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

IRENE SUTTON and MICHAEL A. SIMMONDS

Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London, WC1N 1AX, England

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Abstract—The effects of an acute anaesthetic dose and of chronic administration of pentobarbitone have been investigated on the GABA system in rat brain. This was done by estimating the cerebral γ-aminobutyric acid (GABA) concentration, the activities of both L-glutamate 1-carboxylyase (GAD) and 4-aminobutyrate: 2-oxoglutarate aminotransferase (GABA-T), and also the rate of ^{3}H-GABA disappearance following its intracisternal injection. Neither acute nor chronic barbiturate administration caused any marked changes in the GABA concentrations or the enzyme activities. The exponential disappearance curves observed after injection of a pulse label of ^{3}H-GABA were resolved into "fast" and "slow" components. Acute pentobarbitone was found to significantly decrease the rate constant of the fast component, whilst chronic pentobarbitone significantly decreased the rate constant of the slow component. The possible morphological correlates of these components are discussed. It is concluded that pentobarbitone has distinct actions on the GABA system which are different from those of other central nervous system depressants.

INTRODUCTION

GAMMA-AMINOBUTYRIC acid (GABA) has been suggested as having a role as an inhibitory neurotransmitter in mammalian brain tissue.[1-3] If this is correct, one might expect that some centrally acting drugs would interact with GABA neurones or receptors, altering the turnover of GABA or affecting its concentration and metabolism.

We have recently published the results of an investigation into the effects of acute and chronic ethanol treatment on the GABA system.[4] While acute anaesthetic doses of ethanol were without marked effect, chronic administration of ethanol caused a significant increase in the endogenous content of GABA and an increase in the activity of GABA-T in rat brain. However, the rate of disappearance of a pulse label of ^{3}H-GABA remained unaffected. In an attempt to determine whether these actions are common to all CNS depressants or are specific to ethanol alone, we have now investigated the effects of barbiturates on the GABA system.

The literature concerning the acute and chronic effects of barbiturates on cerebral GABA levels is inconsistent. Some people have reported no change in the GABA concentrations,[5,6] whilst some have observed increases[7] and others decreases.[8] There is little information on the effect of barbiturates on the turnover of GABA, but they have been shown to decrease the turnover of dopamine,[9] 5-hydroxytryptamine[10,11] and noradrenaline[12,13] in the brain. We have, therefore, investigated the effects of

acutely and chronically administered barbiturate on the turnover of GABA in rat brain. At the same time, we have estimated the concentration of GABA and the activities of the enzymes that synthesize and metabolize GABA.

METHODS AND MATERIALS

The effects of acute and chronic barbiturate administrations on the rate of disappearance of a pulse label of ^{3}H-GABA, the endogenous GABA content and the activities 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.

Acute barbiturate experiment. Male Wistar rats weighing 170–200 g were used. They were injected intraperitoneally with sodium pentobarbitone in saline (May & Baker Ltd, Dagenham, England) given as two injections of 25 mg/kg each, 10 min apart. Additional doses of 10 mg/kg were given whenever necessary to maintain a sufficient level of anaesthesia throughout the experiment, so that the righting reflex was completely suppressed. Control rats received the same volume of saline intraperitoneally. In the experiments where the disappearance of a pulse label of ^{3}H-GABA was to be followed, the ^{3}H-GABA was injected 30 min after the first injection of barbiturate or saline and the rats were sacrificed at intervals. Endogenous GABA concentrations were also measured in these experiments. Parallel groups of rats which did not receive the ^{3}H-GABA injections were sacrificed at the same times after receiving the pentobarbitone for estimation of enzyme activities.

Chronic barbiturate experiment. Male Wistar rats weighing 130 g at the start of the experiment were used. Rats were given a normal diet and pentobarbitone dissolved in water as the only available drinking water for 2 weeks. The dose regime used has been shown to be acceptable to rats.[6,14] In the first week each rat drank the equivalent of 100 mg/kg every day, and this was increased to 200 mg/kg in the second week. To camouflage the bitter taste of the barbiturate solution, one saccharin sodium tablet (Hermes Sweeteners Ltd, Zurich, Switzerland) was added for every 100 ml. Control rats received the same number of saccharin tablets in tap water. The motor activity of the rats was assessed by using an activity monitor (C. F. Palmer, High Wycombe, England). Test and control rats were monitored from 7 p.m. to 9 a.m. on alternate nights over the 2 week treatment period. Again, two parallel series of experiments were performed; one to estimate the disappearance of ^{3}H-GABA injected intracisternally and the cerebral GABA concentration, the other to determine the activities of GAD and GABA-T.

Determination of the rate of disappearance of ^{3}H-GABA. Rats were injected intracisternally[15] with 10 μCi of [2,3-^{3}H] GABA (N.E.N. Dreieichenhain, W. Germany) in a volume of 20 μl of Merlis solution.[16] The specific activity of the ^{3}H-GABA used was so high (10 Ci/m-mole), that the amount of GABA injected was only 0·04 per cent of the endogenous GABA content of the brain. This may be regarded as a tracer dose. In all control rats and those receiving chronic barbiturate pretreatment, light ether anaesthesia was used for the injection procedure, but no further anaesthesic was necessary for the rats receiving an acute dose of barbiturate. Animals were killed at 5, 20, 30 and 45 min, 1, 3 and 5 hr after the intracisternal injection of radioactive GABA, and the brains were removed and homogenized within 1 min. The procedure used for isolating ^{3}H-GABA from the radioactive amino acid and acidic metabolites

has been previously described.[4] The only difference in the present experiments was that the brains were homogenized in 0·01 N HCl and, after removing an aliquot for the endogenous GABA assay, sufficient 1·6 N $HClO_4$ was added to the homogenate to make the final solution 0·4 N with respect to $HClO_4$.

Determination of GABA concentration. The determination of endogenous GABA concentration was based on the fluorimetric assay described by Lowe *et al.*,[17] as part of their method for measuring GAD activity. The assay depends on the formation of a fluorescent product from the reaction between GABA and ninhydrin at alkaline pH and in the presence of glutamate. The assay has previously been used satisfactorily to determine GABA concentrations in brain.[18] The reagents used in the assay were 0·05 M glutamic acid in 0·2 M sodium phosphate buffer, pH 6·4; 14 mM ninhydrin in 0·5 M sodium carbonate buffer, pH 9·9–10; and copper tartrate reagent consisting of 1·6 g Na_2CO_3, 329 mg tartaric acid and 300 mg $CuSO_4$, $5H_2O$ all made up in 1 litre of water.

The whole rat brain was homogenized in 4 ml of 0·01 N HCl and 0·25 ml of this was used for the GABA assay, the remainder being used to estimate the rate of disappearance of ^{3}H-GABA. The 0·25 ml of homogenate was diluted with 0·25 ml of 0·01 N HCl and 0·5 ml of 10% trichloracetic acid was added to precipitate the protein. After the samples were centrifuged, 100 μl aliquots of the supernatant were added to 15 μl of the glutamate solution and 200 μl of the ninhydrin solution. This mixture was then incubated at 60° for 30 min and allowed to cool before the addition of 5 ml of copper tartrate reagent. After 15 min the fluorescence of the samples was measured at 450 nm with an activating wavelength of 380 nm. Internal standards were prepared by adding to a sample of homogenate known amounts of GABA (12·5–100 μg) with the trichloracetic acid. No completely satisfactory tissue blank could be prepared. The best blank that could be achieved required the omission of the glutamate solution from the reaction mixture. The glutamate solution is important in the test samples since it selectively increases the amount of fluorescence due to GABA by approximately one hundred times, as well as greatly extending the range of constant proportionality.[17] The endogenous GABA values obtained using this method and this type of tissue blank were in good agreement with those quoted in the literature.[19–21]

Determination of enzyme activities. The activity of GAD was determined by the radiochemical method of Roberts and Simonsen,[22] and the GABA-T activity by the fluorimetric assay of Salvador and Albers,[23] with minor modifications described previously.[4]

RESULTS

Acute barbiturate experiment

The acute administration of an anaesthetic dose of sodium pentobarbitone had only a small effect on the endogenous GABA concentration. It can be seen in Table 1 that there was a significant but small increase in the GABA concentration 1 hr after the barbiturate injection, whilst 30 min after this there was a significant decrease in the GABA concentration when compared with the control values. The cerebral GABA concentrations at 3·5 and 5·5 hr after administration of the barbiturate were not significantly different from the control.

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

	GABA concn (μmoles/g)	GAD activity (m-moles/kg/hr) Acute barbiturate	Added pyridoxal-5'-phosphate	GABA-T activity (m-moles/kg/hr)
Controls	1·50 ± 0·05 (19)	7·04 ± 0·06 (4)	11·45 ± 0·12 (4)	50·18 ± 2·24 (4)
Time after barbiturate administration (hr)				
1	1·80 ± 0·05* (4)	7·05 ± 0·28 (5)	10·73 ± 0·53 (5)	50·08 ± 2·37 (5)
1·5	1·27 ± 0·03* (4)	7·42 ± 0·28 (5)	10·82 ± 0·49 (5)	49·00 ± 1·79 (5)
3·5	1·52 ± 0·09 (4)	7·33 ± 0·26 (5)	11·29 ± 0·64 (5)	48·95 ± 2·27 (5)
5·5	1·45 ± 0·05 (5)	7·85 ± 0·28* (5)	11·62 ± 0·51 (5)	50·19 ± 1·98 (5)
		Chronic barbiturate		
Controls	1·54 ± 0·04 (10)	7·08 ± 0·19 (6)	11·99 ± 0·31 (6)	49·14 ± 1·15 (6)
Chronic barbiturate group	1·52 ± 0·03 (10)	6·63 ± 0·20 (6)	10·63 ± 0·36* (6)	51·36 ± 0·54 (6)

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

GAD activity in the whole brain of rats following an acute dose of sodium pentobarbitone is shown in Table 1. When the assay was performed in the absence of exogenous pyridoxal-5'-phosphate, there was a small but significant increase in the GAD activity apparent at 5·5 hr after the pretreatment. When exogenous cofactor was added to the assay mixture, there were no significant changes in the GAD activity due to the pretreatment. Simultaneous estimates of GABA-T activity (Table 1) showed that the acute barbiturate treatment had no effect on the activity of this enzyme.

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. The disappearance curves of ^{3}H-GABA for control and barbiturate treated rats are shown in Fig. 1. Both of the multiphasic exponential curves show an initial fast rate of disappearance which becomes progressively slower. To facilitate analysis, the curves were mathematically resolved into two exponential components. This was done by linear extrapolation of the slope between the 3 and 5 hr values (slow component) backwards and then subtracting this line from the observed disappearance curve.[24] Where the resultant fast component had a measurable value at 3 hr, the slope of the slow component was reduced and the fast component recalculated until the sum of the two components fitted the original curve. The calculated components of the disappearance curves in Fig. 1a are shown in Fig. 1b and their fractional rate constants (k) in Table 2. It can be seen that there is a significant slowing of the fast component after acute treatment with pentobarbitone, whereas the slow component was unaffected.

Chronic barbiturate experiment

Rats receiving sodium pentobarbitone chronically for two weeks showed no behavioural changes from control rats. There was no significant difference in the night

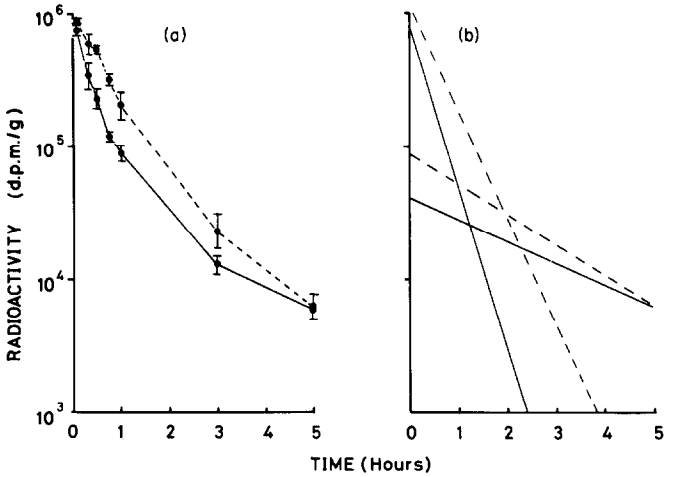

FIG. 1. The effect of acute pentobarbitone on the rate of disappearance of ^{3}H-GABA in rat whole brain, after intracisternal injection of 10 μCi of ^{3}H-GABA. Acute pentobarbitone experiment (– – –); and control experiment (——). (a) Each point is the mean of 4–10 values ± S.E.M. (b) Mathematical resolution of the curves in (a), into two components, which have been calculated by regression line analysis.

time motor activity of the two groups over the 2 week treatment period, neither did visual observation of day time behaviour reveal any differences. Withdrawal symptoms could, however, be elicited 24–48 hr after the last ingestion of barbiturate. These took the form of a catatonic state in response to a 96–100 dB bell for 1 min. Control rats were unaffected. The chronic barbiturate treatment had no effect on the growth rate of the rats as measured by brain and body weights.

Chronic barbiturate administration caused no significant changes in the cerebral GABA concentration nor was the activity of GABA-T affected (Table 1). The activity of GAD was unchanged when the assay was performed in the absence of exogenous pyridoxal-5′-phosphate, but there was a very small but statistically significant decrease of GAD activity in the brains of pretreated rats when the reaction mixture contained added cofactor (Table 1).

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

	Fast component	Slow component
	Acute experiment	
Control	2·74 ± 0·33 (35)	0·38 ± 0·11 (9)
Test	1·82 ± 0·28* (32)	0·53 ± 0·20 (9)
	Chronic experiment	
Control	2·93 ± 0·39 (16)	0·84 ± 0·08 (19)
Test	2·96 ± 0·15 (27)	0·36 ± 0·08* (9)

* Values significantly smaller than corresponding controls at $P < 0·05$.

Numbers in parentheses represent number of points used to determine the k values.

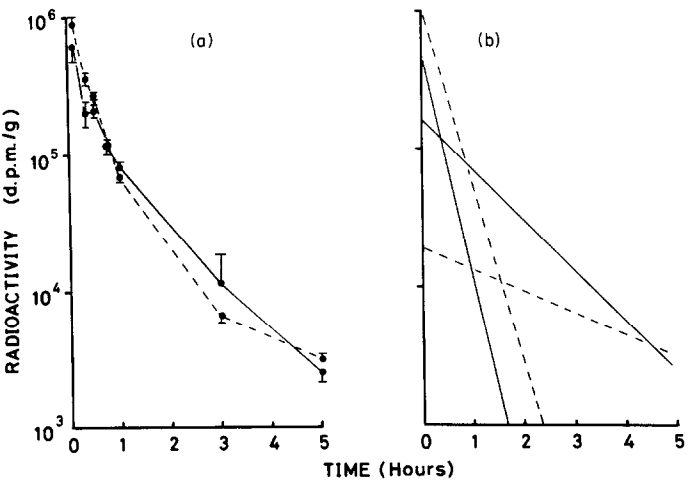

FIG. 2. The effect of chronic pentobarbitone administration on the disappearance of ^{3}H-GABA in rat whole brain. Chronic pentobarbitone experiment (----); and control experiment (——). See Fig. 1 for details.

The rate of disappearance of a pulse label of ^{3}H-GABA from the brains of rats chronically treated with barbiturate and from control rats is shown in Fig. 2a. Again, the curves were mathematically resolved into two components to allow statistical analysis. The calculated components are shown in Fig. 2b and their corresponding fractional rate constants in Table 2. After chronic pentobarbitone treatment, the slow component was significantly slowed compared with the control, whereas the fast component was unaffected.

DISCUSSION

We have attempted, in these experiments, to see whether there is any effect of acute and chronic administration of pentobarbitone on the activity of the GABA system in rat brain. The acutely administered pentobarbitone was sufficient to maintain light anaesthesia. The chronic administration of pentobarbitone, however, caused no behavioural changes and the treatment was probably not long enough for marked dependence to develop.[25,26] The withdrawal symptoms elicited were very mild compared with the audiogenic seizures which can be precipitated in truly dependent rats.[27] Nevertheless, the rats had probably developed some degree of tolerance to barbiturate after 2 weeks of treatment.[28]

Neither the acute nor the chronic administration of barbiturate caused any marked changes in the concentration of GABA in rat brain, or in the enzyme activities of GAD or GABA-T. It is difficult to interpret the transient biphasic change in GABA concentrations following an acute dose of barbiturate, although it may reflect a more substantial change in the activity of the GABA system. The very small changes in GAD activity probably had little effect on the activity of the GABA system as a whole.

Changes in the activity of the GABA system are most likely to be reflected in changes in the rate of disappearance of a pulse label of ^{3}H-GABA from the brain. ^{3}H-GABA injected into the cerebrospinal fluid is actively taken up by brain tissue[29]

and rapidly equilibrates with endogenous GABA.[30,31] The precise site of this uptake appears from autoradiographical[32,33] and cell fractionation studies[34,35] to be both glial and neuronal. There is much evidence for the metabolic compartmentation of GABA into at least two and probably three pools in the C.N.S.[36–40] On the basis of experiments describing the metabolism of GABA in brain tissue[24,30,38,39] and work on the organization of GABA metabolism in the synaptic region,[41] the GABA pools have been allocated morphological sites.[37,38,42] It has been proposed that there is a "small" GABA pool, consisting of 10%[39] to 30%[30] of the total GABA content, which may be concerned with neurotransmission. This pool, which would be situated in nerve terminals has a turnover time in the order of minutes.[24,39] There is also a "large" GABA pool which appears to be involved in metabolism and has a turnover time in the order of hours.[24] It has been suggested that this pool may be located mainly in glial cells and neuronal perikarya.[38] These different GABA pools are not isolated but are dynamically interrelated and in equilibrium with one another.[37–39,43] If the ^{3}H-GABA injected intracisternally labels both pools of GABA,[32–35] then the multi-exponential disappearance of ^{3}H-GABA from brain represents the turnover of both pools.

In the present experiments we have resolved the disappearance curve for ^{3}H-GABA into two exponentials, a "fast component" and a "slow component". The question, therefore, arises whether the fast component predominantly represents the turnover of the neuronal pool of GABA and the slow component predominantly represents the turnover of the glial pool of GABA. It has been shown recently *in vitro* that β-alanine is 200 times more potent as an inhibitor of GABA uptake into glia than it is as an inhibitor of neuronal uptake. Conversely, L-2,4 diaminobutyric acid (DABA) is 20 times more potent as an inhibitor of neuronal uptake than it is of glial uptake.[44] Our own preliminary experiments *in vivo* (unpublished) indicate that, after β-alanine, the ^{3}H-GABA appears mainly in the fast component with little in the slow component. The effects of DABA appear to be the reverse. These preliminary results are compatible with an association of the fast and slow components of ^{3}H-GABA disappearance with the neuronal and glial pools of GABA, respectively, but further experiments are in progress to substantiate this hypothesis.

If we tentatively describe the two components of ^{3}H-GABA disappearance as neuronal and glial, then the effect of acute anaesthetic doses of pentobarbitone is to significantly decrease the turnover of neuronal GABA, leaving the turnover of glial GABA unaffected. Other workers have found that acute pentobarbitone leaves glial metabolism relatively unaffected.[45] The effect on neuronal turnover may be due to an overall reduction of neuronal activity in the brain, since acute barbiturates also cause a decrease in the turnover of dopamine,[9] noradrenaline[12,13] and 5-hydroxytryptamine.[10,11] In contrast, the chronic administration of barbiturate caused a significant decrease in the turnover of glial GABA without affecting the turnover of the neuronal pool of GABA.

These effects clearly differ from those of acute and chronic administration of ethanol.[4] Although acute ethanol, like acute pentobarbitone, was anaesthetic, it did not alter GABA turnover. This suggests that the effect of acute pentobarbitone on neuronal GABA turnover was not simply a consequence of anaesthesia, but due to some specific action of pentobarbitone itself. Chronic ethanol caused a significant increase in the concentration of GABA in brain and increased the activity of GABA-T, but

it did not affect ^{3}H-GABA disappearance.[4] This was interpreted as reflecting an increase in the turnover of the metabolic pool of GABA. The present results with chronic pentobarbitone, however, suggest a reduction in the turnover of the metabolic pool of GABA. Hence, the depressant substances which have been studied, pentobarbitone and ethanol, to which tolerance is developed upon repeated administration, each have distinctly different effects on the GABA system in rat brain.

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