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Pharmacokinetics and pharmacodynamics of gamma-hydroxybutyric acid during tolerance in rats: Effects on extracellular dopamine

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Running Title: PK-PD of GHB during sedative/hypnotic tolerance

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Non-Standard Abbreviations:

ABEC	Area between the baseline and effect curve
I_{max}	Maximum inhibitory effect
IC_{50}	Concentration producing 50% inhibition I_{max}
K_m	Apparent affinity constant for elimination
LRR	Loss in righting reflex
RRR	Regain of righting reflex
V_{max}	Maximal velocity for elimination

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Abstract

Gamma-hydroxybutyrate (GHB) is a potent sedative/hypnotic and drug of abuse. Tolerance develops to GHB's sedative/hypnotic effects. It is hypothesized that GHB tolerance may be mediated by alterations in central nervous system pharmacokinetics or neurotransmitter response. Rats were dosed daily with GHB (548 mg/kg, s.c., QD for 5 days) and sleep time was measured as an index of behavioral tolerance. Plasma and brain GHB pharmacokinetics on days 1 and 5 were monitored using blood and microdialysis sampling. Extracellular (ECF) striatal dopamine levels were measured by microdialysis as a pharmacodynamic endpoint of tolerance. PK/PD modeling was performed to describe the plasma and brain disposition using an indirect response model with inhibition of dopamine synthesis rate to describe the pharmacodynamic response. GHB plasma and brain ECF concentration versus time profiles following acute or chronic exposure were not significantly different. GHB sedative/hypnotic tolerance was observed by day 5. Acute GHB administration resulted in a decrease in striatal ECF dopamine (DA) levels compared to baseline levels. GHB tolerance was reflected by a 60% decrease in dopamine area under the curve (effect and baseline): Acute = 10.1 ± 15.3 % basal DA*min*10^{-3} versus chronic – 4.73±1.49 % basal DA*min*10^{-3} ($p<0.05$, n = 5; unpaired t-test). The PK/PD model revealed an increase in the IC_{50} following chronic exposure indicating decreased dopaminergic sensitivity towards GHB's inhibitory effects. Our findings indicate that GHB pharmacokinetics do not contribute to behavioral tolerance, however, changes in neurotransmitter responsiveness may suggest specific neurochemical pathways involved in the development and expression of tolerance.

Introduction

Gamma-hydroxybutyric acid (GHB), a metabolite of gamma-aminobutyric acid (GABA), is found in mammalian brain and peripheral tissues (Roth and Giarman, 1970; Maitre, 1997). Like GABA and GABAmimetics, GHB is also a central nervous system (CNS) depressant. Administration produces effects ranging from euphoria and sedation to coma, respiratory depression, and death (Wong et al., 2004). GHB's popularity as a recreational drug likely stems from its anxiolytic and euphoric properties. In addition to the illicit use(s) of the drug, it is also used in the FDA approved treatment of narcolepsy with cataplexy, and off-label treatment of alcohol and opiate withdrawal (Poldrugo and Addolorato, 1999; Gallimberti et al., 2000).

The major pharmacodynamic effect of intermediate-to-high doses of GHB is induction of anesthesia, sedation and coma which has been observed in either humans (10 - 60 mg/kg) or rodents (> 200 mg/kg) (Wong et al., 2004). In other animal studies, researchers noted a hypolocomotor effect of GHB (Gianutsos and Moore, 1978; Gianutsos and Suzdak, 1984; Itzhak and Ali, 2002). These behavioral endpoints probably arise as a result of GHB's CNS depression induced either by potentiation of the GABAergic pathway, inhibition of dopaminergic neurotransmission, or both (Roth and Suhr, 1970; Bottiglieri et al., 2001; Carai et al., 2001).

Roth and colleagues (1980) discovered that GHB administration to rats resulted in an inhibition of striatal dopaminergic neuronal firing and possibly reduced release. This inhibition of activity and release is accompanied by increased dopamine biosynthesis and decreased catabolism (Roth and Suhr, 1970; Morgenroth et al., 1976; Nowycky and Roth, 1979; Roth et al., 1980). This compensatory mechanism is likely driven by a decrease in the available pool of extracellular DA that can interact with the presynaptic autoreceptors responsible for regulating synthesis. In 1997, Howard and Feigenbaum showed that systemic GHB administration does result in decreased extracellular striatal dopamine release. The resulting depletion of extracellular dopamine may be a contributing factor involved in the observed sedative and hypolocomotor effects of GHB.

Interestingly, researchers showed that chronic, long term exposure to GHB or its precursor, gamma-butyrolactone (GBL), results in a reduction in the sedative/hypnotic and hypolocomotor effects in rodents (Gianutsos and Moore, 1978; Nowycky and Roth, 1979; Itzhak and Ali, 2002; Bania et al., 2003; Van Sassenbroeck et al., 2003). This phenomenon is quite reproducible despite differences in dosing regimens (e.g. 0.3g/kg, b.i.d. for 10 days to 3g/kg/day for 30 days) or routes of administration (e.g. intraperitoneal or oral) used in the various studies (Nowycky and Roth, 1979; Van Sassenbroeck et al., 2003). Previous studies from our lab have shown that a moderate, 5 day exposure to GHB in rats (548 mg/kg s.c. QD or 5.31 mmole/kg) also results in sedative/hypnotic tolerance development (Bhattacharya et al., 2006a).

Two explanations for the observed behavioral tolerance could be related to changes in GHB pharmacokinetics (PK) or pharmacodynamics (PD). Other researchers suggested that a modest increase in systemic clearance of GHB following long-term GBL exposure could be a contributing factor (Van Sassenbroeck et al., 2003). However, studies from our lab showed no significant changes in GHB systemic pharmacokinetics after five days of administration (548 mg/kg s.c. QD) (Bhattacharya et al., 2006a). While alterations in GHB pharmacokinetics are ruled out as a mechanism of hypnotic tolerance, the time-course of GHB disposition in the CNS had not been well described during behavioral tolerance.

Alterations in GHB pharmacodynamics (e.g. receptor desensitization, changes in neurotransmitter release or re-uptake and altered signal transduction pathways) are other possible mechanisms contributing to the development and expression of tolerance. Following chronic one month GBL exposure, Nowycky and Roth (1979) discovered that the GHB mediated increase in dopamine synthesis and intracellular levels (ie decreased turnover) was significantly diminished with corresponding tolerance to GHB's sedative effects. These same authors also noted that dopamine auto-receptors, which are responsible for regulation of synthesis and release, became super-sensitized (Nowycky and Roth, 1979). Taken together, these findings suggest that the degree of dopaminergic depression following chronic GHB exposure may be diminished and this

neurochemical tolerance may be associated with the observed behavioral tolerance. However, actual changes in the extracellular levels of dopamine during chronic GHB administration have never been determined.

The aim of these studies was to explore the pharmacokinetic/pharmacodynamic relationship of GHB in rats tolerant to the sedative/hypnotic effects of GHB. Using microdialysis sampling, the time-course of GHB disposition in the CNS, and its effects on extracellular dopamine were determined following acute and chronic exposure. Pharmacokinetic-pharmacodynamic (PK/PD) modeling was used to gain a quantitative understanding of the potential mechanisms involved in GHB tolerance.

Materials and Methods

Materials

Dopamine hydrochloride (DA; 3,4-dihydroxyphenethylamine), γ-hydroxybutyric acid sodium salt and Dulbecco's phosphate-buffered saline (artificial cerebrospinal fluid; ACSF) were purchased from Sigma (St. Louis, MO). Perchloric acid 70% (HPLC grade), *ortho*-phosphoric acid 85% (HPLC grade), sodium acetate anhydrous (ACS grade), citric acid anhydrous (ACS grade), 1-octanesulfonic acid (OSA) sodium salt (HPLC grade), EDTA disodium salt (ethylenediaminetetraacetic acid; U.S.P. grade) were obtained from JT Baker (Phillipsburg, NJ). Sodium hydroxide (NaOH) was purchased from Fisher Scientific (Fairlawn, NJ). Deuteriated GHB (GHB-D^6) was obtained from Cerilliant (Round Rock, TX). **Ketamine (**2-(2-chlorophenyl)-2-methylamino-cyclohexan-1-one) and xylazine (2-(2,6-Dimethylphenylamino)-5,6-dihydro-4H-thiazine) were purchased from J.A. Webster (Sterling, MA). HPLC grade acetonitrile, methanol, formic acid and acetic acid were purchased from EM Science (Gibbstown, NJ). All solutions were prepared using deionized water of at least 17.5 MΩ*cm (Millipore; Billerica, Ma). Commercially available CMA/12 concentric microdialysis probes (4 mm polycarbonate membrane, 20 kDa molecular weight cut-off) and guide cannulas were purchased from CMA/Microdialysis (Stockholm, Sweden).

In vivo microdialysis

All procedures involving animals were approved by the University of Buffalo Institutional Animal Care and Use Committee and performed according to the guidelines set forth in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 85-23, revised 1985). Male Sprague-Dawley rats (275-300g) were anesthetized with an intramuscular injection of ketamine (60 mg/kg) and xylazine (5 mg/kg) and placed in a rodent stereotaxic frame. Body temperature was maintained at 37°C with an isothermal warming pad placed under the animal. In brief, a small midline incision on the scalp was made to expose

the reference point on the skull, the bregma, for stereotaxic orientation of the probe placement site. The stereotaxic coordinates for striatal placement of a guide cannula were 0.7 mm anterior, 2.7 mm lateral, with respect to the bregma (Paxinos and Watson, 1986). Small holes were carefully drilled through the skull for the placement of the guide cannula and three stainless steel anchor screws. Two CMA/12 guide cannulas with a dummy probe were then lowered to a depth of 3 mm (from the *dura mater*) into the left and right hemispheres and fixed to the skull using dental acrylic. The skin was sutured around the dental acrylic.

While still anesthetized, rats were surgically cannulated at the jugular vein with micro-renathane® tubing (MRE-040: 0.04 OD x 0.025 ID; Braintree Scientific, Inc. Braintree, MA) for drug administration and blood sampling. Jugular vein cannulas were flushed daily with heparinized saline (50 IU/ml). Animals were allowed to recover for a period of 48 hrs before any experiments were performed.

Dosing, tolerance induction and sleep time determination

Forty-eight hours following surgery, animals were dosed with 548 mg/kg (5.31 mmole/kg) GHB, s.c. once daily (chronic group) or equal volume isotonic saline (acute group) for 4 days. On day 5, all animals received the same subcutaneous dose of GHB (548 mg/kg), establishing chronic and acute exposure groups. The PK/PD study was performed on day 5 following GHB dosing (see below for description of experimental design). On days 1 and 5 of dosing, the time of loss of the righting reflex (LRR; onset) and the time of regaining the righting reflex (RRR; offset) was recorded for both treatment groups. The onset and offset was defined as the time when the animal lost the ability to right itself when placed on its back and the time when it regained that ability, respectively. The difference between the RRR and LRR was defined as the duration of effect or "sedative/hypnotic effect time".

Experimental design

On the day of the study (day 5 of dosing), the dummy probes were removed from the guide cannulas and CMA/12 microdialysis probes were carefully inserted; one for GHB

collection and the other for dopamine collection. The conscious and freely-moving animals were minimally restrained in a large cage (1.5 x 2.5 ft) designed for microdialysis, which was equipped with a multi-directional counter-balance arm (Harvard Apparatus; Holliston, MA) and a neck collar tether (CMA/Microdialysis; Stockholm, Sweden). Rats had free access to food and water throughout the study.

Figure 1 illustrates the experimental design on the day of the study. Both probes were perfused with blank ACSF at a flow rate of 2 µl/min and allowed to stabilize for two-hours. Dialysate fractions were collected over 20 min intervals into empty tubes for GHB sampling or tubes containing 5 µl of 0.1 N $HClO_4$ for dopamine sampling. Following probe stabilization, the GHB probe was perfused with ACSF containing 1 µg/ml GHB for 80 min in order to calculate the *in vivo* probe recovery for GHB using the established method of retrodialysis by drug (Bouw and Hammarlund-Udenaes, 1998). This was followed by perfusion with blank ACSF for an additional 80 min to allow for complete washout of residual GHB. During the retrodialysis and washout period, dialysate samples were collected from the dopamine probe to establish baseline dopamine concentrations. Next, animals received the final, day 5 dose of GHB, and blood and dialysate samples were collected over the next 5 hours. Blood samples were centrifuged at 2000 g for 20 min and the plasma fraction was collected and stored at -80°C until analysis. All GHB and dopamine dialysate samples were stored at -80°C until analysis.

Verification of probe placement in the striatum

Animals were anesthetized with an intramuscular injection of ketamine (60 mg/kg) and xylazine (5 mg/kg) at the conclusion of the experiments. The microdialysis probes were removed from their guide cannulae, dipped in green food dye and re-inserted into the guide, staining the tissue region where the probes were implanted. The probes and the dental acrylic skull cap were removed and the animals were sacrificed by decapitation. Brains were then harvested and stored at -20°C. Tissue was sectioned using a Microm HM 525 cryostat (Richard-Allen Scientific,

Kalamazoo, MI) and correct probe placement was verified by comparison with the coronal brain slices from the Paxinos and Watson craniometric rat brain atlas (Paxinos and Watson, 1986). Any samples obtained from animals with incorrect striatal placement of both probes were excluded from the analysis.

Dopamine sample analysis by ECD-HPLC

Dialysate dopamine concentrations were determined using an HPLC-Electrochemical Detection (ECD) method. In brief, 15 µl of dialysate sample was injected into a Rheodyne 8125 injector (Rheodyne, Rohnert Park, CA) with a 5 µl loop and separated using a BDS-Hypersil C18 column (100*2.1mm I.D., pore size of 100-Å, 5 µm particle size) maintained at 31°C (ThermoHypersil-Keystone, Bellefonte, PA). The mobile phase consisting of 2% acetonitrile with 98% buffer (citrate (30mM), acetate (50mM), EDTA (0.4mM), OSA (0.38 g/l), pH 6.5) was delivered at a flow rate of 0.45 ml/min. Detection was performed using a DECADE amperometric detector with a VT-103 wall-jet flow cell with a 25 µm spacer, a salt bridge Ag/AgCl reference electrode set at an oxidation potential of +670 mV and a 3 mm glassy carbon working electrode (Antec, Leyden, The Netherlands). All electrochemical chromatographic data were integrated using EZCHROM Elite (Scientific Software, Inc., Pleasanton, CA). Dopamine stock solutions were prepared at a concentration of 0.4 µg/ml in deionized water containing 0.1 N $HClO_4$ and stored at -80°C for a period no longer than 3 months. Fresh calibration standard solutions were prepared by dilution of the stock solutions using ACSF and stored at 4°C until injection.

GHB sample analysis by LC/MS/MS HPLC

GHB was extracted from plasma using a strong anion exchange solid-phase extraction procedure and concentrations were determined using an LC/MS/MS assay as described by Fung et al. (2004) with minor modifications. For sample extraction, 5 µl of GHB-D^6 (1 mg/ml) and 5 µl of double distilled water were added to 90 µl of plasma. GHB standards were prepared by

spiking blank plasma with 5 µl of GHB-D^6 (1 mg/ml) and 5 µl of a selected GHB stock solution. Acetonitrile (0.4 ml) was added to precipitate the plasma proteins, followed by centrifugation at 2000 g for 20 minutes. Next, 0.1 ml of supernatant was aspirated and diluted with 0.9 ml double distilled water. Varian Bond Elute SAX cartridges (100 mg resin, 1 ml volume, Palo Alto, CA) were preconditioned with 1 ml of 100% methanol, 6 ml of 10% acetic acid and 1 ml of double distilled water. One ml of diluted supernatant sample or GHB plasma standard was applied to cartridge. The cartridges were washed sequentially with double distilled water (0.5 ml), 50% methanol (0.5 ml) and 100% methanol (0.3 ml). GHB was eluted with 90% acetonitrile / 10% acetic acid (3 ml). The eluent was evaporated by a gentle stream of nitrogen gas, reconstituted in 2.5 ml 0.1% formic acid in double distilled water and 5% acetonitrile and stored at -80°C until analysis.

GHB dialysate samples required little preparation prior to analysis. The first 7 dialysate samples were diluted with 0.16 ml ACSF (1:5) in order to bring them within the linear range of the analytical method. Five µl of GHB-D^6 (0.5 µg/ml) was added to 35 µl of diluted microdialysate sample. Standards were prepared by adding 5 µl of GHB-D^6 (0.5 µg/ml) to GHB stock solutions in ACSF. Samples were stored at -80°C prior to analysis.

For LC/MS/MS HPLC analysis, 10 µl of sample was injected by a Perkin Elmer 200 autosampler (Perkin Elmer, Wellesley, MA) onto an XTerra MS C18 column (250 mm x 2.1mm I.D., pore size of 125-Å, 5 µm particle size; Waters Co., Milford, MA). The mobile phase consisted of 0.1% formic acid in HPLC-grade water with 5% acetonitrile and was pumped through the column at a flow rate of 0.2 ml/min. The retention time of GHB was 4.4 minutes. GHB was detected by a Perkin Elmer Sciex API 3000 triple quadrupole mass spectrometer with a turbo ion spray ionization source set at 400°C and operated in a multiple reaction/positive ion mode. The 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. The peak-area-ratios

of analyte and internal standard were determined using the Analyst 1.3.1 software (Applied Biosystems, Foster City, CA).

Data analysis

The sedative/hypnotic effect time data are presented as mean ± SEM for acute exposure, chronic day 1 exposure and chronic day 5 exposure. Statistical comparisons were performed between the acute and chronic groups using an unpaired Student's t-test and comparison of chronic day 1 and day 5 group was assessed by paired Student's t-test ($p<0.05$) (SAS version 9.0; SAS Institute, Cary, NC).

Retrodialysis by drug was used to determine the relative *in vivo* recovery of the GHB probes. The GHB probe recovery was calculated according to equation 1:

$$\text{Recovery}_{\text{GHB Probe}} = \frac{C_{\text{perf,GHB}} - C_{\text{dial,GHB}}}{C_{\text{perf,GHB}}} \qquad \text{Equation 1}$$

where, $C_{\text{perf, GHB}}$ is the perfusate (inlet) concentration and $C_{\text{dial, GHB}}$ is the dialysate (outlet) concentration. Striatal extracellular fluid GHB concentrations following systemic administration of GHB were obtained by dividing the dialysate concentrations by the experimentally determined GHB probe recovery. The mean *in vivo* probe recovery of GHB determined prior to drug administration was 0.28 ± 0.05 min (mean ± SD; n= 8-10 rats). Dopamine levels were expressed as a percentage of their baseline values.

Pharmacokinetic/Pharmacodynamic Model

Non-compartmental pharmacokinetic analysis was performed on the GHB plasma and brain concentration versus time profiles from each animal using WinNonlin Pro Version 2.1 (Pharsight Corp., Mountain View, CA). The area between the dopamine effect versus time curve and baseline (ABEC) was calculated from the data of each animal in both treatment groups. All data are presented as mean ± SEM and statistical differences between the acute and chronic treatment groups were determined by unpaired Student's t-test at $p<0.05$ (SAS version 9.0; SAS Institute, Cary, NC). Statistical comparison between the dopamine effect versus time series for

acute and chronic groups were made using two-way repeated measures ANOVA (SAS version 9.0; SAS Institute, Cary, NC).

An integrated PK/PD model describing the time course of GHB concentration in the blood and brain ECF and its effects on extracellular dopamine levels was proposed based upon what is known regarding the biodistribution of GHB and its suspected mechanism of action (Figure 2). It is well established that GHB displays nonlinear pharmacokinetics due to capacity-limited elimination processes (Lettieri and Fung, 1979). In the present studies GHB was administered at one dose level by the subcutaneous route. Because of nonlinear kinetics, it is not meaningfully possible to calculate the bioavailability (F) by comparing the ratio of AUCs after extravascular and intravenous input of an equal dose, nor can the absorption rate constant (k_a) be adequately estimated. To determine these parameters, a method for systems with nonlinear elimination (Martis and Levy, 1973) was employed using the subcutaneous data from this study and intravenous data obtained from preliminary studies (unpublished results) after giving the same dose of GHB (data not shown). Also, due to the limited range of doses in our studies we compiled intravenous plasma data from our lab (Bhattacharya and Boje, 2006b) as well as from the literature (Lettieri and Fung, 1979) at doses of 200 and 400-800 mg/kg, respectively, to allow for better approximation of the nonlinear components of the model.

Compartmental analysis was begun by first fitting the pharmacokinetic components of the model (Figure 2). A two-compartment model (non-linear Michaelis-Menten elimination (V_{max}, K_m) from the central compartment (C_P, V_P) and linear distributional clearance into (CL_{in}) and out (CL_{out}) of the brain ECF compartment (C_{ECF}, V_{ECF})) was used to describe plasma and brain GHB concentration versus time profiles following either intravenous (IV) or subcutaneous (SC) input. For GHB administered by the SC route, it was assumed that drug entered the systemic circulation by a linear first order process (k_a). The following differential equations were used for either IV or SC dose:

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$$V_P * \frac{dC_{P,iv}}{dt} = -\left(\frac{V_{max}}{K_m + C_P} + CL_{in} \right) * C_P + CL_{out} * C_{ECF} * \frac{V_P}{V_{ECF}} \quad \text{Equation 2}$$

$$V_{ECF} * \frac{dC_{ECF,iv}}{dt} = CL_{in} * C_p - CL_{out} * C_{ECF} * \frac{V_P}{V_{ECF}} \quad \text{Equation 3}$$

$$\frac{dA_{sc}}{dt} = -k_a * A_{sc} \quad \text{Equation 4}$$

$$V_P * \frac{dC_{P,sc}}{dt} = k_a * A_{sc} - \left(\frac{V_{max}}{K_m + C_P} + CL_{in} \right) * C_P + CL_{out} * C_{ECF} * \frac{V_P}{V_{ECF}} \quad \text{Equation 5}$$

$$V_{ECF} * \frac{dC_{ECF,sc}}{dt} = CL_{in} * C_p - CL_{out} * C_{ECF} * \frac{V_P}{V_{ECF}} \quad \text{Equation 6}$$

where $C_{P, iv}$, $C_{P, sc}$, $C_{ECF, iv}$, $C_{ECF, sc}$ are the GHB plasma and brain ECF concentrations versus time after intravenous or subcutaneous administration, respectively, V_P and V_{ECF} are the apparent volume of distribution in the plasma and brain ECF, A_{sc} is the amount of drug in the subcutaneous absorption site with F being defined as the bioavailability. The initial conditions were set at zero except for $C_{P, iv}(0) = Dose_{iv}/V_P$ and $A_{sc}(0) = Dose_{sc} \bullet F$. All initial parameter estimates were taken from the literature or mathematically calculated (e.g. k_a and F; (Martis and Levy, 1973)). The estimated parameters obtained from simultaneous fitting of the plasma data from the three IV doses and the plasma and brain ECF from the one SC dose included: V_{max}, K_m, CL_{in}, CL_{out}, k_a, V_P, while F and V_{ECF} (physiologic volume of the rat ECF; (Szentistvanyi et al., 1984; Davies and Morris, 1993)) were fixed as constants.

The final parameters obtained from the pharmacokinetic fitting were then fixed and used to fit the pharmacodynamic data. Extracellular striatal dopamine was the biomarker used to monitor GHB's inhibitory effects. While the exact mechanism(s) remain unclear, it is well established that GHB attenuates neuronal dopamine release (Feigenbaum and Howard, 1996; Feigenbaum and Howard, 1997; Howard and Feigenbaum, 1997). Based upon this understanding, an indirect response model (Dayneka et al., 1993) was proposed to describe the

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time course of % basal dopamine release following either acute or chronic subcutaneous GHB administration where $C_{ECF,\ sc}$ of GHB was responsible for inhibition of the zero-order synthesis (k_{in}) of extracellular dopamine. This k_{in} parameter used in the model does not represent the actual biochemical synthesis rate but rather a combination of both synthesis and neuronal release ultimately resulting in the generation of extracellular dopamine. The pharmacodynamic component of this model is also shown in Figure 2, and the differential equation used to describe the rate of change of the dopamine response (R_{DA}) versus time is shown below:

$$\frac{dR_{DA}}{dt} = k_{in} * \left(1 - \frac{I_{max} * C_{ECF,sc}}{IC_{50,ac} + C_{ECF,sc}}\right) - k_{out} * R_{DA} \qquad \text{Equation 8}$$

where k_{out} is the first order rate constant for loss of response with inhibition occurring by capacity (I_{max}) and sensitivity (IC_{50}) parameters driven by $C_{ECF,\ sc}$ and *ac* denotes the IC_{50} constant corresponding to the acute or chronic treatment condition. The initial condition was defined as R_o and set equal to 100% and assumed to be stationary. The zero-order synthesis rate constant, k_{in}, can then be expressed as follows:

$$k_{in} = R_o * k_{out} \qquad \text{Equation 9}$$

This allowed reduction in the total number of parameters to be estimated. All response data, following either acute or chronic exposure, were fitted simultaneously.

All PK/PD model fittings and parameter estimations were performed by nonlinear regression analysis using WinNonlin Pro Version 2.1 (Pharsight Corp., Mountain View, CA). The goodness-of-fit was determined by visual inspection, Akaike Information Criterion, Schwartz Criterion, examination of the residuals and the coefficient of variation of the parameter estimates.

Results

Development of tolerance to GHB sedative/hypnotic effects

Figure 3 illustrates the duration of GHB's sedative/hypnotic effects following acute or chronic GHB exposure. Five days of chronic exposure to GHB resulted in a significant decrease in the duration of effect compared to either the first day of exposure or in animals receiving saline for 4 days followed by a single dose of GHB on the fifth day. There were no differences between the acute group and day 1 of chronic exposure. Table 1 shows the mean onset (LRR) and offset (RRR) times and the corresponding model predicted plasma and brain ECF concentrations of GHB. Based on these data, it appears that the decrease in the duration of the sedative/hypnotic effect in the chronic group is largely due to a significant decrease in the offset time. Furthermore, these behavioral changes following chronic exposure are not related to decreases in either plasma or brain ECF concentrations as these estimated values at the offset were greater in the chronic group versus that of the acute group (Table 1).

Noncompartmental PK/PD analysis

Figure 4A shows GHB concentration versus time in plasma and brain ECF following acute and chronic GHB administration (548 mg/kg, s.c.; 5.31 mmole/kg). With the exception of a modest significant increase in the C_{max} of the chronic group, there were no other significant differences in the noncompartmental pharmacokinetic parameters (Table 2) for either the plasma or brain ECF between treatment groups. Based upon these findings and those in Table 1, changes in either the systemic or CNS pharmacokinetics are unlikely explanations for the observed development of behavioral tolerance.

Figure 4B presents the % basal dopamine release versus time profiles. Following systemic administration of GHB, the % basal dopamine release was decreased compared to baseline (100%) in either treatment group (Figure 4b). However, in animals dosed with GHB for 5 days, the magnitude of extracellular dopamine inhibition is less than rats receiving a single GHB dose, as indicated by a significant decrease in the ABEC following chronic (4.73±1.49 %

basal DA*min*10^{-3}) versus acute treatment (10.1 ± 15.3 % basal DA*min*10^{-3}; n=4, $p<0.05$, unpaired t-test). By two-way repeated measures ANOVA, the time series of dopamine response for acute and chronic GHB exposure were significantly different from one another (n=4 rats/time point/group; $p<0.05$). These data indicate that GHB tolerance includes altered inhibition of extracellular dopamine following chronic exposure.

PK/PD Modeling

Figure 2 presents a mechanistic PK/PD model to assist in developing a quantitative understanding of GHB's attenuation of extracellular dopamine and the development of neurochemical tolerance. To better characterize the pharmacokinetics, GHB plasma data was taken from the literature (Lettieri and Fung, 1979; Bhattacharya and Boje, 2006b) (intravenous administration of a range of doses) and simultaneously fitted along with the plasma and brain ECF data from these studies. Figure 5 A-C shows (a) the observed IV plasma data and the SC plasma and brain ECF after (b) acute and (c) chronic exposure along with the model predicted profiles. Table 3 provides the model predicted parameters and the variance of these estimates.

Figure 6 presents the fitting of the model to the PD data, following either chronic or acute SC GHB exposure. The proposed model captured the trend in the dopamine response data with reasonable precision in parameter estimation (Table 3) for both the treatment groups (acute and chronic). Based on these model predictions, a nearly 10-fold increase in the predicted $IC_{50,\ chronic}$ for the chronic exposure group was observed compared to the $IC_{50,\ acute}$ following acute exposure (Table 3). These data suggest a desensitization of the dopaminergic system towards GHB's inhibitory effects occurs following chronic exposure.

Discussion

Despite the fact that GHB is used chronically for either licit or illicit purposes, little is known regarding the potential for tolerance development in humans (ie development of physical dependence, craving/drug seeking and/or withdrawal syndromes). In contrast, GHB behavioral

tolerance (ie, sedative/hypnotic or locomotor) has been well documented in animals (Gianutsos and Moore, 1978; Nowycky and Roth, 1979; Itzhak and Ali, 2002; Bania et al., 2003; Van Sassenbroeck et al., 2003).

The present studies used an *in vivo* microdialysis animal model to better understand GHB sedative/hypnotic tolerance. Consistent with previous studies from our lab (Bhattacharya et al., 2006a), there were no significant changes in the systemic pharmacokinetics between acute and chronic exposure groups, thereby ruling out alterations in pharmacokinetics as a mechanism of tolerance. While the C_{max} for the chronic exposure group was found to be significantly greater than that of the acute group, this observation is probably related to experimental variability and is not particularly meaningful. If a change in maximal GHB plasma concentrations was the cause for the decreased sleep time, one would predict the opposite trend (ie chronic C_{max} << acute C_{max}). Therefore, even if this alteration was a meaningful result of chronic GHB exposure, it does not account for the shortened sedative/hypnotic effect with multiple dosing.

The findings reported here appear to be in disagreement with those reported by Van Sassenbroeck et al. (2003), where a 20% decrease in systemic GHB AUC, accompanied by a 30% increase in the V_{max} of GHB ($p<0.01$) was noted following chronic administration of the GHB precursor, GBL. Because of differences in the pharmacokinetics of GBL compared to GHB, direct comparisons cannot be made (Lettieri and Fung, 1978).

Regardless of changes in the systemic pharmacokinetics or lack thereof, GHB exerts its pharmacologic effects within the CNS. In the present studies, microdialysis was used to determine the free, unbound GHB concentrations in the extracellular fluid of the striatum. As was observed with GHB's systemic pharmacokinetics, there were no significant differences in the GHB brain pharmacokinetics between the acute and chronic exposure groups.

In support of our observations, Giorgi and Rubio (1981) reported that total GHB brain concentrations in tolerant animals at the RRR (offset) were greater than that measured in non-tolerant rats. The present studies not only confirm their findings but extend the same conclusion

to that of the brain ECF GHB concentrations. As described in Table 1, the estimated drug concentration in the blood or brain at the time of offset of the effect was much greater in the chronic group. Taken together, these data indicate that an alteration in systemic or brain GHB pharmacokinetics does not serve as a valid explanation for the development of sedative/hypnotic tolerance.

Several researchers have shown that acute GHB administration results in a suppression of dopaminergic neuronal activity and an increase in intracellular dopamine content, in addition to its behavioral effects (Roth and Suhr, 1970; Morgenroth et al., 1976; Roth et al., 1980). Early on it was hypothesized that a reduction in neuronal output may result in decreased neuronal release of dopamine. Therefore, the observed increase in dopamine synthesis and intracellular content following acute GHB exposure could be due to compensatory processes responding to the reduction of extracellular dopamine levels (Roth and Suhr, 1970; Morgenroth et al., 1976). The present *in vivo* microdialysis studies clearly show that acute, systemic administration causes a reduction in basal dopamine release. Our data are consistent with findings that have been previously reported in the literature (Howard and Feigenbaum, 1997) and provide sufficient evidence to suggest that depression of dopaminergic neuronal firing will ultimately result in decreased transmitter release.

The question that arises is whether the changes in extracellular dopamine are related to the hypnotic/hypolocomotor effects of GHB, and the tolerance which accompanies chronic exposure. In support of a direct association, Roth and Suhr reported that agents which inhibit dopamine release (e.g. α-methyl-*p*-tyrosine) potentiate GHB induced hypnosis, and the opposite occurs for agents that stimulate (e.g. amphetamine) dopamine release (Roth and Suhr, 1970). Furthermore, other CNS depressants like baclofen and pentobarbital, which induce sedation and hypolocomotion *via* activation of GABAergic neurotransmission, also have effects similar to GHB on dopaminergic neurotransmission (Roth and Suhr, 1970; Gianutsos and Moore, 1978). In

situations of acute GHB exposure, these data suggest a potential correlation between inhibition of the dopaminergic system and the behavioral effects produced by CNS depressants.

If the hypnotic and hypodopaminergic actions of GHB are related, one might also expect to see a change in GHB's effect on the dopaminergic system as a function of chronic exposure. Interestingly, GHB was found to be less effective at inhibiting extracellular dopamine release in GHB tolerant animals. These data indicate that tolerance not only develops towards the behavioral effects of GHB, but also towards its neurochemical depressant effects. Other researchers also arrived at similar conclusions. In 1978, Giantusos and Moore showed that GBL was less effective at causing both an increase in the intracellular content of dopamine and decreasing its turnover in mice that had been pretreated with GBL for 13 days. Similarly, in the same study, the intracellular content of dopamine was less sensitive to the changes induced by acute injections of baclofen in animals who had been pretreated with either itself or GBL for 10 - 12 days. This highlights a commonality amongst other CNS depressants and the effects of GHB. In another study examining the hypodopaminergic effects of GHB following one-month GBL exposure, Nowycky and Roth (1979) had observed that tolerance developed towards the GHB-induced increase in dopamine synthesis and intracellular content. The authors pointed out that dopamine auto-receptors, responsible for regulating dopamine biosynthesis, were super-sensitized. When these receptors are stimulated by dopamine or other agonists, dopamine synthesis is decreased thus serving as a negative feedback loop (Roth, 1975; Roth et al., 1975). This suggests that the intracellular changes in dopamine content and synthesis observed in GHB tolerant animals may be partially explained by the combination of higher extracellular dopamine levels and sensitization of its receptors following chronic GHB exposure.

The PK/PD model was designed to ascribe a mechanistic description of the time course of GHB concentrations and its hypodopaminergic response. This approach provided a quantitative measurement of GHB's depression of extracellular dopamine and the tolerance that developed towards this effect. Assuming an indirect relationship between brain ECF GHB

concentrations and its inhibition of dopamine release, the model predicted a nearly 10-fold increase in the sensitivity parameter, IC_{50}, in GHB tolerant compared to non-tolerant rats. This finding would suggest that the dopaminergic system has become less sensitive towards the inhibitory effects of GHB following chronic exposure.

GHB's hypnotic and hypodopaminergic effects are most likely mediated by potentiation of the GABAergic system. Furthermore, these actions are shared and attenuated by other GABAergic agonists (e.g. baclofen) and antagonists (e.g. CGP 46381, CGP 35348, SCH-50911), respectively (Gianutsos and Moore, 1978; Bottiglieri et al., 2001; Carai et al., 2001). Other researchers showed that cross-tolerance develops towards the behavioral and hypodopaminergic effects of GHB and baclofen (Gianutsos and Moore, 1978; Eckermann et al., 2004). Additionally, biochemical changes specific to the GABAergic system such as GABA-receptor down-regulation (Gianutsos and Suzdak, 1984), and enhanced GABA re-uptake transport activity (Bhattacharya et al., 2006a) occur as a result of chronic GHB exposure. This leads to a working hypothesis that the link which connects the hypnotic and hypodopaminergic effects of GHB and the resulting tolerance may involve GHB's primary action on GABAergic neurotransmission.

In conclusion, the present work demonstrates that short term administration of GHB results in the development of tolerance characterized by a decrease of GHB hypnotic/sedative and hypodopaminergic effects which is not due to changes in the systemic or CNS pharmacokinetics. PK/PD modeling suggests that the development of hypodopaminergic tolerance involves a decrease in the sensitivity of the dopaminergic system towards GHB's inhibitory effects. Whether or not the hypnotic and hypodopaminergic tolerance are directly related to one another require further investigation, but quite possibly involve GHB's action on GABAergic neurotransmission.

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Footnotes

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Legends for Figures

Figure 1. The experimental design used to study brain extracellular GHB pharmacokinetics and pharmacodynamic effects on striatal extracellular dopamine levels in rats.

Figure 2. Integrated PK/PD model for characterizing the time course of GHB plasma and extracellular brain concentrations following intravenous and subcutaneous administration and its inhibitory effect on striatal dopamine release. The parameter descriptions are defined in the text.

Figure 3. Sedative/hypnotic effect time in rats after a single acute dose of GHB (548 mg/kg s.c.; 5.31 mmol/kg) and on days 1 and 5 of chronic administration (548 mg/kg s.c., QD). *p <0.05; significantly different from the single acute dose group (unpaired t-test). **p <0.001; significantly different from chronic day 1 (paired t-test). Data are mean ± S.E.M (n=4-5 rats/group).

Figure 4. (A) GHB concentration versus time profiles in plasma (closed symbols) and brain ECF (open symbols) in rats after receiving either single acute GHB administration (548 mg/kg s.c.; ■) or following five days of chronic exposure (548 mg/kg s.c., QD; ●) and (B) its effects on striatal dopamine release expressed as function of its baseline levels following acute (■) and chronic (●) GHB exposure. Data are mean ± SEM (n=4-5 rats/group).

Figure 5. Time course of GHB concentrations in (A) plasma following single intravenous dosing of 200 mg/kg[a] (▼), 400 mg/kg[b] (◆) and 800 mg/kg[b] (▲); (B) in plasma (■) and brain ECF (□) following single subcutaneous dosing of 548 mg/kg; and (C) in plasma (●) and brain ECF (○) on the fifth day of chronic subcutaneous dosing of 548 mg/kg, QD for 5 days. The solid lines represent the model predicted profiles after simultaneous fitting of all PK data. [a]Data were

extracted from (Bhattacharya and Boje, 2006b). [b]Data were extracted from (Lettieri and Fung, 1979).

Figure 6. Time course of GHB induced inhibition of extracellular striatal dopamine release after receiving either single acute GHB administration (548 mg/kg s.c.; ■) or following five days of chronic exposure (548 mg/kg s.c., QD; ●). Data represent mean ± SEM and are expressed as a function of baseline levels. The solid lines represent the model predicted profiles after simultaneous fitting of all PD data.

Table 1. Onset and offset times of GHB-induced sedation and estimated plasma and brain ECF GHB concentrations in rats

	Onset of Effect (LRR)			Offset of Effect (RRR)		
Group	Time (min)	Plasma GHB (μg/mL)[a]	Brain GHB (μg/mL)[a]	Time (min)	Plasma GHB (μg/mL)[a]	Brain GHB (μg/mL)[a]
Acute (n=4)	21 ± 2	307.3	32.8	199 ± 7	214.0	36.1
Chronic (n=5)	21 ± 2	410.0	32.9	159 ± 9*	369.7	58.9

Onset and offset times are expressed as mean ± SEM.

[a] Values represent estimated GHB concentrations from the PK model at the mean onset and offset time.

* $p<0.05$, significantly different from acute group, unpaired t-test.

Table 2. Noncompartmental analysis of GHB concentration versus time profiles in rats

Exposure	AUC_{0-290} (mg*min*ml^{-1})	Cmax (μg/mL)	T_{max} (min)	V/F (ml/kg)	CL/F (ml*min^{-1}*kg^{-1})	$t_{1/2}$ (min)
Plasma						
Acute	84.8 ± 3.89	479 ± 30.63	50.0	225 ± 25.4	6.52 ± 0.32	24.3 ± 3.54
Chronic	91.3 ± 4.53	602 ± 10.4*	50.0	159 ± 41.5	6.05 ± 0.29	18.8 ± 5.72
Brain ECF						
Acute	11.4 ± 1.59	66.0 ± 8.40	120 ± 5.77	—[a]	—[a]	—[a]
Chronic	10.3 ± 0.74	63.3 ± 3.42	106 ± 4.00	—[a]	—[a]	—[a]

Data are expressed a mean ± SEM.

* $p<0.05$, significantly different from acute group, unpaired t-test.

[a] Not applicable.

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Table 3. Estimated pharmacokinetic and pharmacodynamic parameters

Parameter	Final Estimate	CV%
Pharmacokinetic parameters		
k_a (min^{-1})	0.02	4.96
V_{max} (µg/min/kg)	5324	6.89
K_m (µg/ml)	579	12.5
CL_{in} (ml/min/kg)	0.01	20.1
CL_{out} (ml/min/kg)	0.06	19.7
Vp (ml/kg)	458	2.47
Vecf (ml/kg)	1.16[a]	—[c]
F	0.92[a]	—[c]
Pharmacodynamic parameters		
k_{in}	3.28[b]	—[c]
k_{out} (min^{-1})	0.03	40.0
I_{max}	0.51	22.4
$IC_{50,\ acute}$ (µg/ml)	12.7	103
$IC_{50,\ chronic}$ (µg/ml)	99.1	46.3

[a] Parameter was fixed not estimated.

[b] Secondary parameter calculated as: $k_{in}=R_o*k_{out.}$

[c] Not applicable.

Figure 1

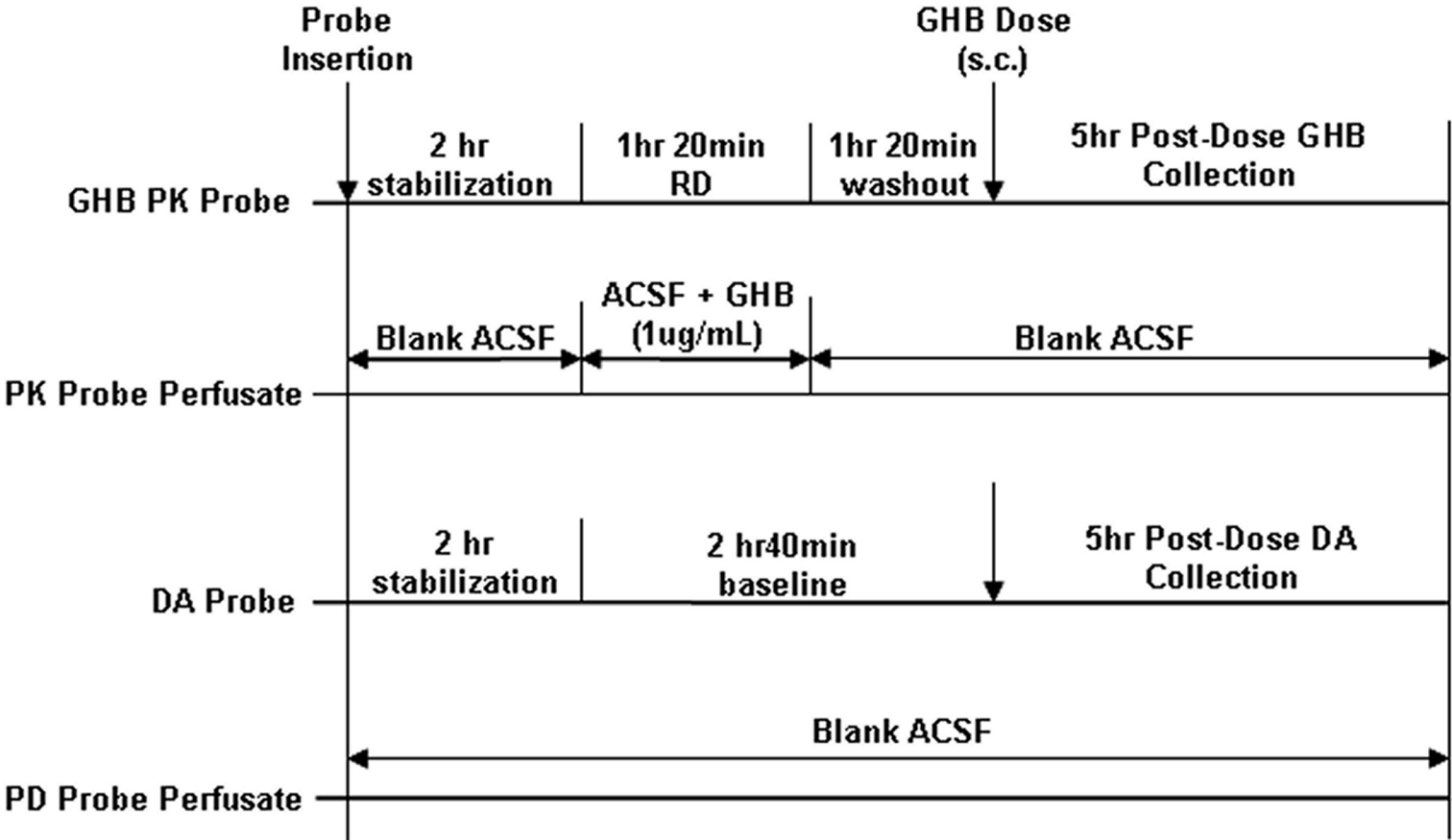

Probe Insertion
GHB Dose (s.c.)
2 hr stabilization
1hr 20min RD
1hr 20min washout
5hr Post-Dose GHB Collection
GHB PK Probe
Blank ACSF
ACSF + GHB (1ug/mL)
Blank ACSF
PK Probe Perfusate
2 hr stabilization
2 hr40min baseline
5hr Post-Dose DA Collection
DA Probe
Blank ACSF
PD Probe Perfusate

Figure 2

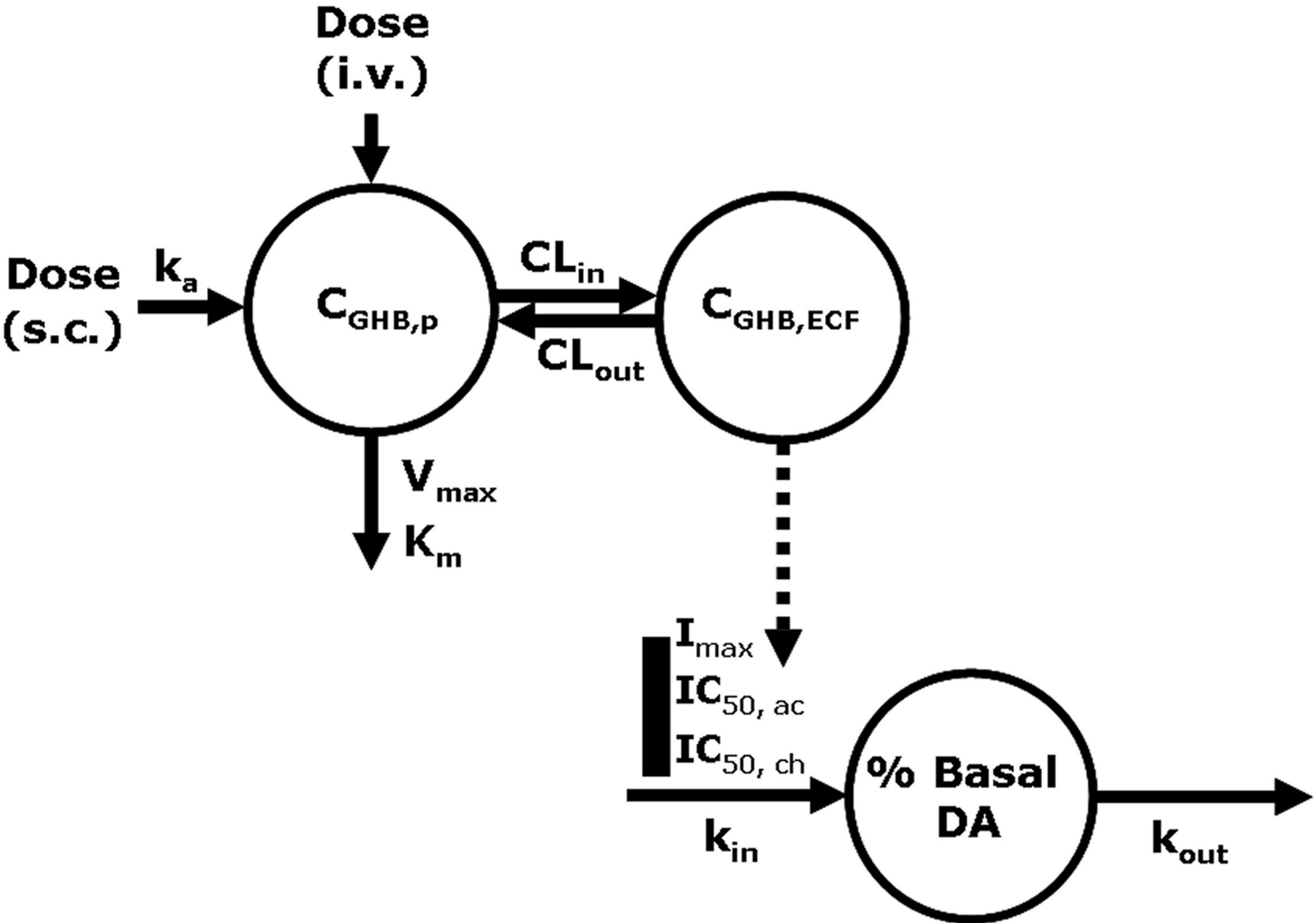

Dose
(i.v.)
Dose
(s.c.)
k_a
$C_{GHB,p}$
CL_{in}
CL_{out}
$C_{GHB,ECF}$
V_{max}
K_m
I_{max}
$IC_{50, ac}$
$IC_{50, ch}$
k_{in}
% Basal
DA
k_{out}

Figure 3

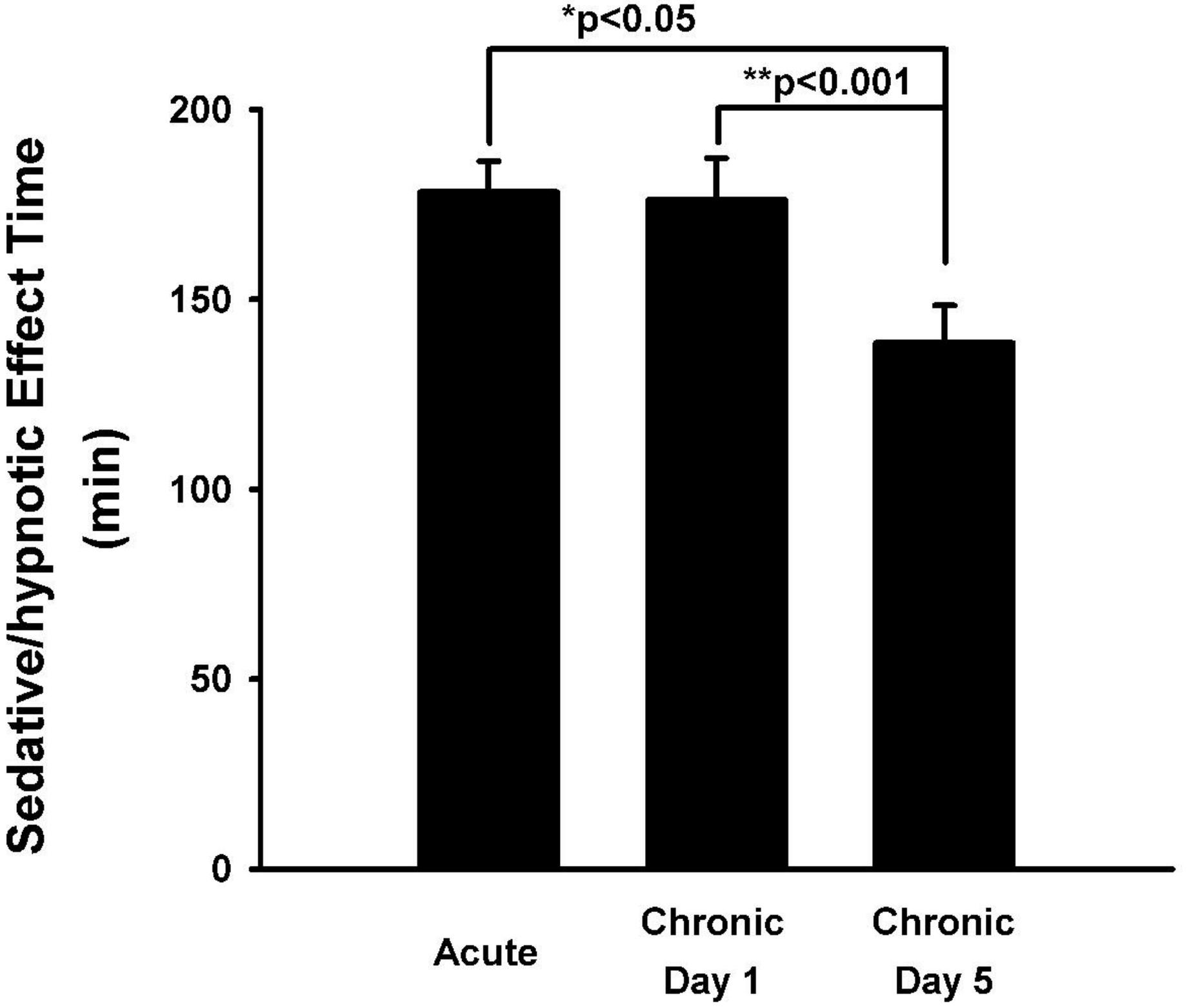

*p<0.05
**p<0.001
Sedative/hypnotic Effect Time
(min)
200
150
100
50
0
Acute
Chronic
Day 1
Chronic
Day 5

Figure 4

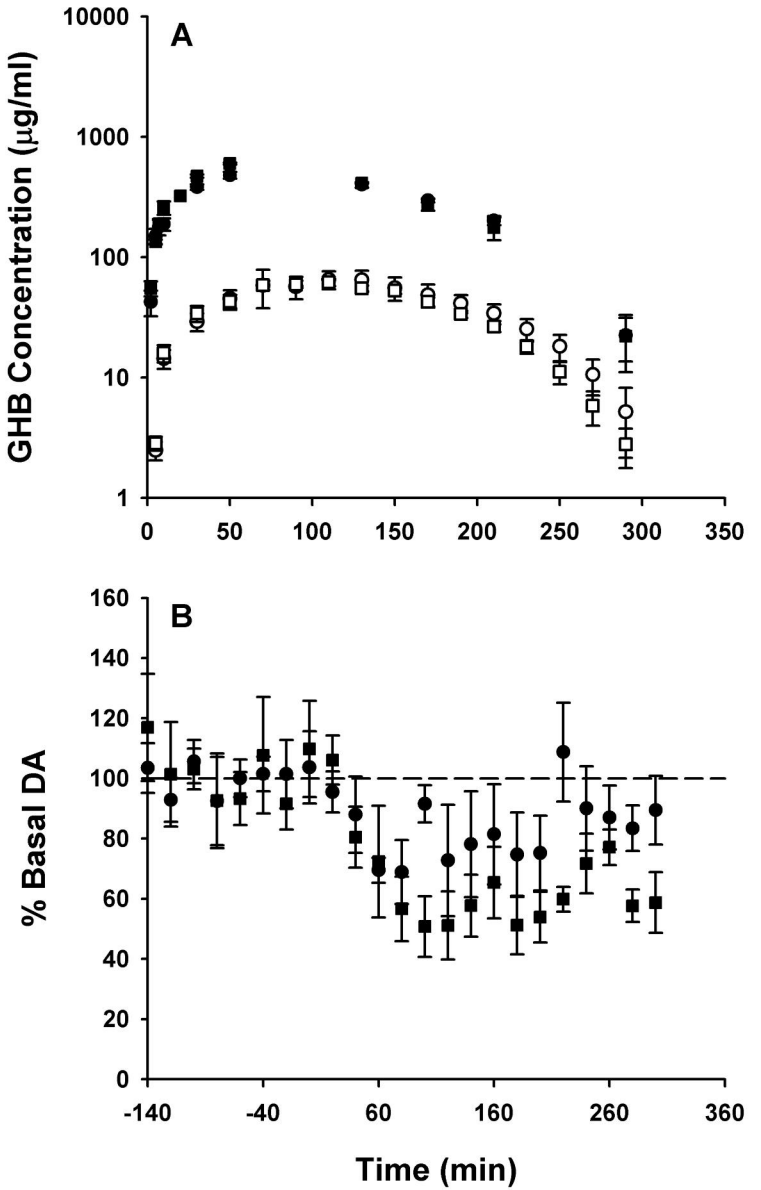

A
GHB Concentration (μg/ml)
10000
1000
100
10
1
0
50
100
150
200
250
300
350
B
% Basal DA
160
140
120
100
80
60
40
20
0
-140
-40
60
160
260
360
Time (min)

Figure 5

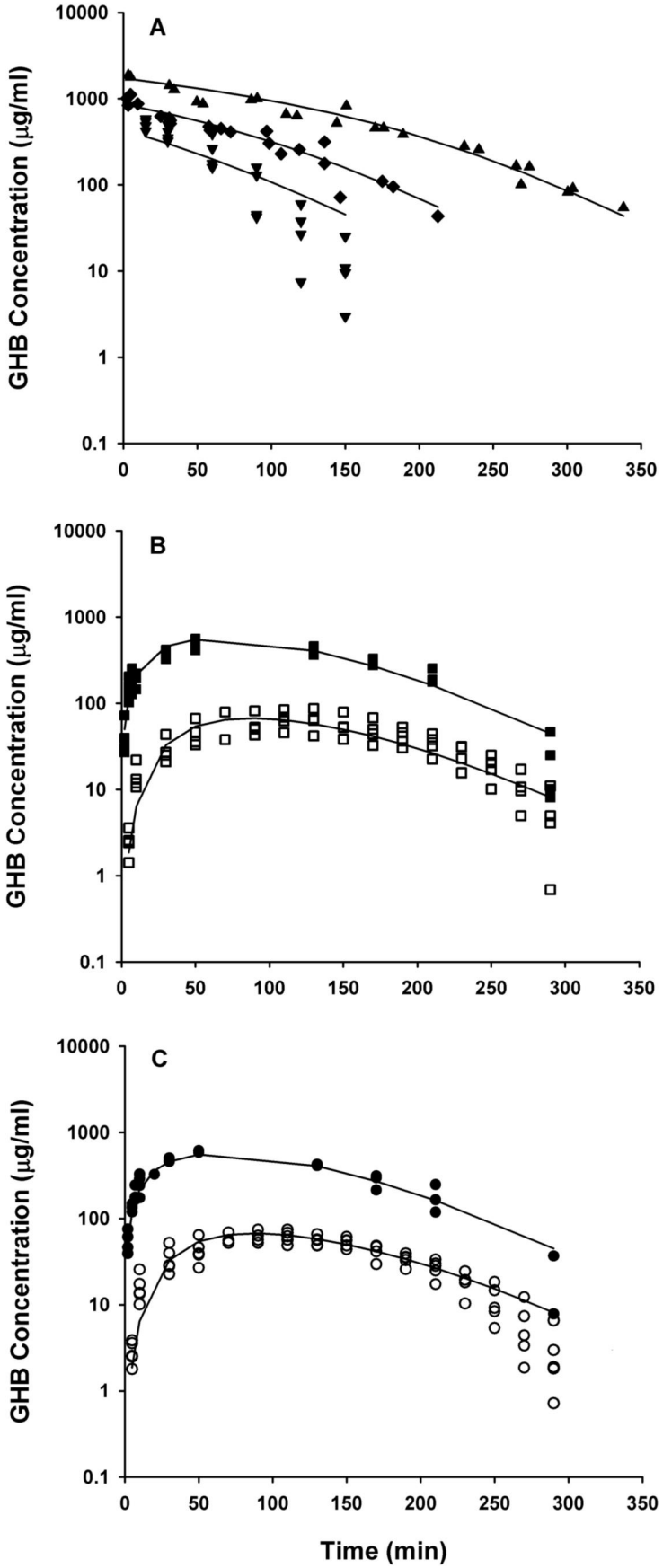
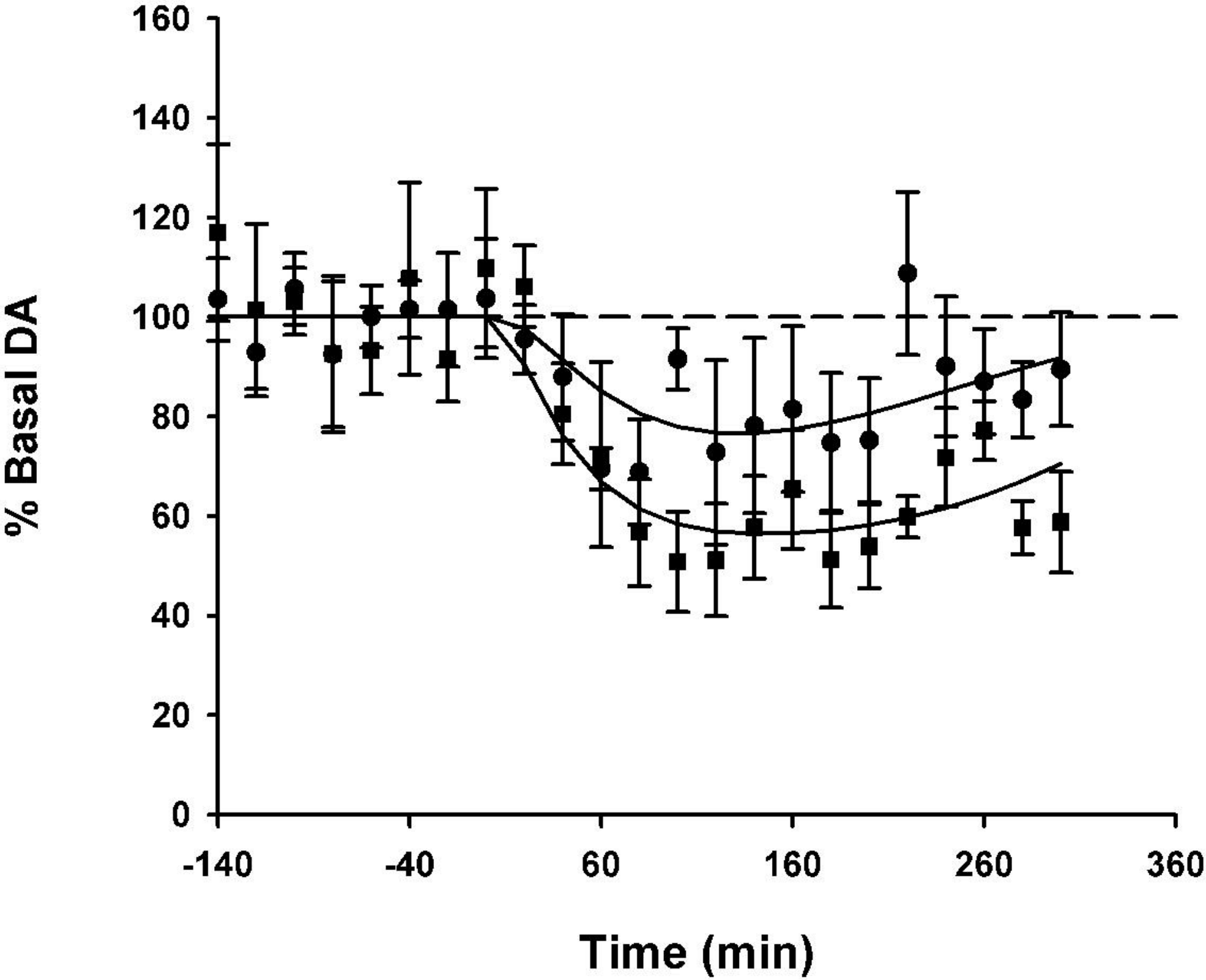

A
B
C
GHB Concentration (µg/ml)
10000
1000
100
10
1
0.1
0
50
100
150
200
250
300
350
Time (min)

Figure 6


160
140
120
100
80
60
40
20
0
% Basal DA
-140
-40
60
160
260
360
Time (min)