)

* Both the control and chronic ethanol group rats began the experiment weighing 130 g.
† Significantly lower than controls at $P < 0·001$.
‡ 14 ml/day of a 14% ethanol solution is equivalent to 2 g ethanol/day.

The effect of chronic ethanol pretreatment on the endogenous concentration of GABA in the whole brain is shown in Table 1. There was a 48 per cent increase in the GABA concentration due to the chronic administration of ethanol and this increase was still statistically significant if the total GABA contents, rather than concentrations, were compared.

In the experiments in which ^{3}H-GABA was injected intracisternally, the disappearance of radioactivity from the brain was analysed in a similar manner to that described for the acute ethanol experiments. Chronic pretreatment with ethanol did not significantly affect the fractional rate constant of disappearance of ^{3}H-GABA (Table 2). Estimates of GAD and GABA-T activities are shown in Table 1. There was no significant change in the GAD activity in rat brains after chronic treatment with ethanol, either in the presence or absence of exogenous pyridoxal-5′-phosphate. The same results were obtained whether the rats were killed in the morning or the afternoon. There was, however, a significant increase in GABA-T activity in brain following the chronic administration of ethanol. This increase was apparent in rats killed in either the morning or the afternoon.

DISCUSSION

After administering ethanol (4 g/kg) intraperitoneally to rats, there was no significant change in GABA concentration or GABA-T activity, but there was a significant increase in the GAD activity of brain. Since this increase was apparent when the assay was performed in the presence of added pyridoxal-5′-phosphate, it suggests that there was an increase in either the specific activity or the amount of apoenzyme present. It is also possible that the amount of pyridoxal-5′-phosphate bound to GAD *in vivo* increased after ethanol treatment, since there was an even greater increase in GAD activity when the assay was performed without exogenous pyridoxal-5′-phosphate.[31]

From the experiments in which the rate of disappearance of ^{3}H-GABA was followed, some idea may be obtained as to whether alcohol pretreatment caused any change in GABA turnover in brain. The intracisternally injected ^{3}H-GABA is rapidly taken up by brain tissue[32] and equilibrates with endogenous GABA[33,34] without significantly increasing the overall amount of GABA present in the brain. In this way, the transmitter pool of GABA in nerve terminals becomes labelled.[35] The subsequent disappearance of ^{3}H-GABA will, therefore, reflect the rate of GABA utilization and resynthesis in nerve terminals. In rats which had received an acute dose of ethanol, there was no significant difference from controls in the fractional rate constants of ^{3}H-GABA disappearance in either of the two phases analysed. Thus, it is unlikely that there was any significant effect on the turnover of the transmitter pool of GABA. The small changes in GAD activity without any significant change in endogenous GABA may reflect a minor increase in the turnover of a metabolic pool of GABA.[8–10]

In rats which had received chronic pretreatment with ethanol, it was necessary to distinguish between acute effects due to the presence of ethanol in the body at the time of death, and chronic effects due to prolonged exposure to ethanol. This was achieved by comparing results of rats killed in the morning with those killed in the afternoon, since rats drink mainly at night and the blood ethanol levels were consequently twice as high in the morning as in the afternoon.

Chronic pretreatment with ethanol had no effect on GAD activity, measured either in the morning or the afternoon, in contrast to the effect of the acute dose of ethanol. This was probably related to the blood levels of ethanol in the latter case being five- to ten-times higher than those seen in chronically treated rats. Thus, GAD activity is increased only by large acute doses of ethanol. The activity of GABA-T, however, was significantly increased by chronic pretreatment with ethanol. This was a chronic and

not an acute effect of ethanol, since the increase in GABA-T activity was present in rats killed either in the morning or the afternoon. Also, the increase was not apparent following an acute dose of ethanol, either 0·5 or 4 g/kg.

Similarly, the significant increase in endogenous GABA concentration following chronic pretreatment with ethanol was a chronic rather than an acute effect, since it was barely apparent at the higher blood levels of ethanol achieved after an acute dose. The significant difference between the GABA concentrations in acute and chronic control animals (Table 1) probably represents a real difference between the groups of animals used for the two series of experiments.

As in the case of rats receiving an acute dose of ethanol, chronic pretreatment resulted in no significant effect on the fractional rate constants of disappearance of ^{3}H-GABA from brain. If there had been a change in the activity of neurones releasing GABA as an inhibitory transmitter, a similar change in the fractional rate constant of ^{3}H-GABA disappearance would have been expected. But this appears not to be the case. On the other hand, the chronic effects of increased endogenous GABA and GABA-T activity probably represent a substantial increase in the turnover of the metabolic pool of GABA. It is unlikely that this was due to increased concentrations of acetate resulting from metabolism of ethanol,[36] since changes in GABA metabolism after large acute doses of ethanol were minimal in the present experiments. Unfortunately, there is little literature concerning the effects of chronic ethanol treatment on cerebral metabolism. The present results suggest that changes in metabolism do occur during long-term treatment with ethanol and that this is an area which should be further investigated.

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