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Tolerance to the hypnotic and electroencephalographic effect of gamma-hydroxybutyrate in the rat: pharmacokinetic and pharmacodynamic aspects

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Abstract

Tolerance to gamma-hydroxybutyrate (GHB) has been suggested in illicit users and has been described for the hypnotic effect in the rat. The aim of this study was to investigate whether tolerance is also observed for the EEG effect, and whether the EEG can give insight into the pharmacodynamic aspects of GHB tolerance. In three series of experiments, rats were pre-treated with either the GHB precursor gamma-butyrolactone (GBL) or saline intraperitoneally twice daily. In the first series, a reduction in sleeping time was observed in the GBL pre-treated rats compared with controls. In the second series, a fast infusion of GHB (300 mg kg^{-1} over 5 min) was given after 10 days pre-treatment. The GHB plasma concentration–time curves showed a slightly faster decrease in GHB concentration in the GBL pre-treated rats, suggesting a small induction of the GHB metabolism ($V_{max} = 2882 \pm 457\ \mu g\ min^{-1}\ kg^{-1}$ vs $2205 \pm 315\ \mu g\ min^{-1}\ kg^{-1}$, $P < 0.01$). In contrast to controls, GBL pre-treated rats did not lose righting reflex. In the third series, a slow infusion of 480 mg $kg^{-1}\ h^{-1}$ was given after 7 days pre-treatment, which allowed fitting a sigmoid E_{max} model to the EEG amplitude versus GHB plasma concentration curve. This showed reduced end-organ sensitivity to GHB in the GBL pre-treated rats (EC50 (concentration required to obtain 50% depression of the baseline effect) $= 653 \pm 183\ \mu g\ mL^{-1}$ vs $323 \pm 68\ \mu g\ mL^{-1}$, $P < 0.001$). In conclusion, chronic pre-treatment with gamma-butyrolactone in the rat results in a reduced sleeping time and this tolerance is reflected by the EEG. This can mainly be explained by reduced end-organ sensitivity.

Introduction

Gamma-hydroxybutyrate (GHB) is a naturally occurring substance with neuromodulating properties (Maitre 1997). After peripheral administration it crosses the blood–brain barrier and in high doses induces behavioural responses including sedation and anaesthesia (Cash 1999). Although GHB is nowadays only sporadically used in anaesthesia (Kleinschmidt et al 1998), it recently gained interest as an investigational sedative in intensive-care patients (Kleinschmidt et al 1999; Soltész et al 2001), and is investigated in the treatment of alcohol dependence (Gallimberti et al 2000) and narcolepsy (Scrima et al 1990). Furthermore, GHB is increasingly misused as a recreational drug with high doses leading to deep coma and even death (Dyer 1991; Chin et al 1998; Zvosec et al 2001). Case studies describe a withdrawal syndrome after chronic illicit GHB use (Sivilotti et al 2001). An increase in dosage during illicit use can lead to an intake around-the-clock (Dyer et al 2001) with doses that are seven times higher than recommended in the treatment of alcohol dependence (Beghe & Carpanini 2000). The increase of GHB intake over time suggests the development of tolerance for which there is already some evidence in animal experiments (Nicholson & Balster 2001). Chronic treatment of mice (Gianutsos & Moore 1978) and rats (Nowycjy & Roth 1979) with the GHB precursor gamma-butyrolactone (GBL) results in the development of tolerance to the hypnotic effect, measured by the time between loss and return of the righting reflex (Nowycjy & Roth 1979). Giorgi & Rubio (1981) found that the GHB brain concentrations upon return of the righting reflex were higher in GBL

pre-treated rats and suggested that the tolerance may be explained by a decrease in end-organ sensitivity to the hypnotic effect of GHB.

We have previously suggested that the changes induced by GHB in the 15.5–30 Hz frequency band of the electroencephalogram (EEG) can be of interest as a surrogate end-point for the hypnotic effect of GHB in the rat (Van Sassenbroeck et al 2001, 2002). The aim of this study was to investigate whether tolerance is also observed for this EEG effect, and to use this parameter to study in more detail the pharmacokinetic and pharmacodynamic aspects of the tolerance to GHB.

Materials and Methods

Three series of experiments were conducted. In the first series, rats were pre-treated with GBL or saline for ten days. At regular intervals during this pre-treatment, the sleeping time induced by GBL was compared between the two groups. In the second and third series, the pharmacokinetic and pharmacodynamic parameters of GHB were compared between rats pre-treated with GBL and control rats, using a fast and slow infusion, respectively. For the pre-treatment of rats, the GHB precursor gamma-butyrolactone (GBL) was used to avoid the repeated administration of a high sodium load with sodium-GHB. GHB is the active component and GBL, which is rapidly and completely hydrolysed in the blood to GHB by a lactonase (Roth & Giarman 1966), has no activity in the brain (Snead 1991). For the pharmacokinetic–pharmacodynamic modelling after the GBL pre-treatment, GHB was used to avoid ongoing conversion of GBL to GHB in the first plasma samples taken during the infusion (Roth & Giarman 1965).

In each experiment, the observer was blinded to the pre-treatment given. The study protocol was approved by the ethical committee for animal research of the Faculty of Medicine of the University of Ghent, Belgium.

Animal instrumentation

Male Wistar rats, 280–430 g, were purchased from Iffa Credo (L'Arbresle Cedex, France) and kept at 21 °C with a 12-h light–dark cycle. In the first series, rats were not instrumented. In the second and third series, polyethylene catheters (PE 10) filled with heparin solution (100 IU mL^{-1}) were inserted into the femoral artery and vein and exteriorised at the nape of the neck one day before the actual experiment. The arterial line was used for blood sampling and the venous line for infusion of GHB. For the third series, five EEG electrodes were implanted as described previously three days before the start of the pre-treatment period (De Paepe et al 1999). All surgery was carried out under pentobarbital anaesthesia (60 mg kg^{-1} i.p.). To minimize restraining stress during the experiment, the rats were put in a restraining cage on several occasions before the actual experiment. During the experiment, the core temperature was measured every hour with a flexible thermistor probe inserted rectally to a depth of 5 cm and a heating lamp externally warmed the rat when the temperature fell below 37 °C. All experiments started between 0800 and 0900 h, after overnight fasting.

The EEG was measured from the right fronto-central lead using a D/EEG Lite digital EEG recorder (Telefactor, Zwolle, The Netherlands) at a sampling rate of 200 Hz. The low-pass and high-pass filter was set at 1 Hz and 70 Hz, respectively.

Experimental protocol

First series of experiments

Twelve rats were randomly allocated to a pre-treatment with either GBL (300 mg kg^{-1}, Sigma Chemical Corporation, Bornem, Belgium) or saline intraperitoneally at 0800 h and 2000 h for a period of 10 days. The GBL was diluted in physiological saline and the GBL concentration of the solution was calculated so as to administer 0.2 mL per 100 g body weight. On day 1, 4, 7 and 10 of this pre-treatment period, the sleeping time, defined as the time between loss and return of righting reflex, was measured in the GBL pre-treated rats and also in the saline pre-treated rats by giving 300 mg kg^{-1} of GBL intraperitoneally at 0800 h.

Second series of experiments

Sixteen rats were randomly assigned to one of two pre-treatment groups. The first group (n = 8) daily received a dose of GBL 300 mg kg^{-1} intraperitoneally at 0800 h and 2000 h for ten days, while the second group (n = 8) served as a control group, receiving the same amount of saline at the same time points. On day ten of the pre-treatment period, all rats received an intravenous infusion of 300 mg kg^{-1} GHB (Sigma Chemical Corporation, Bornem, Belgium) over 5 min. The GHB was dissolved in water and the GHB concentration of the injection solution was calculated so as to administer a total volume of 0.3 mL per 100 g body weight. Arterial blood samples of 100 μL were taken for determination of GHB plasma concentrations at regular time intervals. Sampled blood was replaced with the same amount of isotonic saline solution. To avoid differences in haemodilution between controls and pre-treated rats, the same maximal amount of 16 samples per rat were taken in each group. The timing of the loss and return of the righting reflex were recorded.

Third series of experiments

Three days after the implantation of EEG electrodes, sixteen rats were randomly assigned to one of two pre-treatment groups. The first group (n = 8) daily received a dose of GBL 300 mg kg^{-1} intraperitoneally at 0800 h and 2000 h for a period of seven days (in contrast to the second series), while the second group (n = 8) served as a control group, receiving the same amount of saline at the same time points. On day eight, after a 30-min baseline EEG recording, each rat received an intravenous infusion of GHB of 480 mg kg^{-1} h^{-1} that was stopped when an EEG depression of one second or more was observed in

a burst-suppression pattern. To assess the maximal obtainable EEG suppression, each rat received an infusion of GHB at a rate of $90\,mg\,kg^{-1}\,min^{-1}$ at the end of the experiment, until a burst-suppression pattern was observed with the isoelectric period lasting one second or longer. The timing of the loss and return of the righting reflex and the startle reflex to noise were recorded.

Drug assay

GHB was determined in rat plasma ($20\,\mu L$) by a validated HPLC method as described previously (De Vriendt et al 2001). The calibration curve ranged from 10 to $750\,\mu g\,mL^{-1}$ GHB. Quality control samples at low ($20\,\mu g\,mL^{-1}$), medium ($300\,\mu g\,mL^{-1}$) and high ($700\,\mu g\,mL^{-1}$) concentrations were analysed in duplicate together with the samples. For each quality control sample, the coefficient of variation was < 10% (n = 15) and the accuracy was between 98% and 103% (n = 15). The lower limit of quantitation was $10\,\mu g\,mL^{-1}$.

Analysis of data

In the second series of experiments, the pharmacokinetics of GHB were quantified as described earlier (Van Sassenbroeck et al 2001). In brief, a two-compartmental model with Michaelis–Menten elimination kinetics was fitted to the plasma concentration–time profiles of each individual rat using Winnonlin version 1.5 (Scientific Consulting, Inc):

$$\frac{dC_1}{dt} = \frac{R}{V_C} - \frac{CL_d.C_1}{V_C} + \frac{CL_d.C_2}{V_T} - \frac{V_{max}.C_1}{(K_m + C_1).V_C} \quad (1)$$

$$\frac{dC_2}{dt} = \frac{CL_d.C_1}{V_C} - \frac{CL_d.C_2}{V_T} \quad (2)$$

with

$$CL_d = k_{1,2}V_C = k_{2,1}V_T \quad (3)$$

where dC_1/dt is the rate of decline of drug concentration at time t, V_C the distribution volume of the central compartment, V_T the distribution volume of the peripheral compartment, R the infusion rate, CL_d the intercompartmental clearance, C_1 the concentration in the central compartment, C_2 the concentration in the peripheral compartment, V_{max} the theoretical maximum rate of the elimination, K_m the Michaelis–Menten constant, $k_{1,2}$ the transfer rate constant from the central to the peripheral compartment and $k_{2,1}$ the transfer rate constant from the peripheral to the central compartment.

This was the best fitting model based on the Akaike Information Criterion (AIC) (Akaike 1974) and the visual inspection of the curve and the residual plots (Gabrielsson & Weiner 1997). Using the estimated pharmacokinetic parameters, plasma concentration–time curves were constructed from time zero to a common final time point of 420 min because the time of the last measurable sampling point varied. The area under the curve (AUC) from time 0 to 420 min was then calculated using the trapezoidal rule (Kinetica 2000, Innaphase Co, Philadelphia, PA).

In the third series of experiments, the infusion regimen used was very slow and no hysteresis between the plasma concentrations and the EEG effect was observed. Hence the EEG effect could directly be linked to the plasma concentrations (Van Sassenbroeck et al 2001). A sigmoid inhibitory E_{max} model was used to describe the relationship between the effect-site concentration and the effect:

$$E = E_0 - \frac{E_{max}.C_p^n}{EC_{50}^n + C_p^n} \quad (4)$$

where E_0 is the baseline effect, E_{max} the maximal inhibition of the EEG effect measured after the GHB infusion at a rate of $90\,mg\,kg^{-1}\,min^{-1}$, C_p the GHB plasma concentration, EC50 the concentration required to obtain 50% of the maximal depression and n a constant expressing the slope of the concentration–effect relationship.

The time of loss and return of the startle reflex to noise and the righting reflex were recorded and the effect-site concentration of GHB at the time of loss and return of startle and righting reflex could be directly derived from the plasma concentration–time curve as there was no time-delay between the GHB effect-site and plasma concentration.

Statistical analysis

The results are expressed as mean ± s.d. Sleeping times in the first series of experiments were compared with multivariate regression analysis using a linear mixed model with treatment group, time and their interaction as fixed effects and an unstructured covariance matrix for random effects (SAS Inc, NC). Pharmacokinetic and pharmacodynamic parameters were compared with Student's *t*-test for independent observations (Statistica, Statsoft Inc., Tulsa, OK). $P < 0.05$ was considered as statistically significant.

Results

First series of experiments

Figure 1 shows the individual sleeping time as a function of time in GBL pre-treated rats versus saline pre-treated controls. The sleeping time progressively decreased from 98 ± 5 min to 31 ± 22 min in the GBL pre-treated group at day 10 but did not change in the control group (95 ± 16 min vs 83 ± 6 min, $P = 0.74$). The difference in sleeping time between the two groups was significant already at the fourth pre-treatment day ($P = 0.01$).

Second series of experiments

The infusion of $300\,mg\,kg^{-1}$ of GHB for 5 min induced a loss of righting reflex in the control rats for 42 ± 14 min, while this reflex was preserved in the GBL pre-treated rats. The individual GHB plasma concentration–time

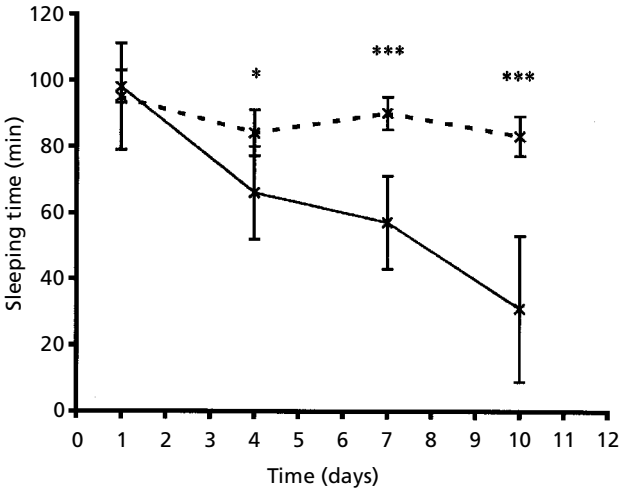

Figure 1 Mean sleeping time in GBL pre-treated rats (300 mg kg^{-1}, i.p., twice daily for 10 days) (n = 6) (—) vs saline pre-treated rats (n = 6) (- - -) as a function of time. Sleeping time was measured every third day after the injection of GBL (300 mg kg^{-1}, i.p.) in both groups. The vertical bars represent the s.d. $*P < 0.05$ and $***P < 0.001$ compared with multivariate regression analysis.

curves in these experiments are shown in Figure 2 and the calculated pharmacokinetic parameters in Table 1. It should be noted that GHB could not be detected before the start of the infusion in the GBL pre-treated rats. The GHB plasma concentrations of the GBL pre-treated rats decreased slightly faster than in the control rats (Figure 2). One GBL pre-treated rat showing the fastest decline in C_p was excluded from further analysis because the V_{max} and K_m could not be estimated independently. A significant difference in V_{max} and area under the plasma concentration–time curve between time zero and 420 min was observed between the GBL pre-treated and the control rats while the other parameters were not statistically different.

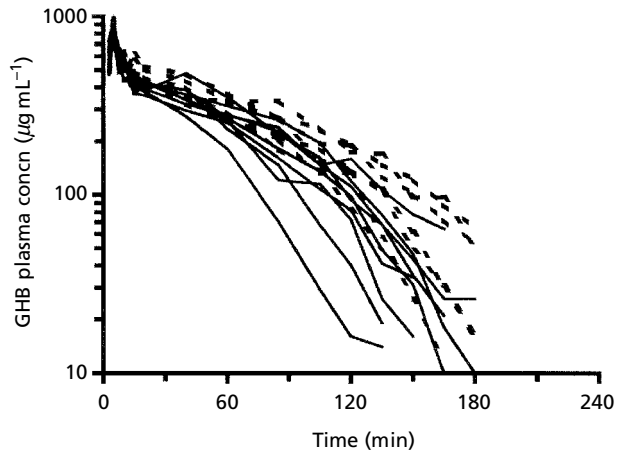

Figure 2 Individual GHB plasma concentration–time curves after intravenous infusion of GHB (300 mg kg^{-1} for 5 min) in eight GBL pre-treated (300 mg kg^{-1}, i.p., twice daily for 10 days) (—) and eight saline pre-treated controls (- - -).

Table 1 Pharmacokinetic parameters after intravenous infusion of GHB (300 mg kg^{-1} for 5 min) in control and GBL pre-treated rats.

	Controls (n = 8)	Pre-treated (n = 7)
V_{max} (μg min^{-1} kg^{-1})	2205 ± 315	2882 ± 457**
K_m (μg mL^{-1})	67 ± 60	95 ± 49
V_C (mL kg^{-1})	173 ± 79	217 ± 49
V_T (mL kg^{-1})	401 ± 56	362 ± 62
CL_d (mL min^{-1} kg^{-1})	85 ± 43	64 ± 24
AUC_{0-420} (mg min mL^{-1})	44 ± 7	35 ± 6*

Results are expressed as mean ± s.d. V_{max} is the theoretical maximum rate of the elimination, K_m the Michaelis–Menten constant, V_C the volume of distribution of the central compartment, V_T the volume of distribution of the peripheral compartment, CL_d the intercompartmental clearance. AUC_{0-420} is the area under the GHB plasma concentration–time curve between time 0 and 420 min. $*P < 0.05$, $**P < 0.01$ vs control (Student's *t*-test for unpaired observations).

Third series of experiments

In this series, one GBL pre-treated rat died during the infusion due to catheter-induced traumatic bleeding, and was excluded from further analysis.

The GHB solution had to be infused over 59 ± 9 min in the control rats and 133 ± 40 min in the GBL pre-treated rats until one second of isoelectric EEG was reached ($P < 0.001$). This corresponds to an administered dose of 475 ± 69 mg kg^{-1} and 1067 ± 317 mg kg^{-1}, respectively ($P < 0.001$). Table 2 shows the times of loss and return of the righting and the startle reflex. As can be derived from the data, the times between loss and return of the

Table 2 Time of loss and return of righting reflex (RR) and startle reflex (SR) and corresponding GHB plasma concentrations (Cp) after intravenous infusion of GHB (480 mg kg^{-1} h^{-1}) until one second of isoelectric EEG in control and GBL pre-treated rats.

	Control (n = 8)	GBL pre-treated (n = 7)
Time (min) of		
loss of SR	29 ± 11	65 ± 14***
return of SR	155 ± 36	193 ± 38
loss of RR	43 ± 7	100 ± 24***
return of RR	104 ± 21	155 ± 33**
Cp (μg mL^{-1}) at		
loss of SR	384 ± 159	723 ± 216*
return of SR	275 ± 124	677 ± 252**
loss of RR	496 ± 51	841 ± 226**
return of RR	455 ± 91	811 ± 261**

Results are expressed as mean ± s.d. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs control (Student's *t*-test for unpaired observations).

startle reflex (126 ± 41 min in control and 128 ± 36 min in GBL pre-treated rats) and of the righting reflex (61 ± 19 min in control and 54 ± 9 min in GBL pre-treated rats) were not significantly different although GBL pre-treated rats received a much higher dose. The GHB plasma concentrations, which reflect the brain concentrations, at loss and return of the startle and the righting reflex were significantly higher in the GBL pre-treated rats.

Figure 3 shows the time course of the EEG parameter during and after the slow infusion of $480\,mg\,kg^{-1}\,h^{-1}$ given until one second of isoelectric EEG was reached. For both the controls and the GBL pre-treated rats, there was no significant difference between the effect of the first infusion ($E_1 = 511 \pm 147\,\mu V\,s^{-1}$ in control and $564 \pm 154\,\mu V\,s^{-1}$ in GBL pre-treated rats, $P = 0.51$) and the second infusion (not shown) ($E_{max} = 543 \pm 137\,\mu V\,s^{-1}$ in control and $621 \pm 156\,\mu V\,s^{-1}$ in GBL pre-treated rats, $P = 0.31$) as the two infusions were both stopped at a one-second EEG suppression. The EEG-effect versus GHB plasma concentration curve showed no hysteresis. Therefore, a sigmoid inhibitory E_{max} model was directly fitted to the EEG effect versus GHB plasma concentration curve (Figure 4).

There was a statistically significant difference between the GBL pre-treated and the control rats for the EC50 ($653 \pm 183\,\mu g\,mL^{-1}$ vs $323 \pm 68\,\mu g\,mL^{-1}$, $P < 0.001$) and for the shape factor, n (3.5 ± 1.3 in the GBL pre-treated vs 2.0 ± 0.7 in the controls, $P = 0.02$). This means that the EEG effect versus GHB plasma concentration curves of the GBL pre-treated rats have shifted to the right and show a less-steep course compared with the curves of the control rats. The differences in E_0 (806 ± 202 vs 729 ± 134, $P = 0.39$) and E_{max} ($621 \pm 156\,\mu V\,s^{-1}$ vs $543 \pm 137\,\mu V\,s^{-1}$, $P = 0.31$) were not significant.

Discussion

Tolerance to GHB has been suggested in illicit users (Dyer et al 2001) and has been described for the hypnotic effect in the rat and the mouse (Gianutsos & Moore 1978). In our experiments, chronic pre-treatment with GBL reduces the sleeping time by GBL or GHB indicating the occurrence of tolerance as described by others (Nowycjy & Roth 1979). This tolerance can theoretically be explained by either pharmacokinetic changes like the induction of GHB metabolism or by a decreased sensitivity of the end-organ (O'Brien 1996).

With regard to the pharmacokinetics, the GHB plasma concentration–time curve showed a slightly faster decrease of GHB concentrations in the GBL pre-treated rats (second series). This can be explained by an increase in elimination, probably due to the induction of the GHB metabolism. Indeed, a higher V_{max} and a smaller AUC_{0-420} were observed.

However, these changes in the pharmacokinetics are rather small and therefore cannot be the primary explanation for the marked reduction in sleeping time. A reduction in end-organ sensitivity must therefore be assumed as suggested by Giorgi & Rubio (1981), who observed that GHB brain concentrations upon return of the righting reflex were higher in GHB-tolerant rats. In our experiments, this decrease in end-organ sensitivity was investigated further by studying an EEG parameter, namely the amplitude in the 15.5–30 Hz frequency band, which we

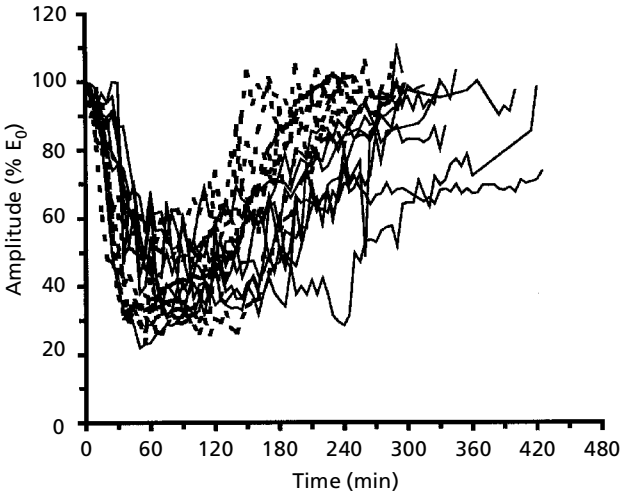

Figure 3 Individual time course of EEG amplitude in the 15.5–30 Hz frequency band, expressed as percentage change of baseline activity (E_0), after intravenous infusion of GHB ($480\,mg\,kg^{-1}\,h^{-1}$ until one second of isoelectric EEG suppression), in seven individual rats pre-treated with GBL ($300\,mg\,kg^{-1}$, i.p., twice daily for 7 days) (—) and in eight saline pre-treated controls (- - -). The infusion started at time zero.

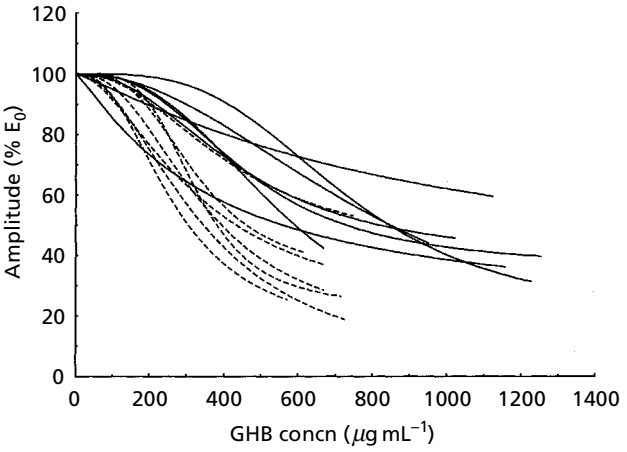

Figure 4 Relationship between the EEG amplitude and GHB plasma concentration in seven individual rats pre-treated with GBL ($300\,mg\,kg^{-1}$, i.p., twice daily for 7 days) (—) and in eight saline treated controls (- - -) after intravenous infusion of GHB ($480\,mg\,kg^{-1}\,h^{-1}$ until one second isoelectric EEG suppression). The EEG amplitude is expressed as a percentage change of baseline activity (E_0). The curves are the result of the fitting of a sigmoid E_{max} model to the data.

have postulated can be used as a surrogate for the hypnotic effect of GHB in the rat (Van Sassenbroeck et al 2001). An advantage of the use of the EEG as a parameter for the depth of hypnosis is that it can be measured continuously and allows studying the tolerance quantitatively. A slow infusion of 480 mg kg^{-1} h^{-1} was administered until one second isoelectric EEG was observed on the raw EEG. This slow infusion allowed linking the EEG effect directly to the GHB plasma concentrations because no hysteresis was observed as has been demonstrated previously (Van Sassenbroeck et al 2001). In these experiments, a model could not be applied to the plasma concentration–time curves due to the limited number of sample points collected in the same rat. Hence, V_{max} and K_m could not be estimated independently with sufficient precision. The values of the EEG parameter were therefore correlated directly to their corresponding plasma concentrations. The EEG effect versus GHB plasma concentration curves of the GBL pre-treated rats showed a clear shift to the right of the EC50 indicating the occurrence of tolerance. Moreover, we estimated that significantly higher GHB concentrations were needed at the time of loss and return of both the startle and the righting reflex. This is in agreement with the results of others who measured higher GHB concentrations in brain homogenates at return of the righting reflex (Giorgi & Rubio 1981). These differences in the EC50 and the shape factor, n, suggest that the observed tolerance is due to changes at the effect site.

Our experiments do not allow clarification of the exact mechanism underlying the tolerance, as it is presently unclear by which mechanism GHB causes hypnosis. However, Carai et al (2001) demonstrated the involvement of $GABA_B$ receptors, which may be activated after the conversion of GHB to gamma-amino butyric acid (GABA) (Hechler et al 1997), or after the stimulation of GABA release (Gobaille et al 1999). Recently, the loading of presynaptic vesicles with both GHB and GABA, resulting in their co-release, has been suggested (Muller et al 2002). However, there are also arguments for a GHB recognition site related to the presynaptic $GABA_B$ receptor (Snead 1996) and a weak direct $GABA_B$ receptor agonism (Lingenhoehl et al 1999). Tolerance at the end organ has been explained by down-regulation of receptors, depletion of endogenous compounds, and systemic adaptations by physiologic systems such as neurohormonal counter-regulation (Sharma et al 1998). These processes can take place at any of the above-mentioned receptor sites.

Conclusions

It is concluded that chronic pre-treatment with GBL in the rat results in a reduction of the sleeping time. This can be explained, to some extent, by the induction of the GHB metabolism, but mostly by a reduced sensitivity of the end organ as demonstrated by the righting and the startle reflex. The EEG parameter, which we have previously suggested can be used as a surrogate measure for the depth of GHB-induced hypnosis, also reflects the observed tolerance. It is, however, presently unclear whether the demonstration of tolerance for GHB after the chronic forced intake of hypnotic doses in the rat is a good model for the chronic intake of non-hypnotic doses in man.

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