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

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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, and 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 pharmacological 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, and β-hydroxybutyric), medium-chain fatty acids (hexanoic and valproic), and organic anions (probenecid, benzoic, salicylic, and α-cyano-4-hydroxycinnamic acid) significantly inhibited GHB influx by 35 to 90%. Dicarboxylic acids (succinic and 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 an 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.

γ-Hydroxybutyric acid (GHB), an endogenous neuromodulator (Cash, 1994) was synthesized by H. Laborit in the early 1960s as a 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 1980s, interest in GHB was rekindled for use in sleep disorders. Recently, the Food and Drug Administration 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 analogs (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 (Ingels et al., 2000; Nicholson and Balster, 2001; Okun et al., 2001). Of interest, GHB's physiochemical properties (colorless, odorless, and 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 homeosta-

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ABBREVIATIONS: GHB, γ-hydroxybutyrate; BBB, blood-brain barrier; MCT, monocarboxylate transporter; BA, benzoic acid; CHC, α-cyano-4-hydroxycinnamic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; OAT, organic anion transport.

sis by restricting the movement of molecules based on size, charge, hydrogen bonding potential, and lipid solubility (Pardridge, 1997; Saunders et al., 1999). Whereas 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 (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.

Materials and Methods

Chemicals. Male Sprague-Dawley rats (250–350 g) were purchased from Harlan (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 (Brea, CA). All test compounds (α-cyano-4-hydroxycinnamic acid; CHC), probenecid, 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 PerkinElmer Life and Analytical Sciences (Boston, MA) and National Diagnostics (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. (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 polyethylene-50 tubing [filled with physiological 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°C, 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 Inc., Holliston, MA). This technique resulted in the perfusion of the left cerebral hemisphere.

After a perfusion period of 15 to 60 s, 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 of Soluene 350 at 50°C. Five milliliters of Soluscint O was added, and the samples were analyzed by liquid scintillation counting using a 1900CA liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences). 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 physiological 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 s and sacrificed. Brain regions were assayed for [^{3}H]GHB as described previously. Based on these studies (see *Results*), a 30-s 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 mM (n = 4), 28 × 10^{-5} mM (n = 3), 5 × 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. 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 in presence or absence of 20 mM (GHB or BA) or 40 mM GHB for 30 s. Brain tissues were harvested and assayed for [^{14}C]BA as described previously.

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 microliters 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 min before analysis. Fluorescence measurements were performed using a PTI fluorometer (Photon Technology International, Lawrenceville, NJ). The excitation and emission wavelengths were 360 and 430 nm, respectively. The study was repeated in triplicate.

Data Analysis. GHB influx clearance (CL_{in}, cubic centimeters per minute per gram) for unidirectional transfer was obtained by fitting eq. 1 to the time course data using WinNonlin Pro version 2.1 (Pharsight, Cary, NC):

$$\frac{Q}{C} = CL_{in} \cdot T + V_{vasc} \quad (1)$$

where Q (dpm per gram) represents the quantity of radiotracer in the brain region normalized for wet brain tissue weight, C (dpm per milliliter) represents the perfusion fluid concentration of [^{3}H]GHB, T (minutes) is the time of perfusion, and V_{vasc} (milliliters per gram) represents the volume of the cerebrovascular capillary bed for each brain region. V_{vasc} data were previously determined in our laboratory 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; and parietal cortex, 6.97 ± 1.19).

For perfusion studies involving single time point analysis (a 30-s perfusion period, determined at different concentrations of GHB), CL_{in} data were converted to cerebrovasculature permeability surface area products (PA, cubic centimeters per minute per gram) using eq. 2:

$$PA = -F \cdot \ln\left(1 - \frac{CL_{in}}{F}\right) \quad (2)$$

where F (cubic centimeters per minute per gram) 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}, nanomoles per minute per gram) were calculated by eq. 3:

$$J_{in} = PA \cdot C \quad (3)$$

where C (millimolar) 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) using eq. 4:

$$J_{in} = \frac{V_{max} \cdot C}{K_m + C} + CL_{ns} \cdot C \quad (4)$$

where V_{max} (nanomoles per minute per gram) is maximal transport rate of GHB influx, K_m (millimolar) is the Michaelis-Menten half-saturation constant, CL_{ns} (cubic centimeters per minute per gram) is the nonsaturable clearance representing passive diffusion, and C (millimolar) 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 version 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 were 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 s. 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-s perfusion time was chosen for additional single time point studies because this was within the linear region, indicating a predominant influx with minimal efflux.

The capillary depletion technique was performed as described previously (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, 0.067 ± 0.008, 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/g, n = 3; without capillary depletion, 0.075 ± 0.052 cm^3/min/g, n = 4). These results with GHB are consistent with the behavior of small, hydrophilic, nonpositively 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

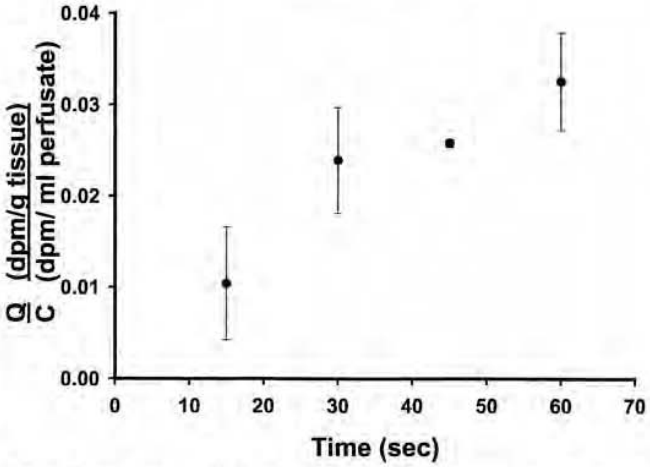

Fig. 1. Time course of GHB influx into rat hippocampus after perfusion with 0.028 μM [^{3}H]GHB. Filled circles represent mean ± S.E.M. (n = 3–4 rats). Similar results were observed for other brain regions (data not shown).

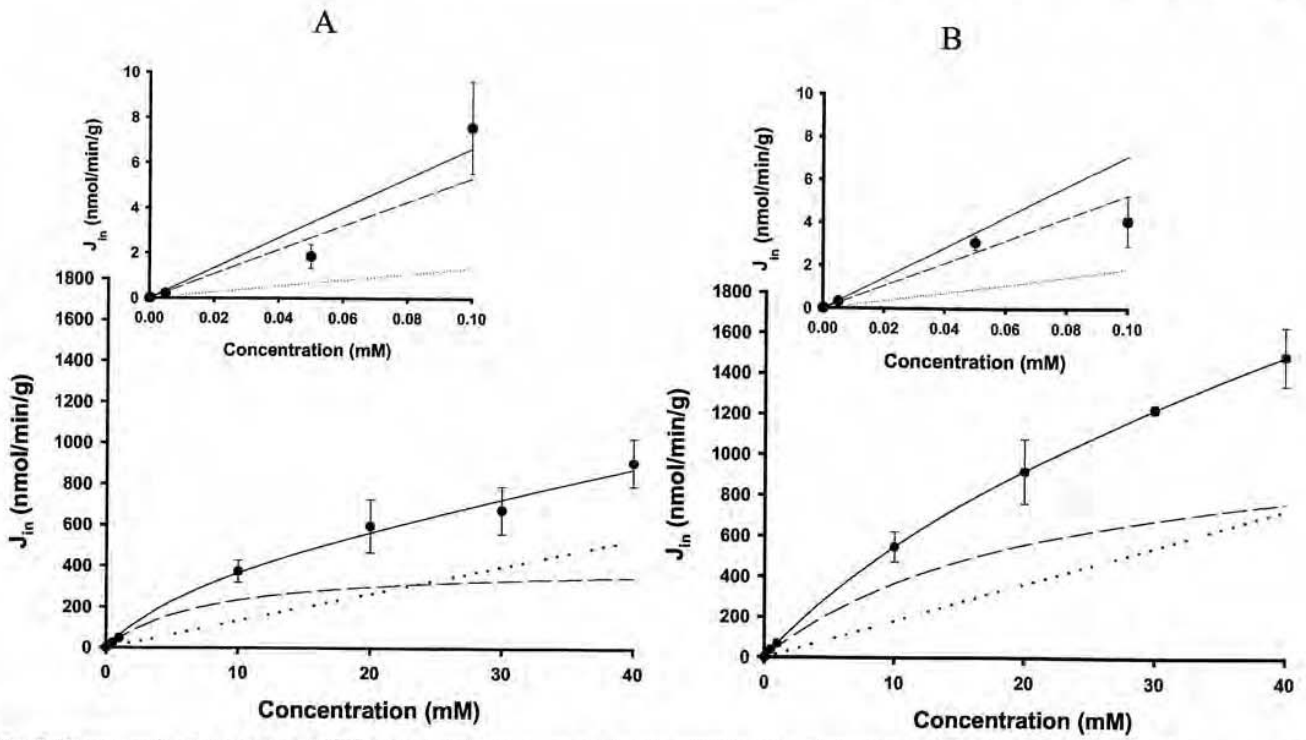

Fig. 2. Concentration dependence of GHB influx into hippocampus (A) and parietal cortex (B) over 0 to 0.1 mM (insets) or 0 to 40 mM (large graphs). The solid line represents the fit of eq. 4 to influx data, the dashed line represents the computer estimated saturable influx, and the dotted line represents 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).

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 seems 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 β-hydroxybutyric acid (four-carbon backbone; C4) each significantly inhibited [^{3}H]GHB BBB influx transport (Table 2).

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}	K_m	CL_{ns}
	$nmol/min/g \times 10^1$	mM	$cm^3/min/g \times 10^{-2}$
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

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 unlabeled 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.

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 nonspecific 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 up-

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

Inhibitor	Conc.	Percentage of Control					
		Hippocampus	Parietal Cortex	Occipital Cortex	Frontal Cortex	Striatum	Thalamus/ Hypothalamus
	mM						
[^{3}H]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*

* $P < 0.05$; significantly different from control by Student's t test (with equal or unequal variances).
† $P < 0.05$; variance significantly different from control by Fisher's variance test.
[a] $n = 8$ for control group.
[b] $n = 4$ for treatment group and [c] $n = 6$ for treatment group.

TABLE 3
Effect of various unlabeled compounds on influx clearance of [^{3}H]GHB across different brain regions
Each value represents mean ± S.E.M. ($n = 8$ for control group, $n = 4$ for treatment group).

Inhibitor	Conc.	Percentage of Control					
		Hippocampus	Parietal Cortex	Occipital Cortex	Frontal Cortex	Striatum	Thalamus/ Hypothalamus
	mM						
[^{3}H]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

* $P < 0.05$; significantly different from control by Student's t test (with equal or unequal variances).
† $P < 0.05$; variance significantly different from control by Fisher's variance test.

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

Inhibitor	Percentage 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*

* $P < 0.05$; significantly different from control by Student's t test (with equal or unequal variances).
† $P < 0.05$; variance significantly different from control by Fisher's variance test.
[a] $n = 4$ for control group and $n = 3$ for treatment group.
[b] $n = 4$ for control group and $n = 9$ for treatment group.

take (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 was 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 concentra-

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