Brain Uptake of the Drug of Abuse γ -Hydroxybutyric Acid in Rats

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ABSTRACT:

γ -Hydroxybutyric acid (GHB) is an endogenous compound and a substrate for the ubiquitous monocarboxylate transporter (MCT) family. GHB is also a drug of abuse due to its sedative/hypnotic and euphoric effects, with overdoses resulting in toxicity and death. The goal of this study was to characterize the distribution of GHB into the brain using in vivo microdialysis and in vitro uptake studies and to determine concentration-effect relationships for GHB in a rat animal model. GHB was administered to rats (400, 600, and 800 mg/kg i.v.), and blood, dialysate, and urine were collected for 6 h post-GHB administration. The GHB plasma and extracellular fluid (ECF) concentration-time profiles revealed that GHB concentrations in ECF closely followed

plasma GHB concentrations. Sleep time increased in a dose-dependent manner (91 ± 18 , 134 ± 11 , and 168 ± 13 min, for GHB 400, 600, and 800 mg/kg, respectively). GHB partitioning into brain ECF was not significantly different at 400, 600, and 800 mg/kg. GHB uptake in rat and human brain endothelial cells exhibited concentration dependence. The concentration-dependent uptake of GHB at pH 7.4 was best-fit to a single-transporter model [$K_m = 18.1$ mM (human), 23.3 mM (rat), $V_{max} = 248$ and 258 pmol $\cdot$ mg $^{-1}$ $\cdot$ min $^{-1}$ for human and rat, respectively]. These findings indicate that although GHB distribution into the brain is mediated via MCT transporters, it is not capacity-limited over the range of doses studied in this investigation.

Introduction

γ -Hydroxybutyrate (GHB) is present endogenously as a metabolite of GABA (Doherty et al., 1978). Administration of GHB causes sedation, respiratory depression, coma, and other effects indicative of central nervous system depression. Numerous studies suggest that many of the pharmacologic effects of GHB at high doses are largely mediated via the GABA $_B$ receptor (reviewed in Carter et al., 2009).

GHB has therapeutic uses as a treatment for narcolepsy (sodium oxybate, Xyrem; Jazz Pharmaceuticals, Palo Alto, CA), and it has been explored to treat alcohol withdrawal. GHB or its precursors 1,4-butanediol and γ -butyrolactone have been abused by bodybuilders for their steroid-like effects (Dyer, 1991), and GHB is often among the drugs ingested at raves and parties (Degenhardt et al., 2002; Halkitis et al., 2007). GHB has also been used in drug-facilitated sexual assaults (Varela et al., 2004). The abuse of GHB has resulted in increased cases of GHB toxicity and overdose (Okun et al., 2001; Wood et al., 2009). The toxic effects of GHB include sedation,

respiratory depression, unconsciousness, coma, and death. Current treatment for GHB overdose is limited to supportive care.

Development of treatments for GHB overdose necessitates a thorough understanding of GHB's toxicokinetics and toxicodynamics. GHB absorption and elimination are capacity-limited (Lettieri and Fung, 1979; Arena and Fung, 1980; Ferrara et al., 1992; Palatini et al., 1993), and GHB renal clearance increases at higher doses (Morris et al., 2005). GHB is a substrate for members of the proton-dependent monocarboxylate transporter family (MCT1, -2, and -4; SLC16A1, A7, A3) (Wang and Morris, 2007; Wang et al., 2007), as well as the sodium-dependent MCT (SMCT1, SLC5A8). L-lactate (an endogenous substrate for MCTs) inhibits MCT-mediated transport of GHB, increases GHB renal clearance, and reduces GHB's sedative/hypnotic effects (Morris et al., 2005; Wang et al., 2008).

We recently reported that GHB concentrations in plasma, whole brain, discrete brain regions, and brain extracellular fluid (ECF) correlate with GHB's sedative/hypnotic effects (Felmlee et al., 2010a). In these studies, we seek to further examine unbound brain concentration-effect relationships by determining the brain ECF concentrations of GHB by microdialysis and correlating ECF concentrations with its sedative/hypnotic effects. In addition, studies were performed to characterize the blood-brain barrier (BBB) uptake of GHB by determining uptake in rat and human brain endothelial cells, in vitro BBB models that expresses MCTs (Smith and Drewes, 2006; Carl et al., 2010), under physiological conditions.

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ABBREVIATIONS: GHB, γ -hydroxybutyrate; MCT, monocarboxylate transporter; SMCT, sodium-coupled monocarboxylate transporter; SLC, solute carrier; ECF, extracellular fluid; BBB, blood-brain barrier; LC/MS/MS, liquid chromatography/tandem mass spectrometry; aCSF, artificial cerebrospinal fluid; GHB-d $_6$, deuterated GHB; HPLC, high-performance liquid chromatography; LRR, loss of righting reflex; RRR, return to righting reflex; CHC, α -cyano-4-hydroxycinnamate; AUC, area under the plasma concentration-time curve; AIC, Akaike information criteria.

FIG. 1. The microdialysis experimental protocol used to study GHB distribution into the frontal cortex and toxicodynamic effects in rats.

Materials and Methods

Chemicals. GHB (sodium salt) was obtained from the National Institute for Drug Abuse (Rockville, MD). Formic acid for liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis and L-lactic acid were purchased from Sigma-Aldrich (St. Louis, MO). Ketamine, xylazine, buprenorphine, and carprofen were all purchased from Henry Schein (Melville, NY). Artificial cerebrospinal fluid (aCSF) was purchased from Harvard Apparatus (Holliston, MA). For the LC/MS/MS assay, deuterated GHB (GHB- d_6) was purchased from Cerilliant (Round Rock, TX), and high-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, water, and acetic acid were purchased from Honeywell Burdick and Jackson (Morristown, NJ). [3H]GHB (purity at least 99% as determined by paper chromatography) was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Animals and Surgery. All animal procedures were approved by the University at Buffalo Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (280–320 g) were used in these studies. Rats were anesthetized with ketamine/xylazine (90/9 mg/kg), implanted with a jugular vein cannula, and subsequently mounted in a stereotaxic frame (Harvard Apparatus). As described previously (Felmlee et al., 2010a), microdialysis guide cannulae (for CMA 11 probes; CMA Microdialysis, North Chelmsford, MA) containing dummy probes were implanted in the frontal cortex (AP + 3.2 mm and ML $\pm$ 2.5 mm from Bregma, and DV -0.5 mm from dura (Paxinos and Watson, 1986) with the incisor bar set 0.5 mm below the interaural line). The side of the brain in which the cannula was placed was alternated across animals. The cannula was fixed in place using jeweler's screws (Plastics One, Roanoke, VA) and dental resin. Rats were allowed at least 6 days for recovery before microdialysis probe implantation.

Evaluation of GHB Concentration-Time Profiles in Brain Using In Vivo Microdialysis. Microdialysis probes (CMA11; CMA Microdialysis) were prepared according to manufacturer's instructions and implanted 24 h before the experiment to allow the BBB to reform after implantation (de Lange et al., 2000). All experiments were conducted in metabolic cages in awake and freely moving rats with ad libitum access to water. The experimental protocol is shown in Fig. 1. On the day of the experiment, microdialysis probes were perfused with aCSF at a rate of 2 μ l/min and allowed to stabilize for 2 h. Microdialysate fractions were collected every 20 min. After stabilization, the probe was perfused with 1 μ g/ml GHB in aCSF for 80 min to calculate in vivo probe recovery of GHB for each rat using the established retrodialysis method (Bouw and Hammarlund-Udenaes, 1998). The retrodialysis period was followed by a washout period in which blank aCSF was perfused for 80 min to remove any residual GHB. No GHB was detected in the final two washout samples; endogenous GHB concentrations are below the detection limits for the LC/MS/MS assay. Rats were then administered GHB (400, 600, or 800 mg/kg i.v.; $n = 4$ /dose), plasma and dialysate samples were collected for 6 h postdose, and times of loss of righting reflex (LRR) and return to righting reflex (RRR) were recorded for each rat. Upon completion of the time course, rats were sacrificed and the probe tracks were stained with dye. Brains were collected to confirm probe location and to ensure probe track condition. Blood samples were centrifuged at 1000g for 15 min at 4°C, and plasma, urine, and dialysate samples were stored at -80°C until analysis.

Plasma, Microdialysate, and Urine Sample Preparation and LC/MS/MS Analysis. Plasma, microdialysate, and urine samples were prepared as described previously (Felmlee et al., 2010a,b). In brief, 5 μ l of GHB- d_6 (1 mg/ml) and GHB stock solution (or double-distilled water for samples) were added to 50 μ l of plasma. Plasma proteins were precipitated with acetonitrile (0.4 ml) followed by centrifugation. Supernatant (0.2 ml) was diluted with 0.8

ml of double-distilled water and extracted using Bond Elut SAX cartridges (Varian, Palo Alto, CA) that were prepared as described previously (Felmlee et al., 2010a). After evaporation, samples were reconstituted in 1.25 ml of 0.1% formic acid in double-distilled water and 5% acetonitrile. Microdialysate samples were diluted with aCSF to bring concentrations within the range of the standard curve. GHB- d_6 (5 μ l of 5 μ g/ml) was added to 35 μ l of microdialysate sample or standard and injected directly onto the LC/MS/MS. GHB- d_6 (10 μ l of 200 μ g/ml) and GHB stock solution (10 μ l) were added to 50 μ l of urine. Double-distilled water (930 μ l) and acetonitrile (1 ml) were then added followed by centrifugation at 10000g for 20 min. The supernatant was collected for LC/MS/MS analysis.

An Agilent 1100 series HPLC with an online degasser, binary pump, and autosampler (Agilent Technologies, Santa Clara, CA) linked to a PE Sciex API triple-quadrupole tandem mass spectrometer with a turbo ion spray (Applied Biosystems, Foster City, MA) were used for all LC/MS/MS analyses. HPLC conditions, mass spectrometer parameters, and linear calibration ranges are detailed in Felmlee et al., 2010a.

GHB Cell Uptake Studies. The immortalized rat (RBE4) and human (hCMEC/D3) brain capillary endothelial cell lines were kindly provided by Prof. P. Couraud (University Rene Descartes, Paris). RBE4 cells (passages 39–44) and hCMEC/D3 cells (passages 28–33) were cultured as monolayers on 75-cm² flasks that were coated with Type I rat-tail collagen (150 μ g/ml; BD Biosciences, San Diego, CA) before plating. Cells were grown at 37°C with 5% CO₂, and medium was changed every 2 to 3 days. RBE4 culture medium was 1:1 α -minimum essentials medium/Hams F10 nutrient mixture supplemented with L-glutamine (2.0 mM), geneticin (300 μ g/ml), human recombinant fibroblast growth factor (1 ng/ml), gentamicin (50 μ g/ml), and 10% v/v qualified fetal bovine serum. hCMEC/D3 culture medium was EBM-2 medium supplemented with 2% fetal bovine serum and growth factors (EGM-2 bullet kit; Lonza Walkersville, Inc., Walkersville, MD). Cells were passaged with 0.25% Trypsin/EDTA and plated on individual collagen-coated, 35-mm wells for uptake studies.

To characterize the uptake of GHB in RBE4 and hCMEC/D3 cells, cells were washed and equilibrated for 30 min at 37°C with uptake buffer containing 138 mM NaCl, 1.8 mM CaCl₂, 5.4 mM KCl, 0.8 mM MgSO₄, 1.0 mM Na₂HPO₄, 5.5 mM D-glucose, and 20 mM HEPES (pH 7.4). Cells were then equilibrated to room temperature for 5 min and subsequently incubated for 0.25, 0.5, 1, 2, 5, and 10 min with [3H]GHB (58 nM) in the same buffer. To determine the concentration-dependent accumulation of GHB in RBE4 and hCMEC/D3 cells, cells were incubated with 0.01, 0.1, 1, 3, 5, 10, 30, and 50 mM GHB for 15 s at room temperature. The 15-s incubation period minimized loss due to metabolism and loss of the radiolabel. Acute inhibition of the uptake of 10 mM GHB was conducted with the MCT inhibitor α -cyano-4-hydroxycinnamate (CHC) (2.5 mM). After incubation, cells were lysed with 0.5 ml of NaOH (1.0 N) for 60 min at room temperature. After lysis, the NaOH was neutralized with 0.5 ml of HCl (1.0 N). [3H]GHB accumulation was determined by liquid scintillation counting, and protein concentration was measured using bicinchoninic acid assay (Pierce, Waltham, MA) with bovine serum albumin as a standard.

Data and Statistical Analysis. To calculate the relative in vivo recovery of GHB from the dialysis probe, retrodialysis recovery was used as described previously (Felmlee et al., 2010a). The recovery of GHB from the probe was calculated using eq. 1 below:

$$\text{Recovery}_{\text{Probe}} = \frac{C_{\text{perfusate}} - C_{\text{dialysate}}}{C_{\text{perfusate}}} \quad (1)$$

FIG. 2. GHB concentration-time profiles in plasma (A) and brain extracellular fluid (B) after intravenous administration of GHB 400 mg/kg ($\square$, dotted line), 600 mg/kg ($\triangle$, dashed line), or 800 mg/kg ($\circ$, solid line). The midpoint time represents the midpoint time for each 20-min fraction collection period. Data are plotted as the mean $\pm$ S.D., $n = 4/\text{dose}$.

where $C_{\text{perfusate}}$ is the perfusate GHB concentration (inlet) and $C_{\text{dialysate}}$ is the dialysate GHB concentration (outlet). To determine GHB concentrations in frontal cortex ECF, GHB dialysate concentrations after intravenous administration of GHB were divided by the experimentally determined GHB probe recovery.

The area under the concentration-time curve (AUC) for GHB in plasma and brain was calculated by the log-linear trapezoidal method using Phoenix WinNonlin 6.0 (Pharsight Corporation, Mountain View, CA). Overall unbound brain ECF/plasma partition coefficients for GHB ($K_{p,u,u}$) were calculated by dividing the brain ECF AUC for GHB by the GHB plasma AUC. Note that GHB exhibits negligible protein binding in plasma (Morris et al., 2005). The time course for partitioning was determined by dividing the respective GHB brain ECF concentration (determined by noncompartmental analysis) with the measured plasma GHB concentration at that time point. Sleep time (minutes) was determined by subtracting the time at which LRR occurred from the time when RRR occurred. Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Inc., San Diego, CA). Statistical differences between group means were assessed using one-way analysis of variance with nonparametric analysis (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

The uptake kinetic parameters Michaelis-Menten constant K_m , maximal velocity V_{max} , and diffusional clearance P were determined using weighted nonlinear regression analysis using Phoenix-WinNonlin 6.0 (Pharsight Corporation) and the following equations:

$$v = \frac{V_{\text{max}} + C}{K_m \times C} \quad (2)$$

$$v = \frac{V_{\text{max}} + C}{K_m \times C} + P \times C \quad (3)$$

$$v = \frac{V_{\text{max}1} + C}{K_{m1} \times C} + \frac{V_{\text{max}2} + C}{K_{m2} \times C} \quad (4)$$

where v is the rate of GHB uptake and C is the GHB concentration. Goodness of fit was determined by the sum of squared derivatives, residual plot, and Akaike information criteria (AIC). Equation 2 yielded the smallest coefficient of variation percentage and AIC value and was subsequently used to estimate kinetic uptake parameters.

Results

GHB Microdialysis in Frontal Cortex. The mean *in vivo* retrodialysis recovery of GHB using the CMA11 probes was $32.5 \pm 7.1\%$ (mean $\pm$ S.D.). The GHB plasma and brain ECF concentration-time profiles increased dose dependently after intravenous administration of 400, 600, and 800 mg/kg GHB [Fig. 2, top (plasma) and bottom (brain ECF)]. Peak GHB plasma and frontal cortex ECF concentrations are reported in Table 1.

To further characterize the distribution of GHB into brain ECF, plasma and brain ECF concentrations were normalized for dose, shown in Fig. 3. The dose-normalized GHB concentrations in plasma and brain ECF at the first six time points (from 5 min, C_{max} , through 120 min) were similar and overlapping for the 400, 600, and 800 mg/kg doses. After 120 min, dose-normalized GHB plasma concentrations from the 400 mg/kg dose were reduced compared with the 600 and 800 mg/kg doses, and this decline was also observed in GHB ECF concentrations at the same time point.

Plasma and brain ECF AUCs (mean $\pm$ S.D.) were determined by noncompartmental analysis and are shown in Table 2. The overall brain ECF/plasma partition coefficients were not significantly different among doses (Table 2). The time course for GHB ECF/plasma partitioning demonstrated a slow increase in partitioning over the first 30 min (consistent with the distribution phase of the plasma and ECF GHB concentration-time curves) after which partitioning remained constant at 0.1 until 180 min, when high variability was observed (Fig. 4).

Higher GHB doses resulted in increased GHB renal clearance for the 800 mg/kg dose (1.55 ± 0.52 , 1.41 ± 0.21 , and $2.18 \pm 0.59 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ for GHB 400, 600, or 800 mg/kg, respectively; $p < 0.01$ for 800 mg/kg versus 400 or 600 mg/kg) and longer sleep times (90.4 ± 14 , 138 ± 12 , $171 \pm 12 \text{ min}$, for GHB 400, 600, or 800 mg/kg, respectively; $p < 0.05$). Sleep time after GHB administration tended to increase as the area under the GHB ECF concentration-time profile increased ($r^2 = 0.557$; data not shown). The concentration of GHB in plasma or frontal cortex ECF at RRR was not significantly different across doses (Fig. 5).

GHB Uptake in Brain Endothelial Cells. The time course for the uptake of 58 nM [^3H]GHB in RBE4 and hCMEC/D3 cells was studied

TABLE 1

Peak GHB concentrations (C_{max}) in plasma and brain ECF observed in rats administered 400, 600, or 800 mg/kg GHB *i.v.*

The peak concentrations were observed at the same time (T_{max}) across all dose groups. Data shown are mean $\pm$ S.D., $n = 4/\text{dose}$.

GHB Dose	Plasma C_{max}		Plasma T_{max}	Brain ECF C_{max}		Brain ECF T_{max}
	$\mu\text{g/ml}$	mM		$\mu\text{g/ml}$	mM	
400 mg/kg	874 ± 114	8.4 ± 1	5	41.3 ± 14	0.39 ± 0.1	50
600 mg/kg	1220 ± 142	11.7 ± 1	5	57.8 ± 30	0.55 ± 0.3	50
800 mg/kg	1620 ± 113	15.6 ± 1	5	69.2 ± 19	0.66 ± 0.2	50

FIG. 3. GHB dose-normalized concentration-time profiles in plasma (A) and frontal cortex ECF (B) after 400, 600, or 800 mg/kg GHB i.v. GHB plasma and ECF concentrations were divided by their respective dose to assess linearity of distribution. The midpoint time represents the midpoint time for each 20-min fraction collection period. Data are plotted as the mean $\pm$ S.D., $n = 4$ /dose.

up to 10 min of incubation at room temperature at pH 7.4. GHB exhibited rapid linear uptake through 30 s (Fig. 6, A and C). The time point of 15 s was used to examine the uptake of various concentrations of GHB.

The concentration-dependence of GHB uptake in RBE4 and hCMEC/D3 cells was determined over a concentration range of 100 μ M to 50 mM at pH 7.4 (Fig. 6, B and D). GHB uptake exhibited classic Michaelis-Menten characteristics and was fitted to eqs. 2 to 4. Uptake data for both RBE4 and hCMEC/D3 cells was best fit to eq. 2 based on sum of squared derivatives, AIC, and residual plots. Data fitting yielded kinetic parameter estimates of $K_m = 23.3 \pm 5$ mM, $V_{max} = 258 \pm 41$ pmol $\cdot$ mg $^{-1}$ $\cdot$ min $^{-1}$ for RBE4 cells, and parameter estimates of $K_m = 18.1 \pm 3$ mM, $V_{max} = 248 \pm 34$ pmol $\cdot$ mg $^{-1}$ $\cdot$ min $^{-1}$ for hCMEC/D3 cells. The uptake of 10 mM GHB was also studied at pH 6.5 and in the presence of the MCT-specific inhibitor CHC. GHB uptake at pH 6.5 was increased to 126% of uptake at pH 7.4 in RBE4 cells. In the

TABLE 2

Noncompartmental analysis of plasma and brain ECF concentration-time profiles for rats administered 400, 600, or 800 mg/kg GHB i.v.

Data shown are mean $\pm$ S.D., $n = 4$ /dose.

GHB Dose	Plasma AUC mg $\cdot$ min/ml	Brain ECF AUC mg $\cdot$ min/ml	Overall Partition Coefficient Brain AUC/Plasma AUC
400 mg/kg	66.0 $\pm$ 13	4.9 $\pm$ 1	0.079 $\pm$ 0.01
600 mg/kg	124 $\pm$ 23*	8.7 $\pm$ 3	0.070 $\pm$ 0.02
800 mg/kg	145 $\pm$ 26*	11.2 $\pm$ 3*	0.070 $\pm$ 0.02

* $P < 0.05$ vs. 400 mg/kg alone.

FIG. 4. Time course for GHB partitioning into brain ($K_{p,u,u}$) was determined from unbound plasma and brain ECF concentrations after GHB doses of 400, 600, and 800 mg/kg i.v. GHB ECF concentrations were divided by GHB plasma concentrations for each plasma sample time point. No significant differences were detected. Data are plotted as the mean $\pm$ S.D., $n = 4$ /dose.

presence of CHC, GHB uptake was inhibited to 60% of control in RBE4 cells and to 66% in hCMEC/D3 cells.

Discussion

This report characterizes the in vivo time course of GHB in plasma and brain (frontal cortex) ECF after intravenous administration of 400,

FIG. 5. GHB concentrations in plasma (A) or frontal cortex ECF (B) at RRR were calculated via noncompartmental analysis from the GHB concentration-time profiles. GHB concentrations at RRR were not significantly different across doses. Data are presented as mean $\pm$ S.D., $n = 4$ /dose.

FIG. 6. Time course for GHB uptake in RBE4 (A) and hCMEC/D3 (C) cells. Cells were incubated with 58 nM [3 H]GHB up to 10 min at room temperature at pH 7.4 to characterize the time course for uptake. Concentration-dependent uptake of GHB in RBE4 (B) and hCMEC/D3 (D) cells. Uptake was done at pH 7.4 by incubating cells with up to 50 mM GHB for 15 s at room temperature. Data are displayed as the mean $\pm$ S.D. of three experiments, each experiment performed in triplicate, and GHB uptake data for each cell line were best fit to the simple Michaelis-Menten equation (eq. 2) as described under *Materials and Methods*.

600, or 800 mg/kg GHB, and it provides *in vitro* kinetic parameter estimates for GHB uptake in rat and human brain endothelial cells.

In this study, the microdialysis probe placement was in the frontal cortex for sampling after intravenous GHB administration. The frontal cortex was used to assess GHB toxicokinetics because GABA_B receptors [a likely site of action for GHB (Mathivet et al., 1997; Nasrallah et al., 2010)] are highly expressed in the cerebral cortex (Bowery et al., 1987). In addition, GABA concentrations can be measured in the frontal cortex, and a previous report indicated a reduction in GABA release in the frontal cortex after the subcutaneous administration of γ -butyrolactone, a precursor of GHB (Hu et al., 2000). Finally, the frontal cortex is connected with other brain regions involved in the mesolimbic dopaminergic pathway that is activated by drugs of abuse (Wise, 2002). For these reasons, GHB ECF levels were measured in the frontal cortex.

Consistent with previous studies (Lettieri and Fung, 1979; Felmler et al., 2010b), nonlinearity was observed in the dose-normalized GHB concentration-time profiles in plasma, most likely due to capacity-limited elimination. The dose-normalized GHB concentration-time profiles in brain ECF also exhibited nonlinearity (the profile of the 400 mg/kg dose did not overlap with the 600 and 800 mg/kg doses). However, GHB concentrations in brain ECF (which are unbound GHB concentrations) appeared to closely follow plasma concentration-time profiles; the nonlinearity observed in ECF concentration-time profiles was not greater than that observed in the GHB plasma concentration-time profiles. Because GHB binding to plasma proteins is negligible (Morris et al., 2005), GHB concentrations in plasma represent unbound concentrations, and the partition coefficients represent the unbound partition coefficient $K_{p,u,u}$. The overall partitioning of GHB into the brain determined from overall AUC ratios are not

significantly different across the doses studied. Calculating the ECF to plasma partition coefficients at each plasma-sampling time point indicated that the partitioning time course overlapped for the three doses studied and peaked at approximately 0.1 during the first 180 min after dosing for the three GHB doses, after which high variability was observed during the rapid elimination phase. Thus, GHB distribution into the brain appears to be linear over the dose range studied.

To further characterize the distribution of GHB into the brain, we studied the uptake of GHB in RBE4 and hCMEC/D3 cells—rat and human brain endothelial cell lines that have previously been characterized as useful *in vitro* models of the blood-brain barrier (Roux et al., 1994; Poller et al., 2008). The uptake of GHB in RBE4 and hCMEC/D3 cells was saturable, concentration- and pH-dependent, and inhibited by CHC, all indicating the role of MCTs as previously demonstrated for GHB uptake in various other cell lines (Wang and Morris, 2007; Wang et al., 2007; Cui and Morris, 2009). The estimated K_m values for uptake, 18.1 ± 3 and 23.3 ± 5 mM, are higher than previously reported values, most likely because the studies here were conducted at pH 7.4 to more closely mimic the physiological conditions at the BBB, rather than a lower pH such as 6.5 or 6 (Wang and Morris, 2007; Wang et al., 2007). The peak plasma GHB concentrations observed here were 1620 ± 113 μ g/ml (15.6 mM) for the 800 mg/kg dose, 1220 ± 142 μ g/ml (11.7 mM) for the 600 mg/kg dose, and 874 ± 114 μ g/ml (8.4 mM) for the 400 mg/kg dose. From 30 to 180 min, GHB plasma concentrations ranged from 1020 to 357 μ g/ml (9.8–3.4 mM), 793 to 249 μ g/ml (7.6–2.4 mM), and 569 to 69 μ g/ml (5.5–0.6 mM) for the 800, 600, and 400 mg/kg doses, respectively. Post-mortem GHB plasma concentrations were recently reported to be 18 to 4400 mg/l, or 172 μ M to 42.2 mM (Zvosec et al., 2011), indicating that the GHB doses used here elicit plasma GHB

concentrations that are relevant to concentrations of GHB observed in clinical cases. These data, in conjunction with the dose-normalized ECF concentration-time profiles, the partitioning time course of brain ECF to plasma, and estimated K_m values from in vitro uptake studies, suggest that the distribution of GHB into the brain is saturated only at very high plasma concentrations such as those seen after toxic doses of GHB [up to 21 mM (Knudsen et al., 2010) or 42 mM (Zvosec et al., 2011)].

GHB has been shown to be a substrate for MCT1, -2, and -4 (Wang et al., 2006; Wang and Morris, 2007). MCT1 is expressed at the BBB and is the predominant MCT in RBE4 cells, whereas MCT2 and MCT4 have been detected in RBE4 cells to a lesser degree (Smith and Drewes, 2006). MCT1 and MCT4 mRNA have both been detected in hCMEC/D3 cells (Carl et al., 2010). The similarity in the in vitro uptake characteristics of GHB in human and rat brain endothelial cells indicates that both serve as useful systems to study GHB brain uptake, but further investigation will be necessary to fully characterize the interspecies differences in transporter expression.

Only MCT1 has been reported to be present in rat BBB, whereas MCT2 and -4, and SMCT1 are present at other sites within the brain (Pierre and Pellerin, 2005). GHB (pK_a 4.72) is over 99% ionized at physiological pH and, therefore, requires facilitated transport across lipophilic membranes. Other potential transporter candidates for GHB brain uptake include the GABA transporters (GAT 1–4), among others. GHB is structurally similar to and a metabolite of GABA, and many of GHB's pharmacological effects are mediated via the GABA_b receptor (Carter et al., 2009). However, GABA did not inhibit GHB brain uptake (Bhattacharya and Boje, 2004), neither does GHB inhibit the brain uptake of GABA (Iversen and Johnston, 1971). Further investigation is necessary to identify other potential transporters of GHB at the BBB.

GHB concentrations in plasma and frontal cortex ECF at the termination of GHB's sedative/hypnotic effect (RRR) were not significantly different across doses, suggesting that GHB ECF concentrations in this brain region are relevant for assessing GHB's sedative/hypnotic effect. This is consistent with recent work in our laboratory measuring total GHB concentrations in plasma, whole brain, hippocampus, striatum, frontal cortex, and frontal cortex ECF at RRR. Whole brain and frontal cortex total concentrations of GHB at RRR (70 and 85 $\mu\text{g/g}$, respectively) were at least double the GHB concentrations in ECF at RRR (35 $\mu\text{g/g}$), indicating that GHB is sequestered or bound to tissue components, perhaps intracellularly. Neurons and glial cells may represent compartments of distribution and accumulation within the brain. MCT1 and -4 are expressed on astrocytes, and MCT2 is expressed on neurons (reviewed in Pierre and Pellerin, 2005), but MCT2 is also expressed on astrocyte endfeet at the apical surface of BBB endothelial cells (Gerhart et al., 1998). SMCT1 is expressed on neurons and may result in increased intracellular GHB concentrations (Martin et al., 2006). In addition, GABA inhibits the uptake of GHB into synaptosomes (Benavides et al., 1982), suggesting that another transporter (potentially one of the GAT family) is involved in the cellular uptake of GHB. Additional investigation is necessary to characterize the uptake of GHB by astrocytes and neurons, and to determine the involvement of other transporters such as neuronal SMCTs or those of the GAT family in the brain distribution of GHB.

In conclusion, the findings presented here indicate that GHB distribution into the frontal cortex is not capacity-limited at the doses studied, and GHB concentrations in frontal cortex ECF are associated with GHB's sedative/hypnotic effect. Further studies will examine how treatments that inhibit the MCT-mediated transport of GHB alter the concentrations of GHB in brain ECF.

Authorship Contributions

Participated in research design: Roiko, Felmlee, and Morris.

Conducted experiments: Roiko and Felmlee.

Contributed new reagents or analytic tools: Roiko, Felmlee, and Morris.

Performed data analysis: Roiko.

Wrote or contributed to the writing of the manuscript: Roiko, Felmlee, and Morris.

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