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The Involvement of GABA-Transaminase in the Blood-Brain Barrier to Radiolabelled GABA*

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Summary. The involvement of GABA-transaminase (GABA-T) in the blood-brain barrier to GABA was investigated using a sensitive radiotracer technique which allows measurement of tracer permeation as a cerebrovascular permeability-area product, PA. In Sprague-Dawley rats subjected to peripheral GABA-T inhibition and occlusion of hepatic circulation, chromatographic (TLC) analysis together with lyophylized plasma samples showed the radioactivity measured in brain parenchyma was derived from the permeation of unchanged [^{3}H] GABA and not its blood-borne metabolites. Permeation (PA) of i.v. injected [^{3}H] GABA was poor in all brain regions examined. Pretreatment of these rats with various compounds known to inhibit endothelial GABA-T activity did not cause any increased permeation of [^{3}H] GABA into the brain. These results suggest that the poor permeation of GABA into the rat brain is unrelated to the activity of GABA-T at endothelial sites.

Key words: Blood-brain barrier – GABA-transaminase – Radiotracer – Rats – Permeability-area product

Introduction

The movement of γ-aminobutyric acid (GABA) between the blood and brain parenchyma is severely restricted (Purpura et al. 1958; Van Gelder and Elliott 1958; Kuriyama and Sze 1971; Frey and Löscher 1980), indicative of the existence of a powerful blood-brain barrier (BBB) to this inhibitory neurotransmitter substance. Indeed, the brain uptake index for GABA is the lowest of all the putative central neurotransmitter substances measured (Oldendorf 1971a).

That such a barrier exists is not surprising since a variety of pathophysiological effects are associated with alterations in "CNS" GABA levels, manifest in a number of neurological and psychiatric disorders including epilepsy (Meldrum 1975), Huntington's chorea (Perry et al. 1973) and schizophrenia (Roberts 1977). In addition, this barrier may be effective in preventing diffusion of "free" GABA from the brain, thereby conserving GABA to its central site of action, and preventing GABA actions at peripheral sites where it may have a neurotransmitter function (Krnjevic 1974; Bowery and Brown 1974; Obata 1976; Krantis 1982; Kerr et al. 1983).

Although a "barrier" to GABA is a generally accepted phenomenon, particularly for the development of effective systemic drug therapies (Lippert et al. 1980), the nature of the mechanism(s) underlying this "barrier" remains to be determined. Under normal conditions the movement of substances through the barrier is transcellular via the endothelial cells, by either lipid or carrier mediation. The poor lipid solubility of GABA characterized by its low partition coefficient (Maggi and Enna 1979) is no doubt a factor. However, a current hypothesis holds that the very poor penetrating cabability of GABA may, in part, be due to the activity of its primary catabolic enzyme 4-aminobutyrate: 2-oxoglutarate aminotransferase (GABA-T) (Van Gelder 1965a, b, 1968) analogous with the blood-brain barrier for monoamines (Björklund et al. 1969; Svengaard et al. 1975). Cerebral endothelial cells are enriched in a large number of enzymes which are proposed to be the regulatory mechanisms for the transport of substances between the blood and brain, including amines, amino acids and monosaccharides (Rapoport 1976). Together with the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD), GABA-T, identical to that present in neural tissue (Wu 1981) is found in high concentrations within cerebral endothelial cells of a number of mammals (Van

Gelder 1965a, b; Djuricic et al. 1978; Krause et al. 1980; Wu 1981).

Evidence that inhibition of this "endothelial GABA-T" results in an increased penetration of GABA into the central nervous system (CNS) is controversial. Systemically applied aminooxyacetic acid (AOAA), a potent inhibitor of GABA-T (Kuriyama et al. 1966) prolonged the righting reflex, increased analgesia to induced pain and caused a marked motor weakness in mice injected with GABA (Van Gelder 1965a, 1966). In a whole-body autoradiographic study of mice, Hespe et al. (1969) observed substantial accumulation of radioactivity in the liver and kidney, but no obvious increase in [^{14}C] GABA penetration into the brain or spinal cord of AOAA-treated animals. Similarly, Kuriyama and Sze (1971) found that pretreatment with AOAA did not significantly increase the amount of radioactivity found in the brain of mice injected with radiolabelled GABA. Furthermore, AOAA treatment did not cause any efflux of intraventricular [^{3}H] GABA. More recent studies suggest that muscimol, a structural analogue of GABA, does not readily penetrate into the CNS of mice (Maggi and Enna 1979; Enna et al. 1980). Although AOAA significantly enhanced the amount of systemically administered muscimol accumulated by brain parenchyma, this may merely reflect a reduction in catabolism of muscimol by peripheral GABA-T resulting in elevated blood levels of unchanged muscimol. Under these conditions, the proportion of unchanged "blood-borne" muscimol available for movement into the brain is increased. Indeed, pretreatment of mice with ethanolamine-O-sulphate (EOS, a specific inhibitor of peripheral GABA-T, Fowler and John 1972; Enna et al. 1980), caused a significant increase in the accumulation of unchanged muscimol in brain parenchyma of mice (Enna et al. 1980). According to Borisenko et al. (1983), this barrier to GABA can be made more permeable by the influence of subnarcotic or narcotic doses of alcohol. The authors suggest these changes are dynamic, related in some way to the activity to central GABA-T since at the doses applied, alcohol is proposed to decrease the activity of this enzyme.

Interestingly, pharmacological and autoradiographic studies Krogsgaard-Larsen et al. 1981; Waszczak and Walters 1980), show 4,5,6,7-tetrahydroisoxazolo-[5,4-c]-pyridin-3-ol (THIP), a potent GABA-agonist, to readily penetrate the BBB. This compound has no significant affinity for GABA-T in vitro and therefore a low susceptibility to metabolism.

Despite the obvious contribution to clinical therapy of patients with "GABA"-related disorders, there is presently no study specifically addressing itself to the nature of the blood-brain barrier to GABA. This report details just such an investigation using systemic administration of GABA-T inhibitors together with quantitative radiochemical techniques which allow for accurate measurement of the permeation of radiolabelled substances across cerebrovascular membranes.

Materials and Methods

Male Sprague-Dawley rats (250–320 g b.wt.) were anaesthetised with 50 mg/kg i.p. sodium thiopental and subjected to surgery. Tapered polyethelene catheters (PE 50) were inserted into both femoral arteries for continuous monitoring of blood pressure or blood sampling, and into a femoral vein for i.v. injections. Following surgical procedures, heparin (2 mg in 1 ml saline) was administered i.v.

In selected experiments, pharmacological inhibition of GABA-transaminase was undertaken in conjunction with occlusion of the peripheral blood supply to selected vascular beds: blood vessels supplying the liver, (the coeliac artery and portal vein) together with the inferior and superior mesenteric arteries were ligated 2 min prior to injection of radiolabelled GABA.

Throughout surgical procedures and subsequent radiotracer experiments, rectal temperature was monitored with the use of a YSI 409 thermistor probe and telethermometer connected to a Grass polygraph, and maintained by infrared warming between 37°–38°C for the course of the experiments.

Measurement of Cerebrovascular Permeability-Area Product (PA)

Rats received an i.v. injection of the radiolabelled, γ-[2,3-^{3}H(N)]-aminobutryric acid (NET-191 38 Ci/mmol), New England Nuclear, dissolved in 1 ml 0.9% NaCl in a dose of 2.5 µCi/100 g b.wt., and for the next 30 min arterial blood was sampled either by continous withdrawal at a constant rate (0.04 ml/min) into a 2-ml syringe mounted to a syringe pump, or by 12 serial samples (100 µl) bled into separate microcentrifuge tubes. At the end of the 30-min sampling period, sodium pentobarbital was injected i.v. to stop cardiac function. The rat was then decapitated and the brain quickly excised, rinsed with saline to remove any external blood, cleared of overlying dura and surface vascularities, and dissected. Samples (20 µl) of plasma from the arterial blood sample were drawn from microcentrifuge tubes and placed in vials containing NCS tissue solubilizer (Amersham/Searle). Brain samples were digested in Soluene-100 solubilizer (Packard) and together with plasma samples prepared for scintillation counting (to 1% error with a Beckman LS9000) by adding 10 ml Dimilume-30 (Packard) to all samples.

Assessment of Blood-Brain Barrier Permeability

Following injection of a single bolus of radiolabelled tracer into the bloodstream, the amount of tracer substance diffusing across the cerebral blood vessel walls into brain parenchyma is assumed to occur in proportion to the area under the curve of arterial plasma concentration vs time (plasma integral):

$$\int_0^{30} C_{\text{plasma}}\,dt \text{ (expressed in units of dpm} \cdot \text{s} \cdot \text{ml}^{-1}\text{)}.$$

For each brain region from each rat, permeation of tracer across the cerebrovascular walls was estimated by the product of permeability (P, cm · s^{-1}) and area (A, cm^2 · g^{-1} tissue), PA, from the ratio of parenchymal tracer concentration accumulated after 30 min, C^{30}_{paren} (dpm · g^{-1}) relative to the plasma integral:

$$PA = C^{30}_{\text{paren}} / \int_0^{30} C_{\text{plasma}}\,dt.$$

For non-perfused animals, *PA* was calculated from the following relationship,

$$PA = \frac{C^{30}_{brain} - (C^{30}_{blood} \cdot RBV)}{\int_0^{30} C_{plasma} dt}.$$

Parenchymal uptake was determined by correcting brain counts (C^{30}_{brain}, dpm/g) for intravascular tracer, estimated as the product of regional blood volume space (RBV, ml blood/g brain) and arterial blood concentration at the time of decapitation (C^{30}_{blood}, dpm/ml). The RBV values used were those calculated in this laboratory on the basis of experiments with ^{51}Cr-erythrocytes: cerebrum and pons-medulla – 0.005 ml · g^{-1}: cerebellum – 0.0078 ml · g^{-1}; Preston et al. 1983. Further details of the *PA* radiotracer technique are available elsewhere (Rapoport et al. 1978; Ohno et al. 1978; Preston et al. 1983).

Metabolism of GABA

To determine the presence of volatile radioactivity, 100-µl portions of the plasma supernatant were lyophilized, digested with Soluene-100 solubilizer, then prepared for scintillation counting by adding 10 ml of dimilume-30. Further analysis of plasma for the presence of non-volatile substances was carried out by thin-layer chromatography (TLC) on cellulose (MN 300) plates. Aliquots of the supernatant and of injectate adjusted for a GABA content of 4 mg/ml by the addition of unlabelled GABA were applied (in triplicate) to the TLC plates. The chromatograms were then developed using the solvent system 2-propanol-2 butanone-1N HCl (60:15:25 v/v) (Zweig and Sherma 1972). Portions of the dried chromatograms were developed with ninhydrin over a GABA reference to identify its final position on the plate. The remainder of the TLC plate was then divided appropriately into 1-cm strips from origin to solvent front and the cellulose powder from each strip scraped into a glass vial containing 2 ml of H_2O. The samples were thoroughly mixed, centrifuged and 1.5-ml aliquots of the supernatant removed into scintillation vials. Samples were then counted in 10 ml of dimilume-30 scintillator. Regions yielding tritium counts could then be related to GABA on the ninhydrin-treated segments.

Drugs

The following drugs were used: γ-Aminobutyric acid (GABA), amino-oxyacetic acid hemihydrochloride (AOAA, Sigma, St. Louis, MO, USA), Ethanolamine-O-sulphate (EOS), *d,l*-gabaculine HCl (Calbiochem, Los Angeles, CA, USA). All drugs were dissolved in saline and the doses given in terms of the drug forms indicated above. Except for AOAA which was most effective when administered s.c. (Löscher 1980), drugs were administered either i.v. or i.p. as indicated.

Statistical Analysis

Results are given in figures and tables as means ± SEM. Student's *t*-test for paired and unpaired samples was used to assess the significance ($P \leq 0.05$) of difference between the mean values.

Results

The profile of radioactivity levels in arterial plasma during 30 min following i.v. bolus injection are shown in Fig. 1. The concentration time curve A represents a control rat, untreated for GABA-T inhibition. In these animals, the proportion of non-volatile vs volatile radioactivity in the plasma was approximately 21.8% (Table 1). Pretreatment with GABA-transaminase inhibitors reduced the formation of volatile radioactivity (Table 1). Specifically, amino-oxyacetic acid (AOAA) proved more effective in preventing formation of volatile tritium than either ethanolamine-O-sulfate (EOS) (Table 1) or gabaculine (GAL, results not shown here). Rats subjected to AOAA (25 mg/kg) pretreatment

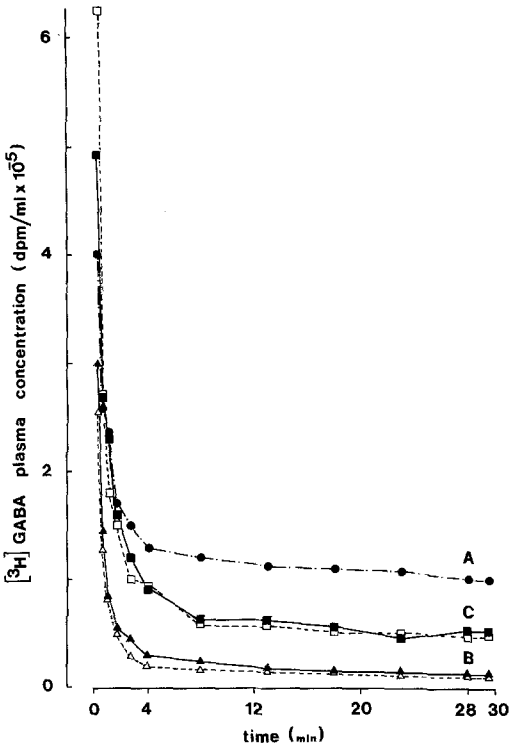

Fig. 1. The arterial plasma concentration profiles for rats administered with [^{3}H] GABA. Each curve represents one experiment. Curve *A* represents control (untreated) rat, Curve(s) *B* represents pretreatment with AOAA 25 mg/kg, and *C* represent pretreatment with AOAA 25 mg/kg together with surgical occlusion. Pretreatment with AOAA 25 mg/kg (s.c.) was begun before the i.v. bolus injection of [^{3}H] GABA (2.5 µCi/100 g) at time zero

Table 1. The effect of GABA-transaminase inhibition of the level of non-volatile tritium in the circulation of rats after i.v. injection of [^{3}H] GABA

Control (%)	EOS (%)	AOAA (%)	AOAA[a] (%)	AOAA[a] EOS (%)
21.82 ± 0.8	71.88 ± 0.4	84.6 ± 0.9	99.09 ± 0.77	97.25 ± 0.25

Each value represents the mean ± SE of plasma samples (100 µl) from four to six experiments (duplicate samples). Results are expressed as percentage of non-lyophylized plasma samples. AOAA 25 mg/kg (90 min s.c.), EOS 300 mg/kg (2.5 h i.p.)
[a] Surgical occlusion

Table 2. The effects of GABA-transaminase inhibitors on cerebrovascular permeability (PA), and the time-integrated ($\int Cpdt$) plasma concentration for the tracer [^{3}H] GABA

	Control	EOS	AOAA	AOAA[a]	AOAA[a] EOS[b]	AOAA[a] GAL
PA	35.5 ± 0.8	40.8 ± 1.2	7.5 ± 0.4	1.77 ± 0.2	2.9 ± 0.56	2.02 ± 0.1
$\int Cpdt$	1.26 ± 0.05	0.7 ± 0.22	0.32 ± 0.1	2.12 ± 0.36	1.37 ± 0.13	1.7 ± 0.1

Each value represents the mean ± SE of measurements from four brain regions of three to eight experiments
PA($s^{-1} \cdot 10^5$), $\int Cpdt$(dpm $s \cdot ml^{-1} \cdot 10^{-8}$)
[a] Surgical occlusion
AOAA 25 mg/kg (90 min s.c.), EOS 300 mg/kg (2.5 h i.p.), GAL 40 mg/kg (16.5 h i.p.),
[b] EOS 500 mg/kg (25 h i.p.)

only, displayed a lower plasma profile (Fig. 1, curve B) and plasma integral (Table 2), as compared to the control. Further experiments using various treatment regimes indicated the use of pharmacological agents alone was inadequate to completely inhibit [^{3}H] GABA metabolism and therefore ^3H_2O formation. However, occluding the blood supply to the liver and mesentery in animals pretreated with AOAA almost completely inhibited the formation of volatile radioactivity (Table 1).

Chromatographic analysis (TLC) of the plasma supernatant from these rats suggested the contribution of metabolites other than ^3H_2O radioactivity measured in plasma is minimal, since the majority of radioactivity located to the cellulose of the chromatograms migrated as authentic GABA. Comparison of the calculated RF values indicated a similar RF (0.524) as identified by ninhydrin. These values are comparable to those already established for GABA using similar chromatographic analysis (Zweig and Sherma 1972). Analysis of the [^{3}H] GABA injectate by chromatography and after lyophilization indicated that radioactivity present in the injectate was only attributable to non-volatile tritium.

Animals pretreated with AOAA (25 mg/kg) together with surgical occlusion displayed a slightly more elevated plasma profile, curve C, compared to animals depicted by curve B; however the curve plateaued below that for the control animals, curve A. Table 2 shows the permeability-area product (PA), for rats subjected to different experimental regimens. The mean PA values were significantly reduced ($df = 6$, $P \leq 0.05$) in the presence of AOAA ($\geq$ 25 mg/kg) alone or together with surgical occlusion of the peripheral blood supply.

At doses up to 500 mg/kg administered in conjunction with AOAA (25 mg/kg) and surgical occlusion, ethanolamine-O-sulphate (EOS) failed to cause any significant alteration in the calculated PA values (Table 2). The slightly higher PA values under these conditions presumably reflect the presence of

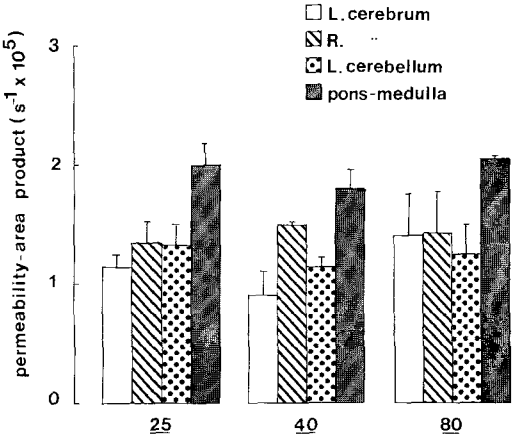

Fig. 2. The effects of amino-oxyacetic (AOAA) on cerebrovascular permeability (PA) to [^{3}H] GABA. Each treatment group represents the mean ± SE of PA calculated from four rats. Four brain regions were sampled from each animal. AOAA (mg/kg) was administered (s.c.) 90 min before the i.v. tracer injection. All animals were subjected to surgical occlusion

^3H_2O, since approximately 3% of the plasma radioactivity could be attributed to volatile tritium (Table 1). Gabaculine (40 mg/kg), an irreversible and specific inhibitor of GABA-T (Schecter and Grove 1980) also failed to cause any significant change in PA relative to those rats treated with AOAA 25 mg/kg only, for any of the brain regions tested. The pons-medulla consistently displayed the highest accumulation of radioactivity (Fig. 2). Increasing the dose, or duration (not illustrated) of pretreatment with AOAA was without any effect.

Discussion

Using a new and sensitive radiochemical technique it was possible to quantitate the permeation of systemically administered [^{3}H] GABA from bloodstream into brain. In the animals untreated with GABA-T inhibitors, a significant amount of radioactivity was located in the brain. A large proportion of this radioac-

tivity could be attributed to substances other than [^{3}H] GABA, since a significant amount of volatile tritium was evident in plasma. The short plasma half-life of GABA (Van Gelder and Elliot 1958) resulting from the rapid metabolism of this neurotransmitter substance in the periphery has presented problems in the investigation of the blood-brain barrier to GABA (Lloyd et al. 1979; Löscher 1980, 1982). To investigate the blood-brain barrier to GABA, it was necessary to completely eliminate the formation of [^{3}H] metabolites, in particular ^3H_2O, since this substance rapidly permeates into brain parenchyma (Oldendorf 1971 a, b). According to Maggi and Enna (1979) there is little spontaneous exchange of [^{3}H] between GABA and H_2O, rather, GABA-transaminase induced breakdown of [^{3}H] GABA yields [^{3}H] which is then exchanged with H_2O giving ^3H_2O, together with a small number of other metabolites. However, under conditions of GABA-T inhibition used here, where the formation of volatile tritium was prevented, there was a substantial reduction in the amount of radioactivity accumulated to brain parenchyma, indicative of a decrease in the proportion of the amount of volatile vs non-volatile radioactivity in plasma. Indeed, chromatographic analysis showed only [^{3}H] GABA to be present in plasma, and it was possible, therefore, to undertake a meaningful study of the blood-brain barrier to this amino acid.

Enzymatic mechanisms are proposed to constitute a major component of the BBB to various systemically administered substances (Dobbing 1961; Björklund et al. 1969; Oldendorf 1971 b; Rapoport 1976; Djuricic et al. 1978; Mrsulja and Djuricic 1980). The presence of GABA-T in cerebral endothelial cells with an activity almost the same as in brain parenchyma (Djuricic et al. 1978) together with the very poor permeability of the BBB to GABA has tempted postulation of an enzymic barrier for GABA (Van Gelder 1965 a, b, 1968). The results of this study do not support this hypothesis. Permeation of radiolabelled GABA, as calculated by the PA, was poor. Pharmacological manipulation of GABA-T with specific inhibitors of this enzyme did not increase the accumulation of radioactivity in brain parenchyma of rats pretreated to prevent the systemic metabolism of [^{3}H] GABA. These results confirm and extend the findings of other studies using mice (Kuriyama and Sze 1971; Maggi and Enna 1979).

Comparing the permeation of GABA measured in this study with that of the non-metabolizable neutral amino acid α-AIB and the less permeant tracer substance sucrose in similar experiments by Preston et al. (1983) and Krantis (1983 a, b), there is good correlation with observations of Oldendorf (1971 a, b) as to their order of ability to enter the brain (α-AIB $\gg$ GABA $>$ sucrose). The amount of radioactivity present in plasma (time-integrated plasma level) was highest in those animals subjected to GABA-T inhibition together with surgical occlusion of portions of the peripheral blood supply. This would result from occlusion of the blood flow to the liver (a primary site of GABA metabolism, Wu et al. 1978), thereby preventing the trapping and subsequent metabolic degradation of GABA by GABA-T. The possibility that high plasma integral values, characteristic of elevated plasma tracer concentrations, spuriously lowered the calculated PA thereby masking the opening of the BBB, seems unlikely. The large reductions in PA evident for the test groups were not always accompanied by significant alterations in the plasma integral. Furthermore, it has been shown that deliberate elevations of the time-integrated plasma tracer concentration did not alter the calculated PA for ^{14}C-sucrose (Preston and Haas 1982).

A possible explanation of the present results is that the GABA-T inhibitor substances failed to penetrate into the cerebral endothelial cells. Under these circumstances the postulated enzymic barrier for GABA would remain intact. However, histochemical analysis in this laboratory of endothelial GABA-T activity (Krantis 1983 a, b) clearly showed AOAA and GAL at the doses used here to significantly inhibit the activity of GABA-T localised to these sites. Furthermore, there is considerable evidence that these GABA-T inhibitors including EOS at doses above 300 mg/kg readily penetrate into the mammalian brain (Löscher 1980, 1982; Ferkany et al. 1979; Enna et al. 1980; Palfreyman et al. 1981). Moreover, the differential PA values evident for the rat brain cannot be due to any differences in the distribution of GABA-T inhibitors to the various brain regions (Walters et al. 1978), since brain tissue from control rats displayed a similar pattern of PA values.

A number of amino acids not taken up to any significant extent by the brain such as glutamate and aspartate, undergo fairly rapid exchange between plasma and brain indicating the participation of mediated transport in the uptake of these substances (Battistan et al. 1971; Pardridge and Oldendorf 1977). Whether a similar mediated transport of GABA across cerebral endothelial cells also occurs is uncertain, although Battistan et al. (1971) suggest the absence of any large net movement of GABA into the brain is due to a "controlled" transport mechanism. In this regard, it has recently been suggested that there exists a low affinity transport system for GABA into the brain (Frey and Löscher 1980). Should such a phenomenon indeed exist for GABA at the endothelial cells it would be interesting to determine whether the transport mechanism(s) involve(s) any of the carriers already established to participate in the bi-directional movement of amino acids across the BBB, including γ-

glutamyl transpeptidase (Pardridge and Oldendorf 1977; Orlowski et al. 1974). Interestingly, the neutral analogue of GABA, α-AIB, is proposed to enter the brain by a specific transport mechanism, the L (leucine preferring) system (Blasberg 1968).

In conclusion, it would appear the barrier to the movement of GABA from blood into brain, under the conditions of this study, is not absolute, instead there is a small permeation across cerebral blood vessel walls. Whether this represents the effects of light anaesthesia, as suggested by Marrazzi et al. (1958) and Van Gelder and Elliot (1958), is uncertain. Nevertheless, this poor ability of GABA to enter the rat brain does not appear to result from the activity of GABA-transaminase localized to the cerebrovascular endothelium. It remains to be determined whether this enzyme is involved in preventing the efflux of GABA from the brain.

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