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Research Paper

Alterations in Neuronal Transport but not Blood-Brain Barrier Transport are Observed during Gamma-Hydroxybutyrate (GHB) Sedative/Hypnotic Tolerance

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Purpose. To investigate if γ-Hydroxybutyrate (GHB) tolerance is mediated by alterations in GHB systemic pharmacokinetics, transport (blood brain barrier (BBB) and neuronal) or membrane fluidity.
Materials and Methods. GHB tolerance in rats was attained by repeated GHB administration (5.31 mmol/kg, s.c., QD for 5 days). GHB sedative/hypnotic effects were measured daily. GHB pharmacokinetics were determined on day 5. In separate groups, on day 6, *in situ* brain perfusion was performed to assess BBB transport alterations; or *in vitro* studies were performed (fluorescence polarization measurements of neuronal membrane fluidity or [^{3}H]GABA neuronal accumulation).
Results. GHB sedative/hypnotic tolerance was observed by day 5. No significant GHB pharmacokinetic or BBB transport differences were observed between treated and control rats. Neuronal membrane preparations from GHB tolerant rats showed a significant decrease in fluorescence polarization (treated — 0.320 ± 0.009, $n = 5$; control — 0.299 ± 0.009, $n = 5$; $p < 0.05$). [^{3}H]GABA neuronal transport V_{max} was significantly increased in tolerant rats (2,110.66 ± 91.06 pmol/mg protein/min vs control (1,612.68 ± 176.03 pmol/mg protein/min; $n = 7$ $p < 0.05$).
Conclusions. Short term GHB administration at moderate doses results in the development of tolerance which is not due to altered systemic pharmacokinetics or altered BBB transport, but might be due to enhanced membrane rigidity and increased GABA reuptake.

KEY WORDS: GABA transport; gamma-hydroxybutyric acid; membrane fluidity; pharmacokinetics; tolerance.

INTRODUCTION

γ-Hydroxybutyrate (sodium oxybate, GHB) is currently a US Food and Drug Administration approved therapeutic agent for cataplexy with narcolepsy. GHB is also under investigation for potential therapeutic indications, including treatment of alcohol withdrawal (1), anxiety (2) and fibromyalgia (3). GHB's proposed therapeutic benefit in the treatment of opioid withdrawal, albeit promising, requires further investigation (4).

Unfortunately, GHB's abuse liability has overshadowed its therapeutic benefits. GHB and its precursors (γ-butyrolactone and 1,4-butanediol) are widely abused as anabolic agents, euphoriants and date rape drugs. GHB, initially synthesized as a GABA (γ-aminobutyric acid) mimetic, likely mediates its physiological and pharmacological effects through GHB and/or $GABA_B$ receptors (5). GHB's physiological effects include neuromodulation and tissue protection (from excessive metabolic demand) (6) whereas its pharmacological effects encompass sedative, hypnotic and anesthetic actions (7). These pharmacological effects follow a steep dose response curve; and as a result, recreational abuse or overdose of GHB (or its precursors) produces dose dependent central nervous system (CNS) effects, ranging from sedation, lethargy and hypnosis to respiratory depression, unconsciousness, coma, and death (8).

Literature evidence on the development of tolerance in man following chronic misuse of GHB is equivocal, and could be dependent on a variety of factors including recreational use (often involving high doses) or therapeutic use (lower doses). Galloway *et al.* (9) published individual cases on recreational use, where individuals self-reported the

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ABBREVIATIONS: AUC, area under curve; AUMC, area under first moment curve; BBB, blood brain barrier; C, perfusion fluid concentration of tracer; CL/F, systemic clearance; CL_{in}, influx clearance; C_{max}, maximum concentration; CNS, central nervous system; DPH, 1,6-diphenyl-1,3,5-hexatriene; GABA, γ-aminobutyric acid; GBL, γ-butyrolactone; GHB, γ-hydroxybutyrate; LRR, loss in righting reflex; MCT, monocarboxylate acid transporter; MRT, mean residence time; P, fluorescence polarization; Q, mass of radiotracer in the brain region normalized for wet brain tissue weight; r, anisotropy; RRR, return in righting reflex; S^2, lipid order; T, time of perfusion; $T_{1/2}$, half life; T_{max}, time to maximum concentration; V/F, volume of distribution; V_{vasc}, regional volume of the cerebrovascular capillary bed.

need to escalate GHB dosage to maintain euphoric and sedative effects. There is a substantial amount of anecdotal reports of GHB tolerance on the internet (via web sites and web logs). Addolorato *et al.* (10) reported a voluntary increase in GHB dosage (6–7 times) in 10% of patients undergoing treatment for alcohol dependence. Conversely, in separate clinical trials of 30 and 48 narcoleptic patients, there were no observations of tolerance development with GHB chronic use (30 weeks and 9 years, respectively) (11,12).

Interestingly, there is consensus in the literature regarding the development of tolerance during chronic GHB administration in rodents (13–17). To date, questions still remain as to how GHB exerts its sedative/hypnotic effects and therefore the exact mechanism(s) underlying tolerance development remains unclear. Possible explanations might involve pharmacodynamic (e.g., receptor desensitization, changes in neurotransmitter re-uptake, altered signal transduction pathways or disruption of protein function due to perturbation of neuronal membranes) and/or pharmacokinetic phenomena (e.g., increased metabolism and elimination or decreased BBB transport).

Evidence in support of pharmacodynamic tolerance is provided by Giorgio and Rubio, wherein they reported significantly higher GHB brain concentrations at the time of return of righting reflex in rats chronically dosed with the GHB precursor, γ-butyrolactone (GBL) (16). Also, Ratomponirina *et al.* observed decreased receptor density (45%) based on binding capacity of radiolabeled GHB after chronic treatment with GHB (500 mg/kg i.p., three times a day for 5 days) (17).

Pharmacodynamic tolerance development to GHB effects might be explained by a direct or indirect interaction of GHB with neuronal membranes leading to downstream changes in protein function. Although controversial, it is postulated that the mechanism of action of some anesthetics and sedative/depressants, such as propofol and ethanol, may involve the perturbation of membrane lipid-protein interfaces or function, thereby influencing the activity/function of certain ion channels, pumps, transport proteins or membrane receptors (18–20). It has been shown that Na+/K+ ATPase and sulfate transporter activities are highly dependent upon membrane fluidity and composition (21–23). As GHB has sedative, hypnotic and anesthetic effects, it is possible that GHB exerts alterations on neuronal membrane fluidity.

Another example of pharmacodynamic tolerance may involve alterations in GABAergic neurotransmission. Much of GHB's depressant effects are believed to involve potentiation of the GABAergic system, which is the primary inhibitory pathway in the CNS. While the exact mechanism(s) remain unclear, systemic high dose administration of GHB increases extracellular neuronal GABA levels in rats (24). Others have hypothesized that the GHB-induced increase is either due to a direct receptor-mediated interaction or metabolic inter-conversion of GHB to GABA (24,25). Moreover, the resulting enhanced extracellular GABA levels are sustained over a period of time, which may result in GABA-receptor desensitization/downregulation or increased GABA reuptake transporter expression (26).

The involvement of a pharmacokinetic tolerance is not clear. Van Sassenbroeck *et al.* reported that GHB showed a slightly increased (~20%) systemic elimination in GBL pretreated *versus* control rats (15). We recently reported that GHB undergoes transport at the blood brain barrier (BBB), likely via a monocarboxylate transporter (27). This suggests yet another mechanism of pharmacokinetic tolerance development involving alterations in GHB transport across the BBB.

The present work had four objectives: (1) To reproduce a robust rat model of GHB tolerance to its sedative/hypnotic effects; (2) To test the hypothesis that GHB tolerance may be due to altered systemic pharmacokinetics; (3) To test the hypothesis that GHB tolerance may be due to altered GHB transport across the BBB; (4) To examine the effects of chronic GHB administration on neuronal membrane fluidity and neuronal GABA reuptake.

MATERIALS AND METHODS

Chemicals and Reagents

[^{3}H]GHB (specific activity, 50.0 Ci/mmol; 98.7% pure) was obtained from Moravek Biochemical Inc. (Brea, CA). [^{3}H]GABA (specific activity, 60.0 Ci/mmol; 99% pure) was obtained from American Radiolabeled Chemicals (St. Louis, MO). GHB (sodium salt form), GABA and benzyl alcohol were procured 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. ScintiVerse BD scintillation cocktail was supplied by Fisher Scientific (Pittsburgh, PA). Deuteriated GHB (GHB-D^6) was obtained from Cerilliant (Round Rock, TX). Ketamine and xylazine were purchased from J.A. Webster (Sterling, MA). The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes, Inc. (Eugene, OR).

Development of Rat Tolerance Model to GHB Effects

All procedures involving animals were approved by the University of Buffalo Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (250–300 g) were randomized into two groups, control (n = 13) and GHB treatment (n = 12). Over a 5 day period, the treatment group was dosed daily with GHB (5.31 mmol/kg) subcutaneously (sc), while the control group received an equal volume of isotonic saline daily. Both groups had free access to food and water. Following daily dosing, subjects were placed individually on absorbent pads and monitored for a loss in righting reflex (LRR), defined as the inability of the rats to right themselves when placed on their back, and a return in righting reflex (RRR), the ability of the rats to right themselves. LRR was assessed every 5 min during the first 3 h after dosing (until LRR was achieved). If LRR was achieved within 3 h, the subjects remained on their backs and were not disturbed until they regained their RRR. Total sedative/hypnotic effect time was calculated as the absolute difference between LRR and RRR. LRR and RRR observations were obtained each day of dosing.

GHB Systemic Pharmacokinetics

It was hypothesized that tolerance to GHB's sedative/hypnotic effects may be due to alterations in systemic

pharmacokinetics, such as altered absorption or clearance. Male Sprague Dawley rats (250–300 g) were randomly divided into two groups (n = 5 per group) and surgically cannulated at the jugular vein. Following a 48 h surgical recuperation period, rats were dosed daily for 4 days with either GHB (5.31 mmol/kg, s.c.) or an equivalent volume of isotonic saline. LRR and RRR for each rat were monitored over the 5 day study period. On day 5, all rats were dosed with GHB (5.31 mmol/kg, s.c.) for a pharmacokinetic study. Blood samples (200 μl) were obtained at predose, 15, 30, 45, 60, 90, 120, 150, 180, 240, and 360 min. Blood samples were centrifuged at 2,000 *g* for 20 min at 4°C and the harvested plasma was stored at −20°C for LC/MS/MS analysis.

In Situ Rat Brain Perfusion

In situ brain perfusion studies were performed on day 6, allowing for a 24 h washout period after the last GHB dose. Control (isotonic saline) and GHB treatment groups (from tolerance studies) were further divided into two subgroups on day 6. Each of these subgroups underwent *in situ* brain perfusion with [^{3}H]GHB (0.02 μM; 1.0 μCi/ml) either in presence or absence of unlabeled GHB (40 mM) to quantify GHB transport across the BBB.

The *in situ* rat brain perfusion technique has been described elsewhere (27). Briefly, adult male Sprague Dawley rats were anesthetized using intramuscularly administered ketamine (90 mg/kg) and xylazine (9 mg/kg) and surgically prepared for perfusion (with either physiological buffer or donor rat plasma) of the left cerebral hemisphere via the left common carotid artery. When used, the physiological buffer consisted of 128 mM NaCl, 24 mM $NaHCO_3$, 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) (27). *In situ* brain perfusion was performed by first preperfusing the cerebral hemisphere with physiological buffer for 10 s at 10 ml/min (Harvard perfusion pump model 55-4150, Harvard Apparatus, Holliston, MA), followed by perfusion with [^{3}H]GHB for 30 s at 10 ml/min. Following decapitation, left cerebral hemispheres were dissected into the following brain regions: the cortices (frontal, parietal and occipital), hippocampus, striatum and thalamus/hypothalamus. Dissected tissue samples were weighed and solubilized overnight with 0.8 ml 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 (Packard Instrument Co., Downers Grove, IL; counting efficiency for [^{3}H] was 61%). An aliquot of the perfusion fluid was assayed by liquid scintillation counting to verify the perfusate analyte concentration.

Effects of Circulating Endogenous Plasma Inhibitors on GHB BBB Transport

Since GHB has potential metabolic effects which could increase endogenous competitors of GHB BBB transport, another set of *in situ* brain perfusion studies were performed to assess GHB Cl_{in} in naive rats using plasma harvested from control or GHB treated rats. Separate groups of rats were dosed with saline (control) or GHB, as previously described. Animal body weight changes were monitored daily and on day 5, total food consumption was measured by weighing of remaining rodent chow. On day 5, treatment (n = 8) and control groups (n = 10) were sacrificed by exsanguination under halothane anesthesia. Plasma was harvested by centrifugation (2,000 ×*g* for 20 min at 4°C) and pooled according to treatment group. The pooled plasma was then used as a vehicle for *in situ* brain perfusion of [^{3}H]GHB (0.02 μM; 1.0 μCi/ml) in naïve rats.

GHB Analysis by LC/MS/MS

GHB plasma concentrations were analyzed using a published HPLC/MS/MS method, with slight modifications, as developed by our collaborators (28). In brief, 25 μl of plasma was mixed with 5 μl of GHB-D^6 (6 mM) and 40 μl of water. Methanol (70 μl) was added to precipitate proteins. Samples were vortexed, shaken (20 min on an autoshaker) and centrifuged (10,000 *g* for 20 min at 4°C). The protein free supernatant was retained for HPLC/MS/MS assay.

HPLC was accomplished using a C18 Aqua column (5 μm, 150 × 4.6 mm; Phenonmenex). The mobile phase consisted of 67% methanol and 33% 5 mM formic acid at a flow rate of 0.75 ml/min. The total run time was 5 min with retention time of 2.5 min for GHB. A Perkin Elmer 200 autosampler (Perkin Elmer, Wellesley, MA) was used to inject 10 μl sample volumes. The detector consisted of a Perkin Elmer Sciex API 3000 triple quadrupole mass spectrometer with a turbo ion spray ionization source (source temperature of 400°C). A splitter from the column outlet to the mass spectrometer regulated the detector inlet flow to 200 μl/min. The mass spectrometer was operated in a multiple reaction / positive ion mode. Mass/charge ratio of GHB precursor and product ion was monitored at 105 and 87, respectively, and for GHB-D^6 (internal standard) at 111 and 93, respectively. Peak height ratios of analyte and internal standard were determined using the Analyst 1.3.1 software (Applied Biosystems, Foster City, CA). GHB calibration curves utilizing peak height ratios of GHB to the internal standard (GHB-D^6) was used to calculate the plasma concentrations.

Neuronal Synaptosomal Preparations and Fluorescence Polarization Analysis

For determination of the *in vivo* and *in vitro* effects of GHB on neuronal membrane fluidity, neuronal synaptosomes were prepared according to the method of Whittaker *et al*. (29) using fresh brain tissue obtained from male Sprague–Dawley rats (250–300 g) following either chronic GHB administration (5.31 mmol/kg QD, s.c. for 5 days), chronic vehicle administration (equivalent volume isotonic saline QD, s.c. for 5 days) or using brain tissue excised from naive untreated rats, respectively.

In brief, rats were anesthetized with halothane, decapitated and the brains were harvested. Cerebral cortices were dissected from other forebrain regions and homogenized in 10 vol. of 0.32 M sucrose using a glass tissue homogenizer. The tissue was centrifuged for 10 min at 1,000×*g*. The resultant supernatant was centrifuged for 20 min at 20,000 ×*g* and the crude P_2 pellet was resuspended in 10 vol of 0.32 M sucrose. The P_2 suspension was layered on top of a

discontinuous sucrose-density gradient (0.32, 0.8 and 1.2 M). The samples were centrifuged in a swinging bucket rotor for 90 min at 60,000 $\times g$ Neuronal synaptosomes were harvested from the 0.8–1.2 M interface and resuspended in oxygenated Krebs–Ringer Bicarbonate buffer with glucose (KRBG; 121 mM NaCl, 25 mM $NaHCO_3$, 10 mM d-glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 0.4 mM K_2HPO_4 pH 7.4) and recentrifuged for 20 min at 20,000 $\times g$. The purified synaptosomes were diluted with KRBG to a final protein concentration of 0.1 mg/ml for fluorescence polarization and 4.0 mg/ml for [^{3}H]GABA uptake studies. DPH (1,6-diphenyl-1,3,5-hexatriene; fluorescent probe) was incorporated into the synaptosomal membrane bilayer during a 30-min incubation at 37°C. The incubation concentration of DPH was set at a final probe-to-lipid molar ratio of ~1:1,000. Protein and lipid concentrations were determined by the Lowry method and the Bartlett method, respectively (30,31).

To measure the direct effects of GHB on membrane fluidity *in vitro*, DPH prelabeled neuronal synaptosomes from naive rats were incubated with various concentrations of GHB (0–10 mM) or benzyl alcohol (0–10 mM) (positive control). Membrane fluidity changes were measured over a temperature range of 25–45°C by fluorescence polarization, with excitation at 355 nm and emission at 430 nm, using 4 nm excitation and emission slit widths. The equipment used for the fluorescence polarization consisted of a PTI fluorometer with a xenon arc lamp, motorized Glan–Thompson polarizing prisms and Peltier unit (Photon Technology International, Lawrenceville, NJ). To measure the effects of GHB on membrane fluidity following chronic administration *in vivo*, synaptosomal preparations were obtained from GHB treated and control (vehicle alone) animals. Fluorescence polarization was measured as described, but without addition of GHB or benzyl alcohol.

Neuronal Synaptosomal [^{3}H]GABA Uptake

For the determination of the effects of chronic *in vivo* GHB exposure on neuronal [^{3}H]GABA reuptake, synaptosomes were prepared from treated or control rats as previously described. Brain tissues from each animal permitted an evaluation of 8 concentrations, in triplicate, at 37 and 4°C. Synaptosomal uptake was initiated by adding 50 µl of the synaptosomes to 950 µl of KRBG buffer containing 0.6 µCi of [^{3}H]GABA and various concentrations of unlabeled GABA, as necessary. Based on published data showing that [^{3}H]GABA synaptosomal uptake is linear over 3–5 min (32–34), uptake incubations were performed at 0 or 37°C for 2 min. The reactions were terminated by rapid dilution with 5 ml ice-cold KRBG buffer and vacuum filtration over GF/B filters using a Brandel M-24R Cell Harvester. The filters were rinsed with 5 ml ice-cold KRBG buffer and placed in vials containing 5 ml of ScintiVerse BD scintillation cocktail for liquid scintillation counting.

Data Analysis

Because GHB pharmacokinetics were previously determined to be nonlinear at the doses employed in this study (35), pharmacokinetic parameters for each animal were determined using the noncompartmental analysis module of WinNonlin Pro Version 2.1 (Pharsight Corp., Mountain View, CA). The area under curve (AUC) and area under the first moment of the curve (AUMC) were calculated using the linear trapezoidal rule up to the time to maximum concentration (T_{max}) after which log trapezoidal method was used. The apparent terminal half life ($t_{1/2}$) was obtained by $0.693/k$, where k is the apparent terminal elimination rate constant determined by log linear regression analysis of the terminal phase of the plasma concentration profile. The mean residence time (MRT) was calculated as the ratio of AUMC and AUC. Apparent clearance (CL/F) was calculated as ratio of dose to AUC. The apparent volume of distribution (V/F) was determined as CL/F divided by k.

GHB influx clearance determined from the *in situ* brain perfusion studies, (CL_{in}, cm^3/min/g), was calculated as (Eq. (1)):

$$\frac{Q}{C} = \mathrm{CL}_{\mathrm{in}}^{*} T + V_{\mathrm{vasc}} \tag{1}$$

where, Q (dpm/g) represents the quantity of radiotracer in the brain region normalized for wet brain tissue weight, C (dpm/cm^3) represents the perfusion fluid concentration of [^{3}H]GHB, T (min) is the time of perfusion (0.5 min) and V_{vasc} (cm^3/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 (27).

Neuronal synaptosomal membrane fluidity changes were assessed by fluorescence polarization (P), anisotropy (r) and lipid order (S^2) parameters (Eqs. (2), (3), and (4)):

$$P = \frac{I_{II} - I_{\perp}}{I_{II} + I_{\perp}} \tag{2}$$

$$r = \frac{2(P)}{(3 - P)} \tag{3}$$

$$S^2 = \frac{(4r/3) - 0.1}{r_0} \tag{4}$$

where I_{II} and $I_{\perp}$ are the parallel and perpendicular intensities, and $r_0 = 0.4$ (maximal fluorescence anisotropy value) (36). A decrease in the parameter value as a function of increased drug concentration or increased temperature indicates an increase in membrane fluidity. The polarization and anisotropy values are indicative of changes in the rotational movement of DPH in the bilayer and the lipid order parameter gives information about the fluid phase of the bilayer.

For neuronal [^{3}H]GABA accumulation studies, the specific activity of the radioisotope of interest was used to convert dpm to mass and normalized for protein mass and incubation time. Net synaptosomal uptake was determined as the difference between the total uptake (determined at 37°C) and non-specific uptake (determined at 0°C). The synaptosomal uptake *versus* concentration data was analyzed using Eq. (5), which describes a saturable uptake process.

$$v = \frac{V_{\mathrm{max}}^{*} C}{K_m + C} \tag{5}$$

where v is rate of synaptosomal accumulation during the incubation period, V_{max} is the corresponding maximal rate of accumulation, C represents drug concentration and K_m is the Michaelis–Menten dissociation constant. For each animal, parameter estimates of $V_{max,}$ and K_m were obtained by nonlinear regression analysis using the computer program, WINONLIN (Pharsight Corp., Mountain View, CA).

Statistical Analysis

All statistical analyses were performed using SAS version 9.0 (SAS Institute, Cary, NC). Results were considered statistically significant at $p < 0.05$. Data are presented as mean ± S.D. or S.E.M., as specified.

All LRR, RRR and duration of sedative/hypnotic effect data were analyzed using repeated measures ANOVA followed by Bonferroni's *post hoc* analysis. Significant differences in weight gain over the 5 day study were analyzed with repeated measures ANOVA, followed by Dunnett's *post hoc* analysis. If a rat did not lose its LRR after GHB dosing, then for the purposes of statistical analysis it was designated as a non-responder and its LRR, RRR and duration of sedative/hypnotic effect were set as missing values. Comparative differences in sedative/hypnotic effect time between the treatment groups and control groups for the first GHB dose (Day 1 for treatment; Day 5 for control) was assessed with one way ANOVA with Bonferroni's *post hoc* analysis. Differences in pharmacokinetic parameters were assessed by Student's *t*-test (two-tailed). Significant differences in brain CL_{in} between the treatment and control groups were evaluated using either Student's *t*-test (when donor plasma was used as perfusate) or one way ANOVA with Bonferroni's *post hoc* analysis (when physiologic buffer used as perfusate).

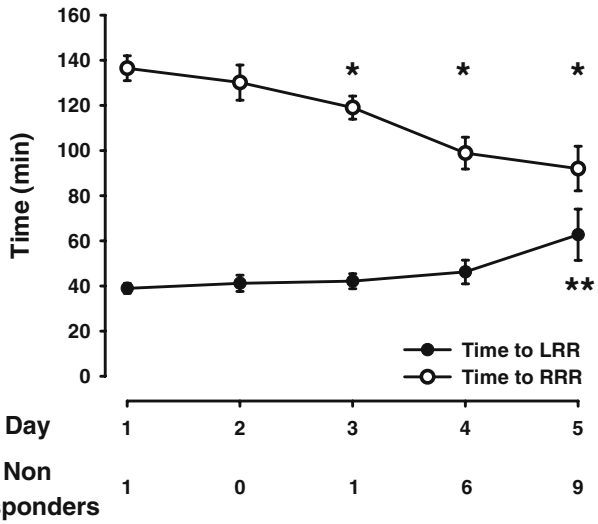

Fig. 1. Time to loss in righting reflex (LRR) and return of righting reflex (RRR) for rats responding to a dose of 5.31 mmol/kg GHB s.c. over 5 days. *Closed circles* represent LRR while *open circles* represent RRR. ** $p < 0.05$; significantly different from Day 1 (LRR), * $p < 0.05$; significantly different from Day 1 (RRR). Total number of animals used was 12 per day. Data represents mean ± S.E.M. of responders.

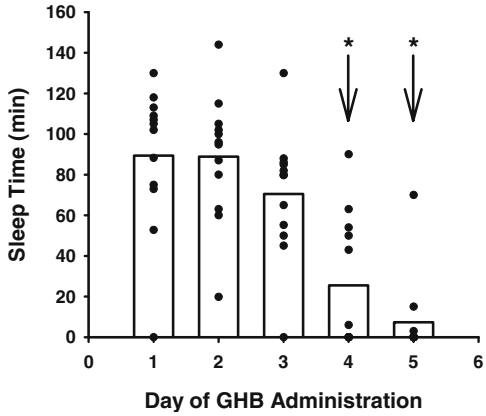

Fig. 2. Total sedative/hypnotic effect time (absolute difference between LRR and RRR) for rats dosed with 5.31 mmol/kg GHB s.c. for 5 days ($n = 12$). *Closed circles* represent sedative/hypnotic effect time of each rat and *bars* represent average sedative/hypnotic effect time per day. *$p < 0.05$; significantly different from Day 1.

Differences in fluorescence polarization values in the presence and absence of GHB *in vitro* were assessed using one-way ANOVA with Bonferroni *post-hoc* analysis. Differences in polarization values between control and GHB treated groups were determined using repeated measures ANOVA with Bonferroni *post hoc* analysis.

An unpaired Student's *t*-test was used to assess the statistical significance of transport parameter estimates (V_{max} and K_m) for control *versus* chronic treatment groups.

RESULTS

Development of Tolerance to GHB Sedative/Hypnotic Effects

Figures 1 and 2 illustrate the time course of GHB tolerance development. Repetitive GHB dosing resulted in a significant increase in time to LRR on day 5 *versus* day 1 and a significant decrease in time to RRR on days 3, 4 and 5 *versus* day 1 (Fig. 1). The number of rats non-responsive to GHB's sedative/hypnotic effects increased with repetitive dosing (Fig. 1). The sedative/hypnotic effect time (difference between RRR and LRR) also decreased with repeated daily GHB dosing (Fig. 2). There was a significant decrease in the sedative/hypnotic effect time on days 4 and 5 when compared to day 1 (Fig. 2).

Systemic GHB Pharmacokinetics

Altered systemic pharmacokinetics is one potential mechanism of tolerance to GHB's sedative/hypnotic effects. Figure 3 depicts day 5 GHB plasma concentration-time profiles of two groups of rats: single GHB dose (GHB administered s.c. on day 5 following 4 days of saline) and short-term, daily GHB dosing (GHB administered s.c. for 5 days). The profile of the short-term, daily GHB dosing is similar to that from a single GHB dose. Since GHB pharmacokinetics are nonlinear at the doses employed in this study (35), the data were analyzed by noncompartmental methods; Table I presents the results of this analysis. None of

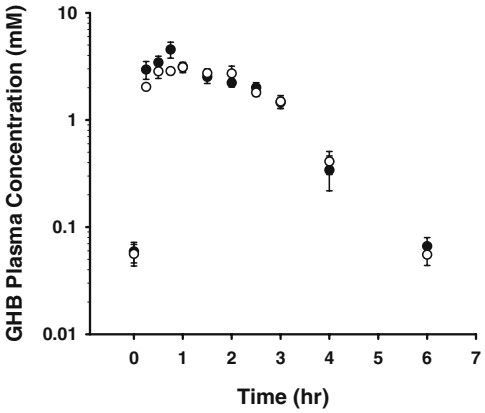

Fig. 3. GHB plasma pharmacokinetics of single and short-term daily (5 day) GHB dosing (5.31 mmol/kg s.c.). Data represents mean ± S.E.M. (n = 5 each group). *Closed circles* represent short-term GHB dosing while *open circles* represent single dose. Time zero represents endogenous GHB concentrations (0.056 ± 0.012 mM for single dosing and 0.059 ± 0.013 mM for short-term dosing).

the noncompartmental parameters were significantly different between the single dose group and the short-term dosing group. These data exclude a tolerance mechanism based on altered systemic pharmacokinetics after s.c. administration.

In Situ Rat Brain Perfusion

GHB is transported across the BBB by a carrier-mediated transporter, most likely by an isoform of the monocarboxylate acid transporter (MCT) (27). Alterations in BBB transport properties can represent another mechanism of tolerance to GHB's sedative/hypnotic effects. Table II reports [^{3}H]GHB BBB transport CL_{in} values for the GHB treatment and control groups in presence or absence of unlabeled 40 mM GHB. No significant differences were observed for the CL_{in} values obtained from perfusion with [^{3}H]GHB alone for the control and treatment groups. However, significant decreases in CL_{in} values were observed within each group when unlabeled GHB was added, consistent with a saturable transport mechanism. However, this decrease in CL_{in} in the presence of unlabelled GHB was not different between the GHB treated and control groups. The finding that 40 mM GHB decreases the BBB transport of GHB influx confirms our previous findings (27), and also rules out altered GHB BBB transport as a mechanism of tolerance to GHB sedative/hypnotic effects.

Table I. Noncompartmental Pharmacokinetic Parameter Estimates in Single and Short-term (5 day) GHB Dosing (5.31 mmol/kg s.c.)[a]

Pharmacokinetic parameter	Single dose group	Short-term dosing group
AUC (mmol*h*l^{-1})	8.26 ± 0.182	8.79 ± 0.913
V/F (l*kg^{-1})	0.824 ± 0.217	1.01 ± 0.254
CL/F (l*h^{-1}*kg^{-1})	0.644 ± 0.014	0.636 ± 0.079
AUMC (mmol*h^2*l^{-1})	15.2 ± 0.816	15.3 ± 1.65
MRT (h)	1.85 ± 0.057	1.74 ± 0.018
T_{max} (h)	1.35 ± 0.312	0.800 ± 0.05
C_{max} (mM)	3.71 ± 0.214	4.58 ± 0.745
Half life (h)	0.887 ± 0.235	1.09 ± 0.266

[a]Four days of isotonic saline preceded GHB dosing in the single dose group. GHB pharmacokinetics were determined on day 5 in the short-term group. Data represent mean ± S.E.M. (n = 5).

During the course of these studies, the saline control group gained weight by day 5 (280 ± 6.18 g on day 1, 305 ± 5.12 g on day 5, n = 16, $p < 0.05$), while the body weights of the GHB treatment group neither increased nor decreased (278.8 ± 5.64 g on day 1, 279.4 ± 5.50 g on day 5, n = 21). A significant difference in rat chow consumption was observed between the two groups, with the treatment group consuming less food (72.06 ± 2.49 g, n = 9) than the control (95.6 ± 2.98 g, n = 16; $p < 0.05$). These data suggest that GHB treated rats could also be in a semi-starved condition which can result in an increased level of endogenous ketone bodies in the blood. Alternatively, GHB is proposed to increase growth hormone secretion (37), which in turn is known to induce ketogenesis probably through lipolysis (38). Thus, an increased circulating concentration of ketone bodies could competitively inhibit MCT mediated GHB transport across the BBB and thus contribute to tolerance development. However, no significant differences were observed in CL_{in} (Table III) obtained by perfusion of naive rats with donor plasma obtained from control or GHB treated rats. The CL_{in} values derived using plasma as a perfusion vehicle were significantly lower (unpaired t-test, $p < 0.05$) than those values observed using

Table II. GHB Influx Clearance Values (10^{-2} × cm^3/min/g) across the Rodent BBB following Saline (control) or GHB (treatment; 5.31 mmol/kg, s.c.)[a]

	Control		Treatment	
Brain region	Tracer only (n = 6)	Tracer + 40 mM GHB (n = 7)	Tracer only (n = 6)	(Tracer + 40 mM GHB) (n = 6)
Hippocampus	5.92 ± 0.547	3.65 ± 0.418*	7.04 ± 0.623	4.19 ± 0.359†
Striatum	7.08 ± 0.835	4.63 ± 0.866	7.31 ± 0.921	6.19 ± 0.564
Frontal cortex	8.39 ± 0.834	4.93 ± 0.498*	8.78 ± 0.524	5.51 ± 0.444†
Parietal cortex	8.00 ± 0.805	5.05 ± 0.617*	8.07 ± 0.651	5.45 ± 0.435†
Occipital cortex	8.29 ± 0.998	4.74 ± 0.595*	8.39 ± 1.13	4.99 ± 0.332†
Thalamus/hypothalamus	6.29 ± 0.883	3.97 ± 0.587	7.42 ± 0.842	4.11 ± 0.485†

[a]Data are mean ± S.E.M.
*$p < 0.05$; Significantly different from control (tracer only).
†$p < 0.05$; Significantly different from treatment (tracer only).
V_{vasc} data (× 10^{-3} cm^3/g) were previously determined in laboratory (27): Hippocampus—10.0; striatum—7.59; frontal cortex—6.16; parietal cortex—6.97; occipital cortex—6.08; thalamus/hypothalamus—7.97.

Table III. GHB Influx Clearance Values ($10^{-2} \times cm^3/min/g$) across Naive Rodent BBB Following *In Situ* Perfusion[a]

Brain region	Control ($n = 5$)	Treatment ($n = 4$)
Hippocampus	1.87 ± 0.448	1.61 ± 0.289
Striatum	2.01 ± 0.502	2.45 ± 0.279
Frontal cortex	2.68 ± 0.329	2.92 ± 0.366
Parietal cortex	2.51 ± 0.427	2.29 ± 0.269
Occipital cortex	3.10 ± 0.412	2.69 ± 0.464
Thalamus/hypothalamus	2.17 ± 0.638	0.687 ± 0.2

[a]Perfusions were done using donor plasma from either control (saline dosed) or GHB treated (5.31 mmol/kg, s.c.) rats. Data are mean ± SEM.

physiologic buffer. The discrepancy of the striatum data in Table II and the thalamus/hypothalamus data in Table III (though not statistically significant, even though the reduction in influx clearance is 68%) is probably due to the small tissue samples obtained of these areas which adds to the analytical variability. We previously showed that there were no brain regional differences regarding GHB BBB transport (27).

It is likely that various endogenous substrates of MCT transport (e.g., small chain monocarboxylic acids, ketone bodies, medium chain fatty acids and other organic anions) were present in the plasma of the donor rats, thereby accounting for the lower Cl_{in}.

Neuronal Synaptosomal Membrane Fluidity Studies

DPH fluorescence polarization was measured at various concentrations of either GHB or benzyl alcohol as a function of increasing temperature in neuronal synaptosomes obtained from naive rats (Fig. 4A and B). As temperature is expected to have an effect on membrane fluidity, there was a consistent decrease in DPH fluorescence polarization by adding GHB or benzyl alcohol with increased temperature (Fig. 4A and B and Table V). As expected from a known positive control, DPH fluorescence polarization was consistently decreased as a function of increasing benzyl alcohol concentration (Fig. 4B), indicative of enhanced membrane fluidity. In contrast to the findings obtained using benzyl alcohol, there was no clear evidence of concentration dependence of GHB's effects on DPH fluorescence polarization (Fig. 4A). Moreover, fluorescence polarization values at 37°C (Table IV) in the presence of GHB were not significantly different from that observed in the absence of GHB (0.344 ± 0.015, one-way ANOVA with Bonferroni *post hoc*, $p > 0.05$). A similar lack of GHB concentration effects were observed for anisotropy and lipid order parameters at various concentrations of GHB at 37°C (Table IV).

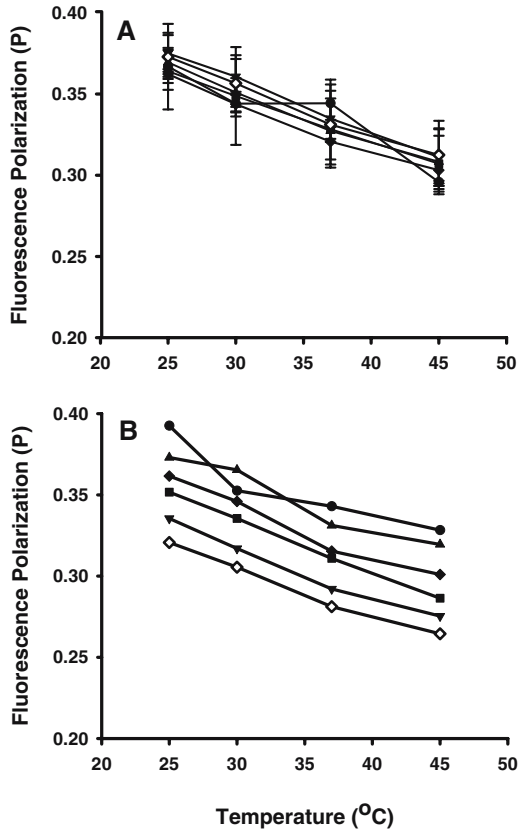

Fig. 4. Fluorescence polarization of DPH as a function of temperature in neuronal synaptosomes obtained from naive rats and incubated with varying concentrations of (A) GHB ($n = 5$ measurements per temperature at each concentration in duplicate preparations) or (B) benzyl alcohol ($n = 5$ measurements per temperature at each concentration) *in vitro*. *Closed circles* represent 0 mM GHB or 0 mM benzyl alcohol, *closed triangles* represent 0.0125 mM GHB or 1 mM benzyl alcohol, *closed squares* represent 0.1 mM GHB or 2 mM benzyl alcohol, *closed diamonds* represent 1 mM GHB or 3 mM benzyl alcohol, *open triangles* represent 5 mM GHB or 5 mM benzyl alcohol and *open diamonds* represent 10 mM GHB or 10 mM benzyl alcohol. Data represent mean ± S.D.

Since there were no GHB-concentration effects on membrane fluidity in preparations from naive rats, we assessed membrane fluidity temperature effects on neuronal synaptosomes from rats dosed with GHB (Table IV). DPH fluorescence polarization at 30 and 37°C was found to be significantly increased in neuronal synaptosomes harvested

Table IV. Fluidity Parameters from Naive Neuronal Synaptosomes in the Presence of Added GHB Concentrations at 37°C[a]

GHB concentration (mM)	Polarization (P)	Anisotropy (r)	Lipid order parameter (S^2)
0	0.344 (0.015)	0.259 (0.012)	0.614 (0.042)
0.0125	0.327 (0.007)	0.245 (0.006)	0.566 (0.019)
0.1	0.328 (0.024)	0.246 (0.020)	0.570 (0.066)
1	0.321 (0.024)	0.239 (0.009)	0.548 (0.031)
5	0.335 (0.012)	0.251 (0.011)	0.588 (0.035)
10	0.331 (0.025)	0.248 (0.021)	0.586 (0.069)

[a]Values expressed as mean ± S.D. ($n = 5$ measurements per temperature from duplicate preparations).

Table V. Fluidity Parameters of Neuronal Synaptosomes Obtained after Repeated *In Vivo* GHB Exposure[a]

Group	Temperature (°C)	Polarization (P)	Anisotropy (r)	Lipid order parameter (S^2)
Control	25	0.349 (0.007)	0.264 (0.006)	0.629 (0.021)
	30	0.328 (0.009)	0.246 (0.008)	0.569 (0.025)
	37	0.299 (0.009)	0.222 (0.008)	0.489 (0.026)
	45	0.285 (0.018)	0.210 (0.015)	0.450 (0.049)
Treated	25	0.355 (0.012)	0.268 (0.011)	0.644 (0.036)
	30	0.343 (0.009)*	0.258 (0.008)*	0.611 (0.027)*
	37	0.320 (0.009)*	0.239 (0.008)*	0.545 (0.026)*
	45	0.295 (0.015)	0.218 (0.012)	0.478 (0.041)

[a]GHB dose 5.31 mmol/kg QD, s.c. for 5 days. Values expressed as mean ± S.D. (n = 5 preparations group).
*$p < 0.05$ compared to corresponding control.

from GHB treated animals (0.343 ± 0.009 at 30°C and 0.320 ± 0.009 at 37°C) *versus* the control group (0.328 ± 0.009 at 30°C and 0.299 ± 0.009 at 37°C; repeated measures ANOVA, $p <$ 0.05;). The anisotropy and lipid order parameter values followed a similar trend as the fluorescence polarization at 30 and 37°C (Table V).

Neuronal Synaptosomal [^{3}H]GABA Uptake

[^{3}H]GABA synaptosomal uptake was saturable and temperature dependent (temperature dependency data not shown). Figure 5 shows a comparison of the rate of specific uptake *versus* concentration for [^{3}H]GABA from control and chronic GHB treated rats. [^{3}H]GABA V_{max} was significantly different ($p < 0.05$) from the treated group (1,612 ± 176 pmol/mg protein/min; n = 7) *versus* control (2,110 ± 91 pmol/mg protein/min; n = 7). No differences were detected for [^{3}H]GABA K_m (GHB treated—10.30 ± 0.85 μM *versus* control—8.97 ± 0.60).

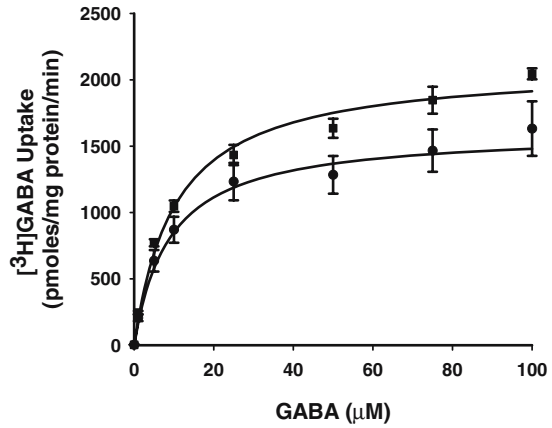

Fig. 5. Uptake of [^{3}H]GABA by rat cerebral cortical synaptosomes. Synaptosomes were incubated with [^{3}H]GABA prepared from control animals (*closed circles*) or chronic GHB treated animals (*closed squares*) for 2 min. Net synaptosomal uptake was determined as the difference between the total uptake (determined at 37°C) and non-specific uptake (determined at 0°C). The data were best described by saturable process (Eq. (5)). The *solid lines* represent the predicted mathematical fit of the data using WINONLIN (Pharsight Corp., Mountain View, CA). Data are mean ± S.E.M.; n = 7 rats/group.

DISCUSSION

Evidence in support of the development of tolerance to GHB's sedative/hypnotic effects in humans is equivocal. However, a time dependent tolerance development to the sedative/hypnotic properties of GHB (measured as a decrease in hypolocomotion, catalepsy or loss in righting reflex) is well characterized in rodent species (13–15). Consistent with the literature on rodents, we report that short-term (5 days) GHB administration to rats (5.31 mmol/kg, s.c., daily) results in the reproducible development of tolerance to GHB's sedative/hypnotic effects.

One potential mechanism of tolerance to GHB's effects in rodents involves alterations in pharmacokinetics, which, until this study, was not rigorously investigated. This paper reports that systemic GHB pharmacokinetics did not change following short term administration (five daily doses of 5.31 mmol/kg). The systemic exposure of GHB, as judged by the AUC, was not different between single dose and short-term daily dosing groups. T_{max} and C_{max}, approximate indicators of the absorptive phase following subcutaneous dosing, were not significantly different between the two groups. Thus, it can be inferred that the increase in time to LRR upon repeated dosing was not due to a change in the absorption of GHB from the injection site. Similarly, Cl/F, as well as the apparent terminal half life, were not significantly different, ruling out enhanced elimination as a mechanism of GHB tolerance. The lack of significant changes in GHB systemic pharmacokinetic parameters with 5 day GHB dosing excludes a systemic pharmacokinetic mechanism in the development of GHB tolerance.

Van Sassenbroeck *et al.* (15) assessed GHB plasma pharmacokinetics following chronic dosing with γ-butyrolactone (GBL), a precursor of GHB. Following 10 days of twice a day dosing with GBL (3.484 mmol/kg), the authors observed a 20% decrease in GHB AUC, accompanied by a 30% increase in the V_{max} of GHB ($p < 0.01$). In companion pharmacokinetic–pharmacodynamic electroencephalographic (EEG) tolerance studies, Van Sassenbroeck *et al.* concluded that these pharmacokinetic changes did not account for a rightward shift in the GHB concentration–EEG effect curve (15). Our pharmacokinetic study design differed from Van Sassenbroeck *et al.* in that (1) we dosed GHB, whereas they dosed GBL, and (2) our chronic dosing regimen utilized once-a-day administration using a high GHB dose. Compared to Van Sassenbroeck, a greater percentage of our

animals developed full tolerance to GHB's effects, i.e., 75% of our animals were not responding by day 5 (Figs. 1 and 2).

While the pharmacokinetic–pharmacodynamic study of Van Sassenbroeck *et al*. is illuminating, several issues merit critical evaluation. Testing for GHB pharmacokinetic tolerance following GBL dosing is not ideal. The authors assume that GBL undergoes rapid hydrolysis to GHB. However, there are documented differences in GHB's and GBL's neurochemical, behavioral properties (39,40) and pharmacokinetic properties (41). For example, compared to GHB, GBL undergoes rapid absorption, has a slower initial elimination, has a larger volume of distribution (41) and has negligible affinity to the GHB receptor (42). Appropriate assessment of GHB pharmacokinetics following GBL dosing requires a pharmacokinetic metabolite model that incorporates the formation of GHB from GBL as well as GHB elimination. The studies of Van Sassenbroeck *et al*. are confounded by the lack of an appropriate pharmacokinetic metabolite model, as well as different pharmacokinetic–pharmacodynamic properties of GHB and GBL.

We have previously demonstrated that GHB undergoes carrier mediated transport at the BBB (27). One cause of tolerance development could be altered transport across the BBB through a change in transporter expression or transport characteristics (e.g., V_{max}, K_m). Using an *in situ* brain perfusion, rats pretreated with GHB (5.31 mmol/kg s.c. for 5 days) did not have altered transport across the BBB. The pretreated rats developed tolerance to the hypnotic/sedative effects of GHB as revealed by Figs. 1 and 2, however the CL_{in} values showed no significant difference between the treated and control groups (Table II). Consistent with our previously published paper on GHB BBB transport (27), unlabeled GHB (40 mM) significantly inhibited the CL_{in} of tracer GHB in both treated and control groups (Table II).

GHB was marketed to bodybuilders and athletes to promote weight loss (43). One reason for the abuse of GHB as an anabolic agent is due to its proposed growth hormone secretion effect (37). Both growth hormone and starvation can lead to weight loss and subsequent ketogenesis (38). In the present study, GHB dosed animals neither gained nor lost body weight, in contrast to the weight gain observed in control animals and GHB dosed animals consumed less rat chow than the control animals. However, it is possible that GHB, through anabolic, metabolic or body weight effects, may induce increases in endogenous substrates of MCT BBB transport, which might represent an alternative mechanism of GHB tolerance. This increased production of ketone bodies could have an impact on the BBB transport of GHB through two processes. First, ketone bodies can act as a secondary source of energy during starvation; an increased expression of MCT transporters would facilitate ketone body uptake as an adaptation to ketonemia. Gjedde and Crone (1975) and later, Pollay and Stevens (1980) showed that starvation induced the BBB transport of beta-hydroxybutyrate (as measured using brain uptake index studies) (44,45). Also, Leino *et al*. reported that diet induced ketosis caused an increased MCT1 expression in the rat brain (46). In the present study, if MCT expression were increased, then one would predict an increased uptake of GHB across the BBB. However, this prediction is not supported by the present *in situ* brain perfusion studies (Table II). Secondly, ketone bodies are MCT substrates and therefore can competitively inhibit GHB transport across the BBB. However, Table III shows that endogenous ketone bodies, if produced and present in the plasma of GHB treated rats, do not alter GHB brain influx. Hence, a mechanism involving GHB induced increases in ketone bodies, with subsequent inhibition of GHB BBB transport is ruled out. Thus, taken together from the above discussion, it can be deduced that the tolerance to GHB's hypnotic/sedative effects is not due to altered transport at the BBB, either through altered transporter expression or endogenous competitive substrates.

While it has been shown here that the observed tolerance development is not explained by altered GHB disposition, another possibility is that tolerance may involve physical alterations of neuronal membrane fluidity or neurotransmitter reuptake processes. In contrast to benzyl alcohol, a compound which is known to enhance fluidity by direct partitioning into the lipid bilayer, GHB does not directly result in perturbation/disruption of neuronal membranes. However, increased fluorescence polarization was measured in the presence of neuronal synaptosomes harvested from GHB tolerant animals (compared to naive rats) suggesting that chronic GHB exposure *in vivo* results in a decrease in neuronal membrane fluidity. Of interest, consistent with the findings reported here, chronic ethanol exposure in rats results in a net decrease in basal fluidity (i.e., without added fluidizer) of rat synaptic membranes. Behavioral tolerance and membrane fluidity alterations arising from chronic ethanol are also associated with changes in phospholipid and membrane-bound carbohydrate composition (47). Additionally, several studies have shown that changes in membrane fluidity lead to altered activities of Na+/K+ ATPase and the sodium/sulfate co-transporters (21–23). *In toto*, these findings suggest that GHB tolerance may lead to changes in protein function/activity in a more rigid membrane environment, potentially altering neuronal function or excitability. Whether this speculation is a mechanism or consequence of GHB tolerance requires additional study to understand the molecular mechanisms involved in these membrane adaptations and the resultant impact on membrane-protein function.

GABAergic neurotransmission plays a key role in mediating GHB's hypnotic effects and may be involved in GHB tolerance development. GHB itself undergoes limited metabolism to GABA under physiologic conditions; during overdose or chronic exposure, this pathway may play a more important role (48). Interestingly, GHB administration elicited an increase in the neuronal GABA reuptake. Gobaille and colleagues reported that high dose GHB administration resulted in an increase in extracellular GABA levels in the rat frontal cortex as measured by microdialysis sampling *in vivo* (24). Furthermore, many of the CNS depressant effects of GHB have been shown to be attenuated by administration of GABAb-receptor antagonists. These lines of evidence support the hypothesis that GHB's sedative/hypnotic properties are mediated either by direct or indirect potentiation of the GABAergic system (24,25,49).

Chronic extracellular GABA induces an increase in GABA reuptake in primary hippocampal cultures (26), illustrating that GABA modulates its own reuptake clearance from the synaptic cleft through the GABA transporter GAT1. The increased activity of GAT1 is likely due to a

decrease in transporter internalization arising from mechanisms involving G-protein-coupled receptors that activate protein kinase C, syntaxin 1A, and SNARE proteins (26,50,51). Taken together, these data indicate the existence of a feedback mechanism for the regulation of GABA levels at the synapse and provide a possible explanation for one of the mechanisms involved in the development of tolerance to GHB's sedative/hypnotic effects.

CONCLUSIONS

The present work demonstrates that short term administration of GHB results in development of tolerance characterized by a decrease of GHB hypnotic/sedative effects. Noncompartmental analysis of GHB plasma pharmacokinetic profiles of pretreated *versus* control rats shows that GHB tolerance is not due to a change in the systemic pharmacokinetics of GHB. Furthermore, using *in situ* brain perfusion, GHB BBB transport is not altered in GHB tolerant rats. While observed physical changes in neuronal membranes and enhanced neuronal GABA reuptake have been observed consequent to GHB tolerance, further studies are required in order to provide a more detailed explanation and insight of the potential mechanism(s) of GHB tolerance development.

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