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γ-Hydroxybutyrate (GHB) Carrier Mediated Transport Across the Blood Brain Barrier

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Nonstandard Abbreviations: **DPH**, 1,6-diphenyl-1,3,5-hexatriene; **BA**, benzoic acid; **BBB**, blood brain barrier; **C**, total concentration of GHB; **CHC**, α-cyano-4-hydroxycinnamic acid; **CL_{in}**, influx clearance; **CL_{ns}**, nonsaturable clearance; **CNS**, central nervous system; **C_r**, perfusion fluid concentration of tracer; **F**, perfusion fluid flow through each brain region; **GABA**, γ-aminobutyric acid; **GHB**, γ-hydroxybutyrate; **J_{in}**, mass transfer influx; **K_m**, Michaelis-Menten half saturation constant; **MCT**, monocarboxylate transporter; **OAT**, organic anion transport; **PA**, permeability surface area product; **Q**, mass of radiotracer

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in the brain region normalized for wet brain tissue weight; **T**, time of perfusion; $\mathbf{V_{max}}$, maximum transport rate; $\mathbf{V_{vasc}}$, regional volume of the cerebrovascular capillary bed

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ABSTRACT

γ-Hydroxybutyrate (sodium oxybate, GHB) is an approved therapeutic agent for cataplexy with narcolepsy. GHB is widely abused as an anabolic agent, euphoriant and date rape drug. Recreational abuse or overdose of GHB (or its precursors: γ-butyrolactone or 1,4-butanediol) results in dose dependent central nervous system (CNS) effects (respiratory depression, unconsciousness, coma, death) as well as tolerance and withdrawal. An understanding of the CNS transport mechanisms of GHB may provide insight into overdose treatment approaches. The hypothesis that GHB undergoes carrier mediated transport across the BBB was tested using a rat *in situ* brain perfusion technique. Various pharmacologic agents were used to probe the pharmacological characteristics of the transporter. GHB exhibited carrier mediated transport across the BBB consistent with a high capacity, low affinity transporter; averaged brain region parameters were V_{max} = 709 ± 214 nmol/min/g, K_m = 11.0 ± 3.56 mM and CL_{ns} = 0.019 ± 0.003 cm^3/min/g. Short chain monocarboxylic acids (pyruvic, lactic, β-hydroxybutyric), medium chain fatty acids (hexanoic, valproic) and organic anions (probenecid, benzoic, salicylic, CHC) significantly inhibited GHB influx by 35-90%. Dicarboxylic acids (succinic, glutaric) and γ-aminobutyric acid did not inhibit GHB BBB transport. Mutual inhibition was observed between GHB and benzoic acid, a well-known substrate of the monocarboxylate transporter (MCT1). These results are suggestive of GHB crossing the BBB via a MCT isoform. These novel findings of GHB BBB transport suggest potential therapeutic approaches in the treatment of GHB overdoses. We are currently conducting “proof-of-concept” studies involving the use of GHB brain transport inhibitors during GHB toxicity.

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γ-Hydroxybutyric acid (GHB), an endogenous neuromodulator (Cash, 1994) was synthesized by H. Laborit in the early 1960s as a γ-aminobutyric acid (GABA) mimetic agent (Laborit, 1964). GHB was studied for potential use as an anesthetic agent, however, its adverse effects outweighed its therapeutic effects. In the late 1980's, interest in GHB was rekindled for use in sleep disorders. Recently the FDA granted orphan drug status to GHB (sodium oxybate; Xyrem®) as a controlled substance with restricted distribution for the treatment of narcolepsy with cataplexy. GHB is currently under investigation for potential therapeutic use in alcohol and opioid withdrawal (Gallimberti et al., 2000), and in other conditions such as depression, anxiety and fibromyalgia (Scharf et al., 1998; Ferrara et al., 1999).

However, GHB derives notoriety from its current popularity as a recreational drug of abuse. GHB and its analogues (γ-butyrolactone and 1,4-butanediol) are currently abused for their recreational and pleasurable properties (heightened sexual pleasures, stress reduction, sedative, antianxiety and antidepressant effects {http://www.projectghb.org/}) by dance club attendees (rave parties); anabolic effects by body builders; and disinhibitory and sedative effects by sexual predators (Nicholson and Balster, 2001; Okun et al., 2001). Of interest, GHB's physiochemical properties (colorless, odorless, slightly salty taste) have been exploited as an "ideal" date rape drug (ElSohly and Salamone, 1999; Smith, 1999).

The surge in GHB (as well as γ-butyrolactone and 1,4-butanediol) abuse by the drug counterculture has led to a substantial increase in drug overdoses and fatalities (Okun et al., 2001; Zvosec et al., 2001). Adverse events associated with GHB overdose include seizures, respiratory depression and impaired consciousness leading to coma and death. Presently the treatment of GHB overdose includes empirical interventions and symptomatic treatments (Nicholson and Balster, 2001; Okun et al., 2001).

The blood brain barrier (BBB) maintains brain homeostasis by restricting the movement of molecules based on size, charge, hydrogen bonding potential and lipid solubility (Pardridge, 1997; Saunders et al., 1999). While many compounds penetrate the BBB by passive diffusion, many other agents undergo active influx or efflux by transport proteins (Tamai and Tsuji, 2000).

An understanding of the transport mechanisms of GHB across the BBB may provide insight into rationale treatment approaches for GHB toxicity. A review of the older literature suggests that GHB may cross the BBB by a transport protein. Roth and Giarman (Roth and Giarman, 1966) reported that preadministration of β-hydroxybutyrate to rats resulted in decreased brain and blood concentrations of exogenously administered GHB. A careful reexamination of Roth and Giarman's data reveals a decrease in the GHB brain to blood ratio in the presence of β-hydroxybutyrate (0.438 without β-hydroxybutyrate, n= 3-4 versus 0.323 without β-hydroxybutyrate, n= 3-4). β-Hydroxybutyrate was recently identified as a substrate for the monocarboxylate transporter (MCT) (Enerson and Drewes, 2003). GHB also inhibits MCT substrate uptake in erythrocytes and cardiac myocytes (Poole and Halestrap, 1993). In addition, there is substantial evidence of MCT expression at the BBB (Kang et al., 1990; Terasaki et al., 1991; Kido et al., 2000).

Considered in toto, this evidence suggests the hypothesis that GHB undergoes MCT carrier mediated transport across the BBB. Using an *in situ* brain perfusion technique, we report that GHB undergoes both carrier mediated transport and passive diffusion, and that the carrier mediated processes are pharmacologically inhibited by known inhibitors of MCT.

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MATERIALS AND METHODS

Chemicals

Male Sprague-Dawley rats (250-350 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). [^{3}H]GHB (specific activity, 35.5 Ci/mmol]) and [^{14}C]benzoic acid ([^{14}C]BA; specific activity, 60 mCi/mmol) were purchased from Moravek Biochemical Inc. (Brea, CA). All test compounds (α-cyano-4-hydroxycinnamic acid; CHC), probenecid, γ-aminobutyric acid (GABA), succinic acid, glutaric acid, L-lactic acid, glycine, sucrose and the sodium salt forms of GHB, BA, salicylic acid, β-hydroxybutryic acid, hexanoic acid, valproic acid and pyruvic acid were purchased from Sigma-Aldrich (St. Louis, MO). Soluene 350 and Soluscint O were purchased from Packard Corp. (Meriden, CT) and National Diagnostics Inc. (Atlanta, GA) respectively. Ketamine and xylazine were purchased from J.A. Webster (Sterling, MA).

In Situ Rat Brain Perfusion Protocol

All procedures involving animals were approved by the University of Buffalo Institutional Animal Care and Use Committee. The transport of GHB across the BBB was quantified using the *in situ* rat brain perfusion. This technique was first developed by Takasato *et al* (Takasato et al., 1984) and later modified which included a change in the surgical procedure and perfusion flow rate (Allen and Smith, 2001). Briefly, adult male Sprague Dawley rats (250 – 350 g) were anesthetized using a mixture of ketamine (90 mg/kg) and xylazine (9 mg/kg), administered intramuscularly and placed on a warming pad to maintain body temperature. Electrocardiograms were continuously monitored (Snap-Master, version 3, Hem Data Corporation, Southfield, MI) throughout the surgical procedure. The thoracic cavity of the rat was opened and the left common carotid artery exposed. This was followed by the ligation of the external carotid artery and cauterization of the superior thyroid and occipital arteries. The common carotid artery was cannulated proximal to the bifurcation of the external and internal carotid arteries with a 25 gauge hypodermic needle affixed to PE-50 tubing (filled with physiologic perfusate**:** 128 mM NaCl, 24 mM $NaHCO_2$, 4.2 mM KCl, 2.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 0.9 mM $MgSO_4$ and 9 mM glucose

(oxygenated with 95%/5% O_2/CO_2, 37 ^oC, pH ~7.4; (Mahar Doan et al., 2000)). A syringe containing oxygenated perfusate was attached to the cannula. The left ventricle of the heart was quickly severed to arrest blood flow to the brain. The left common carotid artery was ligated below the cannula insertion point. *In situ* brain perfusion was immediately initiated at a flow rate of 10 ml/min with a perfusion pump (Model 55-4150, Harvard Apparatus, Holliston, MA). This technique resulted in the perfusion of the left cerebral hemisphere.

Following a perfusion period of 15-60 seconds, rats were sacrificed by decapitation. Brains were removed, placed on ice chilled glass plates and the left hemispheres were dissected into the following brain regions: the cortices (frontal, parietal and occipital), hippocampus, striatum and thalamus/hypothalamus. Dissected tissue samples were placed in pretared liquid scintillation vials, weighed, and solubilized overnight with 0.8 ml Soluene 350 at 50 ^oC. Five ml of Soluscint O were added and the samples were analyzed by liquid scintillation counting using a1900CA Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL). The counting efficiencies for [^{3}H] and [^{14}C] were 0.61 and 0.95 respectively. An aliquot of the perfusion fluid was similarly assayed by liquid scintillation counting to verify the perfusate analyte concentration.

In separate experiments, the capillary depletion technique was used to determine the distribution of [^{3}H]GHB between brain vasculature and brain parenchyma (Triguero et al., 1990). For experiments where high concentrations of GHB or inhibitors (>5 mM) were required, the sodium chloride concentration of the perfusate was adjusted to maintain physiologic osmolality.

Experimental Protocols

Linear influx of GHB

Pilot studies were first performed to determine linear permeability conditions, i.e. the time course over which [^{3}H]GHB influx was linear and unidirectional (Takasato et al., 1984; Smith, 1999; Mahar Doan et al., 2000). Animals (n = 3-4) were perfused with [^{3}H]GHB (0.028 μM; 1.0 μCi/ml) for 15, 30, 45 or 60

sec and sacrificed. Brain regions were assayed for [^{3}H]GHB as described previously. Based on these studies (see Results), a 30 second perfusion period was selected for all subsequent studies.

GHB Concentration Dependent Study

GHB concentration dependent influx studies were performed to assess the extent of saturable transport in the presence of the following concentrations of unlabeled GHB in separate groups of rats: 0.028 μM (n=4), 28 x 10^{-5} mM (n=3), 5 x 10^{-3} mM (n=3), 0.05 mM (n=3), 0.1 mM (n=3), 0.5 mM (n=3), 1 mM (n=3), 10 mM (n=3), 20 mM (n=4), 30 mM (n=4) and 40 mM (n=4). Brain tissues were assayed for [^{3}H]GHB as described previously.

Substrate Inhibitor Studies

Substrate inhibitor studies were performed to determine the substrate specificity of the GHB transporter and to aid in the pharmacological characterization of the transporter. The compounds used for the inhibition studies were selected based on their known transport characteristics and/or chemical structures. Test inhibitors (1-20 mM) were individually coperfused with [^{3}H]GHB (0.028 μM; 1.0 μCi/ml). Short chain monocarboxylic acids (L-lactic, pyruvic and β-hydroxybutyrate acids), dicarboxylic acids (succinic and glutaric), medium chain fatty acids (hexanoic and valproic acids), and organic acids (benzoic and salicylic acids) were coperfused at 20 mM. CHC, a specific inhibitor of the MCT was coperfused at 1 mM. Other organic anions that were tested for inhibitory effects on GHB transport included GABA (10 mM) and probenecid (10 mM). Negative controls for transport included substances that undergo minimal to moderate passive diffusion, e.g. sucrose (20 mM) and glycine (20 mM). The concentrations of potential inhibitor compounds were selected either based on K_m values (if known) or the limit of solubility in the perfusate.

Inhibition of Benzoic Acid Transport

Benzoic acid (BA), a known substrate for MCT at the BBB (Kido *et al*, 2000) was used to further probe the role of MCT in GHB transport across the BBB. [^{14}C]BA (8.33 μM ; 0.5 μCi/ml) was perfused

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in presence or absence of 20 mM (GHB or BA) or 40 mM GHB for 30 sec. Brain tissues were harvested and assayed for [^{14}C]BA as previously described.

Self-Association Studies

1,6-Diphenyl-1,3,5-hexatriene (DPH) was used as a fluorescence probe for substance self-association in the perfusate. Sodium dodecyl sulfate, with a critical micellar concentration of 0.83 mM (Kumar Sau et al., 2002), was used as a positive control. Four µl of a freshly prepared solution of DPH (5 mM, solubilized in tetrahydrofuran) was added to separate test tubes containing either 2 ml of valproic or hexanoic acids (10-50 mM) or sodium dodecyl sulfate (0-5 mM). The solutions were kept in dark for 30 minutes prior to analysis. Fluorescence measurements were performed using a PTI fluorometer (Photon Technology International, Lawrenceville, New Jersey). The excitation and emission wavelengths were 360 nm 430 nm respectively. The study was repeated in triplicate.

Data Analysis

GHB influx clearance ($CL_{in,}$ cm^3/min/g) for unidirectional transfer was obtained by fitting Equation 1 to the time course data using WinNonlin Pro version 2.1 (Pharsight Corp, Cary, NC):

$$\frac{Q}{C} = CL_{in} * T + V_{vasc} \quad \text{Equation 1}$$

where, Q (dpm/g) represents the quantity of radiotracer in the brain region normalized for wet brain tissue weight, C (dpm/ml) represents the perfusion fluid concentration of [^{3}H]GHB, T (min) is the time of perfusion and V_{vasc} (ml/g) represents the volume of the cerebrovascular capillary bed for each brain region. V_{vasc} data were previously determined in our lab using [^{3}H]inulin for each brain region (10^{-3} cm^3/g (n=5): thalamus / hypothalamus: 7.97 ± 0.96; hippocampus: 10.0 ± 2.08; striatum: 7.59 ± 0.85; frontal cortex: 6.16 ± 0.78; occipital cortex: 6.08 ± 0.52; parietal cortex: 6.97 ± 1.19).

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For perfusion studies involving single time point analysis (a 30 sec perfusion period, determined at different concentrations of GHB), CL_{in} data were converted to cerebrovasculature permeability surface area products (PA, cm^3/min/g) using Equation 2:

$$PA = -F * \ln(1 - \frac{CL_{in}}{F}) \qquad \text{Equation 2}$$

where F (cm^3/min/g) is the perfusion fluid flow through each region; these values were obtained from the literature (Takasato et al., 1984; Allen and Smith, 2001).

GHB mass transfer influx data (J_{in}, nmol/min/g) were calculated by Equation 3:

$$J_{in} = PA * C \qquad \text{Equation 3}$$

where C (mM) is the total perfusate concentration of GHB (labeled and unlabeled).

To determine the saturability of GHB BBB influx, parameter estimates of V_{max}, K_m and CL_{ns} were obtained by iterative nonlinear regression analysis (WinNonlin Pro version 2.1, Pharsight Corp, Cary, NC) using Equation 4:

$$J_{in} = \frac{V_{max} * C}{K_m + C} + CL_{ns} * C \qquad \text{Equation 4}$$

where V_{max} (nmol/min/g) is maximal transport rate of GHB influx, K_m (mM) is the Michaelis-Menten half saturation constant, CL_{ns} (cm^3/min/g) is the nonsaturable clearance representing passive diffusion and C (mM) is the total concentration of GHB (labeled and unlabeled). A weighting scheme was used for the nonlinear regression analysis (iterative reweighting - $1/ Y^2_{predicted}$).

Statistical Analysis

Statistical analyses were performed using SAS V 8.0 (SAS Institute, Cary, NC). A two sample test for equal or unequal variance (Fischer's test)was initially used to analyze the CL_{in} data, comparing the control (without inhibitors) versus test (with inhibitor). Depending on these results, statistical significance (p = 0.05) of the Cl_{in} data was assessed using the appropriate student's t-test (with equal or unequal variances) to test for statistically significant differences ($p<0.05$).

RESULTS

Pilot GHB BBB Transport Studies

Pilot studies revealed a linear influx of [^{3}H]GHB (0.028 μM) into various brain regions over 60 sec. Figure 1 presents a representative concentration - time course for the hippocampus. Similar linear time courses were observed for other brain regions (data not shown). A 30 sec perfusion time was chosen for additional single time point studies since this was within the linear region, indicating a predominant influx with minimal efflux.

The capillary depletion technique was performed as described (Triguero et al., 1990) to investigate the capillary sequestration of [^{3}H]GHB. The [^{3}H]GHB distribution volume (n=3 rats) in the homogenate, supernatant and pellet (capillary) fractions were 0.071 ± 0.009 cm^3/g, 0.067 ± 0.008 cm^3/g and 0.003 ± 0.001 cm^3/g respectively, indicating that less than 5% of the total [^{3}H]GHB was sequestered within the capillaries. Moreover, there were no significant differences in the [^{3}H]GHB CL_{in} determined with versus without the capillary depletion procedure (with capillary depletion: 0.119 ± 0.027 cm^3/min/gm; n=3; without capillary depletion: 0.075 ± 0.052 cm^3/min/gm; n=4). These results with GHB are consistent with the behavior of small, hydrophilic, non-positively charged molecules, such as sucrose and urea (Triguero et al., 1990), which undergo minimal capillary sequestration. Accordingly, the capillary depletion step was not performed in subsequent experiments.

For all brain regions and concentrations, GHB CL_{in} values were at least 40 fold lower than the cerebrovascular flow values obtained from the literature (Takasato et al., 1984; Allen and Smith, 2001), which suggests that GHB BBB transport is flow independent (data not shown).

GHB BBB Transport Concentration Dependence

Figure 2 shows GHB concentration dependent influx for two representative regions, the hippocampus and parietal cortex. Similar data were observed for the other brain regions (data not shown). Michaelis-Menten BBB transport parameters for each region are shown in Table 1. Because the concentration – BBB influx data were pooled from multiple animals and subjected to nonlinear regression

analysis as a single data set, the regional parameter estimates cannot be statistically compared against each other due to an inability to estimate the true variability associated with each parameter estimate. However, it appears that the cortices show greater V_{max} estimates relative to the other regions; this is perhaps due to the higher capillary density of the cerebral cortex relative to other regions (Klein et al., 1986).

GHB BBB Transport Inhibition Studies

Tables 2 and 3 illustrate the effects of various compounds on the BBB transport of GHB. Consistent with the concentration dependent transport studies, self-inhibition of GHB (40 mM) influx was observed. Short chain monocarboxylic acids are known substrates of MCT (Enerson and Drewes, 2003). L-Lactic acid (three carbon backbone; C3), pyruvic acid (C3) and beta-hydroxybutyric acid (four carbon backbone, C4) each significantly inhibited [^{3}H]GHB BBB influx transport (Table 2). The dicarboxylic acids, succinic acid (C4) and glutaric acid (five carbon backbone, C5), did not inhibit [^{3}H]GHB BBB transport (Table 2), although an unexplained stimulation of [^{3}H]GHB BBB transport was observed for succinic acid in the striatum and thalamus/hypothalamus regions. The medium chain fatty acids, hexanoic acid (six carbon backbone, C6) and valproic acid (eight carbon backbone, C8), significantly inhibited [^{3}H]GHB BBB transport (Table 2). The inhibition of [^{3}H]GHB BBB transport by other organic anions is shown in Table 3. Benzoic acid and salicylic acid significantly inhibited [^{3}H]GHB uptake. CHC, a specific inhibitor of MCT (Wang et al., 1996; Enerson and Drewes, 2003) showed significant inhibition of [^{3}H]GHB BBB transport. Probenecid, which has a broad specificity for multiple transporters (Deguchi et al., 1997), significantly inhibited [^{3}H]GHB BBB influx. GABA did not significantly inhibit [^{3}H]GHB transport.

The BBB influx of [^{14}C]BA, a known MCT substrate, was significantly inhibited ($p<0.05$) by unlabelled BA (20 mM) and GHB (40 mM) as shown in Table 4. Interestingly, 20 mM GHB did not significantly inhibit [^{14}C]BA, suggesting that GHB has a lower affinity for the transporter than benzoic acid.

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The inhibition of [^{3}H]GHB influx by a number of compounds required the use of high concentrations (20 mM) of inhibitors. Several control studies were performed to ascertain that the observed inhibition of [^{3}H]GHB influx was not due to non-specific physicochemical interactions that would impede [^{3}H]GHB access to the transporter. Sucrose and glycine were selected as negative controls as these compounds undergo minimal to moderate passive diffusion. Neither sucrose (20 mM) nor glycine (20 mM) inhibited [^{3}H]GHB uptake (Table 3), suggesting that high millimolar concentrations of substances do not necessarily physiochemically interact with [^{3}H]GHB to sequester it from access to the transporter.

Another potential artifact that might explain the inhibition [^{3}H]GHB influx by medium chain fatty acids could be the entrapment of [^{3}H]GHB in self-associative structures formed by the fatty acids. Such self-associative structures would have the effect of reducing [^{3}H]GHB influx clearance. The formation of self-associative structures were studied using fluorescence. DPH, a fluorescence probe for self-association, inserts itself into the self-associated structure, resulting in an increased fluorescence signal. The positive control, sodium dodecyl sulfate showed a steep concentration - dependent increase reaching a plateau phase at higher concentrations, consistent with the formation of micelles saturated with DPH probe (data not shown). Valproic and hexanoic acids, medium chain fatty acids, did not show any concentration increase in fluorescence with increasing concentrations (data not shown), suggesting that these compounds do not self-associate. These results rule out a fatty acid inhibition mechanism based on self-association.

DISCUSSION

A careful reexamination of data published in 1966 by Roth and Giarman (Roth and Giarman, 1966) revealed a decrease in the GHB brain to blood ratio in the presence of β-hydroxybutyrate. Since β-hydroxybutyrate is a MCT substrate (Enerson and Drewes, 2003) and GHB inhibits MCT in erythrocytes and cardiac myocytes (Poole and Halestrap, 1993), it was hypothesized that GHB undergoes carrier mediated transport via BBB MCT.

Using a rat *in situ* brain perfusion preparation, the present study observed that the kinetics of GHB BBB influx is characterized as a saturable, carrier mediated process (average K_m ~ 11 mM, range of 2.62 – 22.4 mM among the various brain regions; average V_{max} ~ 709 nmol/min/g, range of 225 – 1520 nmol/min/g range among the various brain regions) and a nonsaturable, diffusional process (average CL_{ns} ~ 0.019 cm^3/min/g, range of 0.0089 – 0.0299 cm^3/min/g). These K_m and V_{max} values are consistent with low affinity, high capacity transport and are comparable to those values observed for other substrates of the MCT (Pollay and Stevens, 1980; Kido et al., 2000) and the medium chain fatty acid transporter (Adkison and Shen, 1996). At low GHB concentrations (~ 1 mM), the saturable transport pathway contributes an estimated 67 - 89 % of the total influx transport across the various rodent brain regions. In humans (where endogenous plasma GHB concentrations are typically less than 10 μM (Fieler et al., 1998)), it is likely the GHB carrier mediated process will predominate over the diffusional process i.e., the V_{max}/K_m is 3.3 fold greater than CL_{ns} assuming that the average V_{max} and K_m estimates in rats are similar to humans.

The characteristics of the BBB transport protein responsible for GHB influx was pharmacologically probed using a diverse set of potential inhibitors. Short chain monocarboxylic acids known to be transported by the BBB MCT1, e.g. lactic, pyruvic and β-hydroxybutyrate (Tildon and Roeder, 1988; Kang et al., 1990; Enerson and Drewes, 2003), significantly inhibited [^{3}H]GHB influx clearance suggesting that MCT1 contributes to BBB GHB transport. Salicylic acid (a MCT and organic anion transport (OAT) substrate), benzoic acid (a well known MCT substrate (Kang et al., 1990; Terasaki

et al., 1991; Kido et al., 2000) and CHC (a specific inhibitor of MCT (Wang et al., 1996; Enerson and Drewes, 2003)) showed significant inhibition of GHB influx, again implicating a MCT isoform in the BBB transport of GHB.

Since MCT substrates inhibited GHB influx, it was necessary to determine if GHB inhibited the BBB transport of a known MCT substrate across the BBB, e.g., benzoic acid. GHB, as well as unlabelled benzoic acid, significantly inhibited [^{14}C]benzoic acid influx. The mutual inhibitory interaction of GHB and benzoic acid on each other's influx implicates a role of MCT in the BBB transport of GHB. Presently it is known that the endothelial cells of the BBB express MCT1 but little MCT2. However review of literature suggests that other isoforms of MCT (MCT6 and 7) are also expressed in the brain (Price et al., 1998), with the transporter localization yet to be defined. Thus the possibility that more than one isoform of MCT could be involved in GHB transport across the BBB cannot be ignored.

Substrates of other transporters were studied for their effects on GHB transport. The dicarboxylic acids, glutaric and succinic, which are substrates of OAT and not MCT (Lee et al., 2001), did not inhibit GHB influx clearance, thereby implying that OAT is not likely involved in GHB influx.

The role of the medium chain fatty acid transporter in GHB transport was studied using hexanoic and valproic acids (Adkison and Shen, 1996). Both valproic and hexanoic acids significantly inhibited GHB brain influx, which may implicate a fatty acid transporter for GHB influx. Adkison and Shen (Adkison and Shen, 1996) observed that valproic acid inhibited the BBB influx of MCT substrates, but MCT substrates failed to inhibit valproic acid BBB influx. This lack of mutual inhibition suggested that valproic acid, while not transported by MCT, can interact with MCT to prevent substrate transport.

Probenecid inhibits a wide array of transport systems including isoforms of MCT, OAT and N'-methylnicotinamide sensitive organic cation transporters (Deguchi et al., 1997). The broad specificity of probenecid on various transport systems often confounds interpretation of the data. For example, divergent effects of probenecid on substrates of the medium chain fatty acid transporter, octanoic acid and valproic acid, were observed. Probenecid was found to inhibit the brain influx of octanoic acid (Spector,

1988) but enhance influx of valproic acid (Adkison and Shen, 1996). There are conflicting reports as to whether probenecid does / does not inhibit brain lactic acid transport (via the MCT) (Yuwiler et al., 1982; LaManna et al., 1993). Although GHB transport into rat brain is probenecid sensitive (Table 3) this information does not lend insight into the identity of likely transport protein(s).

GHB transport systems were identified in synaptosomes (K_m = 46.4 μM, V_{max} = 154.9 pmol/min/mg protein) (Benavides et al., 1982) and synaptic vesicles (K_m = 10 mM, V_{max} = 27 nmol/min/mg protein) (Muller et al., 2002), providing evidence that GHB reuptake mechanisms exist in the synaptic cleft. The *in situ* brain perfusion design allows one to measure influx across blood brain barrier, whether additional transport occurs or not is beyond the resolution of the experimental method. GABA was found to significantly inhibit GHB synaptosomal uptake (Benavides et al., 1982; Muller et al., 2002). However, in the present GHB BBB transport studies, GABA failed to inhibit GHB influx, thereby suggesting that the GHB transport protein at the BBB is different from that at the synaptic cleft.

The composite view of the pharmacologic inhibition data suggests that a MCT isoform, rather than a medium chain fatty acid transporter, is likely involved in GHB transport at the BBB. The pharmacologic evidence is summarized as follows: (1) All tested MCT substrates inhibited GHB influx. (2) A known specific inhibitor of MCT (i.e., CHC) inhibited GHB influx. (3) Pyruvic acid inhibited GHB influx to a greater extent than lactic acid (decreases of ~ 51% versus ~ 24%, respectively). This is in agreement with the finding that 2-oxo acids (e.g., pyruvate) have a higher affinity for MCT than 2-hydroxy acids (e.g., lactate) (Enerson and Drewes, 2003). (4) Significant cross inhibition between benzoic acid (a MCT substrate) and GHB was observed. Lastly, (5) dicarboxylic acids have no effect on MCT substrates (Terasaki et al., 1991; Kido et al., 2000) but enhance medium chain fatty acid uptake (Adkison and Shen, 1996). However, no inhibitory effectof dicarboxylic acids on GHB BBB influx was observed.

In summary, the present work identifies the BBB transport mechanisms for GHB, which involve a carrier mediated process (saturable and inhibitable, likely via a MCT isoform), and a passive diffusion process. This information provides important insights into GHB therapy, overdoses and drug-drug

interactions. In drug overdoses, post-mortem GHB blood concentrations range from 27-121 μg/ml (0.26 - 1.16 mM), and may reach as high as 330 μg/ml (3.17 mM) (Kalasinsky et al., 2001). Using the transport parameter estimates from our rat *in situ* brain perfusion preparation, the GHB carrier mediated mechanism is 1.4 to 6.9 times greater than the passive diffusion mechanism at the reported post-mortem GHB concentrations. This suggests that administration of GHB transport inhibitors may reduce brain GHB concentrations in cases of overdose. We are presently engaged in proof of concept studies to assess the feasibility of utilizing GHB transport inhibitors in the treatment of GHB toxicity.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Time course of GHB influx into rat hippocampus following perfusion with 0.028 μM [^{3}H]GHB. Filled circles represent mean ± S.E.M. (n = 3-4 rats). Similar results were observed for other brain regions (data not shown).

Figure 2. Concentration dependence of GHB influx into (A) hippocampus and (B) parietal cortex over 0–0.1 mM (insets) or 0-40 mM (large graphs). The solid line represents the fit of equation 4 to influx data; the dashed line represents the computer estimated saturable influx and the dotted line represents the computer estimated nonsaturable (passive diffusion) influx. Filled circles represent mean ± S.E.M. (n = 3-4 rats). Similar results were observed for other brain regions (data not shown).

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Tables

Table 1. Brain regional parameter estimates of GHB transport at the BBB. Values are computer estimates obtained through nonlinear regression analysis.

Brain Region	V_{max} (nmol/min/g x 10^{1})	K_m (mM)	CL_{ns} (cm^3/min/g x 10^{-2})
Hippocampus	41.3	7.68	1.33
Striatum	31.0	3.97	2.43
Frontal Cortex	59.9	7.98	2.34
Parietal Cortex	119	22.4	1.82
Occipital Cortex	152	21.5	0.89
Thalamus/Hypothalamus	22.5	2.62	2.99

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Table 2. Effect of various unlabeled compounds on influx clearance of [^{3}H]GHB in two representative brain regions. Each value represents mean ± S.E.M.

Inhibitor	Conc (mM)	% of Control					
		Hippocampus	Parietal Cortex	Occipital Cortex	Frontal Cortex	Striatum	Thalamus / Hypothalamus
[3H]GHB (Control)[a]		100 ± 13.3	100 ± 6.00	100 ± 5.14	100 ± 7.51	100 ± 10.2	100 ± 12.6
Short Chain Monocarboxylic Acids							
Pyruvic[b]	20	38.3 ± 13.8*****	51.4 ± 16.9*****	52.0 ± 12.5*****	46.6 ± 12.6*****	48.3 ± 18.2*****	38.3 ± 13.3*****
Lactic[b]	20	61.4 ± 2.31†*****	75.3 ± 2.47*****	71.5 ± 5.62*****	70.5 ± 8.76*****	87.6 ± 4.84	87.4 ± 5.79
β-Hydroxybutyric[b]	20	51.5 ±7.46*****	72.2 ± 2.12†*****	64.8 ± 3.61*****	67.9 ± 4.51*****	115 ± 12.1	90.5 ± 10.5
γ-Hydroxybutyric[c]	20	75.8 ± 7.86	87.8 ± 7.57	85.5 ± 5.77	87.7 ± 5.04	87.1 ± 9.98	80.6 ± 11.7
	40	59.8 ± 3.66†*****	72.7 ± 3.16*****	72.7 ± 5.59*****	74.9 ± 3.95*****	75.6 ± 13.8	86.6 ± 6.69
Dicarboxylic Acids							
Succinic[b]	20	85.6 ± 9.24	93.3 ± 9.89†	108 ± 8.43	119 ± 6.85	152 ± 6.41*****	219 ± 38.8*****
Glutaric[b]	20	84.4 ± 7.17	101 ± 11.5	101 ± 11.5	97.7 ± 17.8	127 ± 25.3	122 ± 12.7
Medium Chain Fatty Acids							
Hexanoic[b]	20	11.8 ± 4.83†*****	38.3 ± 6.08*****	43.5 ± 3.56*****	40.4 ± 2.44†*****	66.4 ± 5.37*****	45.1 ± 8.88*****
Valproic[b]	20	24.2 ± 13.1*****	44.8 ± 8.96*****	49.7 ± 12.7*****	39.9 ± 9.22*	58.3 ± 12.0*****	37.3 ± 16.2*

† $p<0.05$; Variance significantly different from control by Fischer's variance test.

* $p<0.05$; Significantly different from control by Student's t test (with equal or unequal variances).

[a] n = 8 for control group, [b]n = 4 for treatment group, [c]n=6 for treatment group.

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Table 3. Effect of various unlabeled compounds on influx clearance of [^{3}H]GHB in two representative brain regions. Each value represents mean ± S.E.M.

Inhibitor	Conc (mM)	% of Control					
		Hippocampus	Parietal Cortex	Occipital Cortex	Frontal Cortex	Striatum	Thalamus / Hypothalamus
[3H]GHB (Control)		100 ± 13.3	100 ± 6.00	100 ± 5.14	100 ± 7.51	100 ± 10.2	100 ± 12.6
Organic Anions							
Salicylic	20	31.9 ± 7.05*	50.7 ± 5.48*	50.7 ± 5.48*	48.0 ± 7.72*	57.9 ± 5.23*	54.6 ± 15.1*
Benzoic	20	29.2 ± 8.23*	44.6 ± 6.18*	46.5 ± 6.59*	40.1 ± 5.97*	56.4 ± 15.7*	47.3 ± 10.8*
CHC	1	58.7 ± 5.73*	72.2 ± 7.78*	72.2 ± 7.77*	72 ± 8.12*	89.9 ± 18.5	89.2 ± 7.22
Probenecid	10	18.4 ± 5.54*	33.8 ± 1.09†*	28.7 ± 2.69*	31.4 ± 0.55†*	51.5 ± 6.81*	45.2 ± 3.28†*
GABA	10	65.8 ± 7.35	87.5 ± 2.87	83.8 ± 6.50	80.1 ± 8.59	94.9 ± 12.2	112 ± 9.09
Negative Controls							
Sucrose	20	92.8 ± 4.78†	106 ± 3.16	111 ± 1.58	111 ± 2.56†	110 ± 17.8	86.6 ± 12.3
Glycine	20	105 ± 10.1	88.7 ± 12.7	113 ± 15.7	104 ± 7.10	106 ± 17.0	100 ± 20.1

† $p<0.05$; Variance significantly different from control by Fischer's variance test.

* $p<0.05$; Significantly different from control by Student's t test (with equal or unequal variances).

n = 8 for control group, n = 4 for treatment group

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Table 4. Effect of various unlabeled compounds on influx clearance of [^{3}H]GHB in two representative brain regions. Each value represents mean ± S.E.M.

Inhibitor	% of Control					
	Hippocampus	Parietal Cortex	Occipital Cortex	Frontal Cortex	Striatum	Thalamus / Hypothalamus
[^{14}C]BA (Control)	100 ± 6.09	100 ± 3.67	100 ± 4.65	100 ± 3.79	100 ± 6.42	100 ± 5.20
[^{14}C]BA + BA 20 mM[a]	59.7 ± 6.12*****	58.1 ± 3.96*****	64.6 ± 7.09*****	53.3 ± 2.88*****	67.3 ± 7.52*****	54.2 ± 4.08*****
[^{14}C]BA + GHB 20 mM[b]	89.3 ± 6.16	88.1 ± 4.29	86.2 ± 4.70	89.1 ± 4.32	91.1 ± 7.29	84.6 ± 5.20
[^{14}C]BA + GHB 40 mM[a]	70.9 ± 1.11†*****	73.7 ± 2.16*****	76.1 ± 3.37*****	68.4 ± 1.18*****	96.5 ± 7.26	66.1 ± 2.70*****

† $p<0.05$; Variance significantly different from control by Fischer's variance test.

* $p<0.05$; Significantly different from control by Student's t test (with equal or unequal variances).

[a] n = 4 for control group, n = 3 for treatment group

[b] n = 8 for control group, n = 9 for treatment group

Figure 1

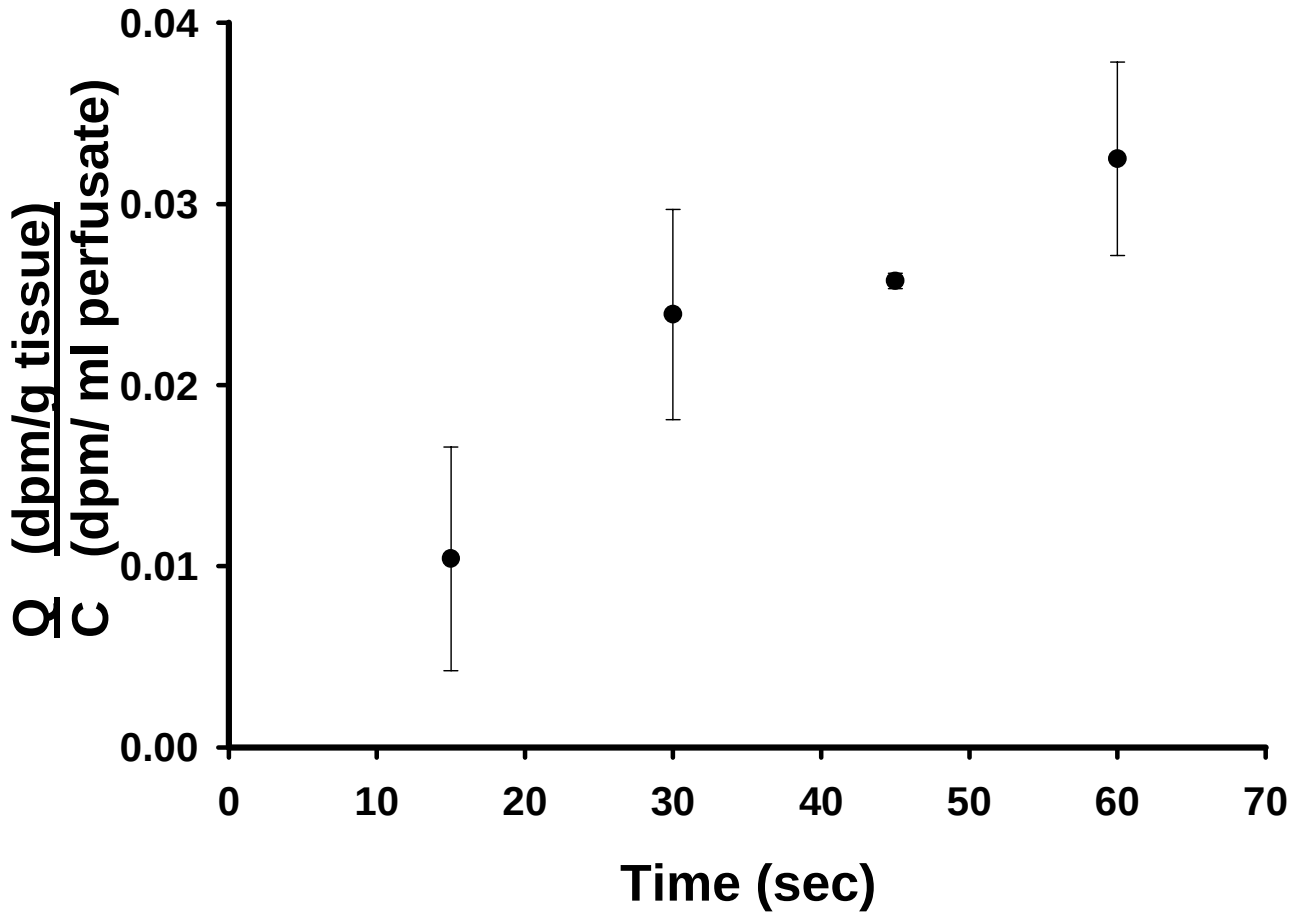

Figure 2