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# Sulpiride, but not haloperidol, up-regulates γ-hydroxybutyrate receptors in vivo and in cultured cells

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**Abstract**

Five days of γ-hydroxybutyrate (GHB) administration ($3 \times 500$ mg $kg^{-1}$ $day^{-1}$ i.p.) to rats resulted in a significant decrease in the density of GHB receptors measured in the whole rat brain without modification of their corresponding affinity. Similar administration of (−)-sulpiride ($2 \times 100$ mg $kg^{-1}$ $day^{-1}$ i.p. for 5 days) induces an up-regulation of GHB receptors without change in their dissociation constants ($K_d$). Haloperidol ($2 \times 2$ mg $day^{-1}$ i.p. for 5 days) showed no effect. Administered chronically via osmotic minipumps directly into the lateral ventricles, (−)-sulpiride (60 μg $day^{-1}$ for 7 days) and GHB (600 μg $day^{-1}$ for 7 days) up-regulated and down-regulated rat brain GHB receptors, respectively. Finally, in a mouse hybridoma cell line (NCB-20 cells) expressing GHB receptors, the treatment of these cells with 1 mM GHB, 100 μM (−)-sulpiride or 1 mM GABA decreases, increases and induces no change, respectively, in the density of GHB receptors after 3 days of treatments. These results indicate that chronic GHB treatment modifies the expression of its receptor and that sulpiride also induces plastic changes in GHB receptors perhaps via antagonistic properties. © 1998 Elsevier Science B.V.

*Keywords:* γ-Hydroxybutyrate receptor; Sulpiride; Haloperidol; Chronic administration; NCB-20 cells; Brain, rat

## 1. Introduction

γ-Hydroxybutyrate (GHB) is a normal brain metabolite which possesses several properties of a classical neurotransmitter (Vayer et al., 1987b; Maitre, 1997). In particular, it is thought to trigger directly or to potentiate by metabolism some GABAergic mechanism in brain (Della Pietra et al., 1966; DeFeudis and Collier, 1970; Vayer et al., 1985; Banerjee and Snead, 1995). However, the mesolimbic and nigrostriatal dopaminergic pathways appear to be the main targets of the neuroregulatory effects of GHB (Gessa et al., 1966; Roth et al., 1980; Hechler et al., 1991). These effects are mediated through GHB receptors located in the dopaminergic structures of the brain ($A_9$, $A_{10}$ and $A_{12}$, striatum and olfactory tracts) and also in the cortex and hippocampus (Benavides et al., 1982; Snead and Liu, 1984; Hechler et al., 1987, 1992). GHB enters the brain freely and peripheral administrations of GHB are often used in order to potentiate the GHB endogenous system.

In particular, high and repeated doses of GHB are administered to man not only for the therapeutic benefits in various diseases such as narcolepsy (Mamelak et al., 1986), alcohol or heroin addiction and withdrawal (Fadda et al., 1989; Gallimberti et al., 1993), but also for the induction of anaesthesia (Laborit, 1973; Hoes et al., 1980). Chronic GHB use has also been reported by drug abusers (Chin et al., 1992). Similarly, chronic treatment with benzamide neuroleptics, including sulpiride, could affect the sensitivity and/or density of GHB receptors since these compounds bind with high affinity to GHB sites in vitro (Maitre et al., 1994). GHB receptors are most probably regulated after prolonged treatment with agonists or antagonists and up to now, no result has been reported concerning the possible modulation of the expression of these receptors in vivo or in cell culture. We examined the effects of chronic GHB or (−)-sulpiride administration on rat brain GHB receptors and compared them with chronic haloperidol treatment. This last neuroleptic does not interfere with GHB binding. The in vivo results have been confirmed by in vitro experiments using a neuronal cell line expressing GHB high affinity binding sites.

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## 2. Materials and methods

### 2.1. GHB, haloperidol or sulpiride treatments

For intraperitoneal (i.p.) treatment, male Wistar rats, weighing 350–400 g at the end of drug administration, were housed individually in cages with a 12-h light/dark cycle. Food and water were given ad libitum. Three daily i.p. administrations of 500 mg $kg^{-1}$ GHB ($Na^+$ salt, purchased from Sigma) were given for 5 days, at 9:00 AM, 2:00 PM and 7:00 PM, respectively. (−)-Sulpiride was administered i.p. twice a day (9:00 AM and 7:00 PM) at doses of 100 mg $kg^{-1}$ for 5 days. Under the same conditions, other rats were injected with haloperidol (two daily administration of 2 mg $kg^{-1}$ i.p.). Saline solution was administered to control rats. The animals were killed 14 h after the last injection for the preparation of brain membranes.

For intracerebroventricular infusions, male Wistar rats (400 g) were anaesthetized with Imalgène 500 (100 mg $kg^{-1}$ i.p.) and a stainless steel cannula (30-gauge) was stereotaxically implanted into the right lateral ventricle, 1.8 mm lateral from bregma at a depth of 4.1 mm from the skull. The cannula was fixed to the skull with two stainless-steel screws and methacrylic cement. Then the cannula was connected via vinyl tubing to an osmotic minipump (model $2ML_2$ Alzet, Charles Rivers, France) implanted subcutaneously in the interscapular region of the rat.

Concentrations of the drugs were 0.5 mg $ml^{-1}$ saline (60-$\mu$g doses 24 $h^{-1}$) for (−)-sulpiride and 5 mg $ml^{-1}$ saline (600 $\mu$g 24 $h^{-1}$) for GHB. Drugs were infused at 5 $\mu$l $h^{-1}$ ($4.89 \pm 0.23$ $\mu$l $h^{-1}$) for 8 days. After surgery and during the infusion period, the rats were housed individually in cages. The animals were killed after 8 days, and the correct placement of the cannula in the lateral ventricle was immediately confirmed for each rat.

### 2.2. Assay of GHB receptors in brain homogenates

Total rat brain hemispheres (i.e., total brain without cerebellum and pons–medulla) of Wistar adult rats either treated with saline or with GHB, (−)-sulpiride or haloperidol were used to prepare brain membranes. Animals were killed by decapitation and their brains, rapidly extracted and dissected, were homogenized in 10 volumes 0.32 M sucrose containing 5 mM EDTA, adjusted to pH 6.0 and maintained at 0°C. $P_2$ fractions were obtained after subsequent centrifugations in this medium. Crude membranes were prepared according to a previously described method (Maitre et al., 1994). Briefly, $P_2$ pellets were homogenized with a polytron in 70 volumes of distilled water at 0°C containing 5 mM EDTA. After centrifugation, pellets were washed with cold water (4°C) containing 0.5% CHAPS (3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) and 5 mM EDTA. Membranes were centrifuged and washed again with 50 mM potassium phosphate buffer pH 6.0, then stored for 1 day at −80°C. Saturation curves were performed as described by Benavides et al. (1982) but using a rapid filtration assay with GF/B filters (Whatmann) in order to separate bound from free [$^3$H]GHB. Fitting saturation binding curves by non-linear regression allowed the determination of $B_{max}$ and dissociation constants ($K_d$) (GraphPad Prism Program, San Diego, CA). Protein measurements were performed using the BCA protein assay (Pierce, USA).

### 2.3. NCB-20 cell culture

NCB-20 cells were cultured at 37°C in a humidified $CO_2$ (5%) incubator in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, penicillin (50 U $ml^{-1}$) and streptomycin (50 $\mu$g $ml^{-1}$). NCB-20 cells were subcultured in 75 $cm^2$ flasks (Falcon) and media were changed every 3–4 days. For experiments, cells were seeded (30 000 cells per dish) in 35 mm Petri dishes which were first treated for 2 h at 37°C with polyethylenimine (20 mg% in sterile distilled water). One day after seeding, differentiation was induced by treatment of the cells for up to 3 days with 1 mM dibutyryl cyclic AMP. Control cells were cultured in parallel in the absence of dibutyryl cyclic AMP for the same period of time.

In some experiments, the cell medium was supplemented with 1 mM GHB or with 100 $\mu$M (−)-sulpiride for 48 h, just before measurements of the [$^3$H]GHB binding in the presence of 100 nM radioactive GHB.

### 2.4. [$^3$H]GHB binding experiments on NCB-20 cells

Cells were washed three times with 100 mM potassium phosphate buffer, pH 6.0 (solution A), then incubated for 15 min in the same solution at room temperature. Binding experiments were carried out in solution A containing 100 nM [$^3$H]GHB (100 Ci $mmol^{-1}$, CEA, France) for 30 min at 0°C. Cells were rapidly washed twice for 10 s with 2 ml of solution A at 0°C. Non-specific binding was determined in the presence of 1 mM non-radioactive GHB. Saturation curves were constructed with radioactive GHB varying from 10 to 1100 nM. The cells were scraped from the dishes using distilled water and counted for radioactivity after vigorous vortexing in 5 ml of scintillation fluid (Rotiszint, Roth, Germany). Protein concentrations were determined by the BCA protein assay (Pierce). Binding affinities and $B_{max}$ values were calculated using the GraphPad Prism Program.

### 2.5. Data analysis

$B_{max}$ and $K_d$ values used for statistics were calculated from the non-linear regression analysis which provides more reliable estimations (GraphPad Prism Program). However, linear (Scatchard plot) regression analyses were

presented for rapid visual interpretation of the data. Deviation from the model (non-linear regression analysis) was checked with the runs test and was never significant for all experiments. The $r^2$ were always better than 0.96 in all cases. The significance of differences between treated and control groups for both $B_{max}$ and $K_d$ was assessed by means of Student's *t*-test.

## 3. Results

### 3.1. GHB receptor binding after chronic GHB in vivo

Scatchard analysis of [$^3$H]GHB binding to brain membranes from GHB-treated rats showed that, as in controls, GHB bound to a single population of recognition sites (Maitre et al., 1994). The $B_{max}$ of [$^3$H]GHB binding to brain hemisphere membranes from rats treated for 5 days i.p. with GHB was reduced by 45% (Table 1 and Fig. 1A; $p < 0.02$, $n = 3$ rats measured in triplicate at each concentration). But the $K_d$ remains unaltered compared to controls.

Similar to the effects of 5 days i.p. treatment, the continuous infusion of GHB into the brain lateral ventricle reduced the $B_{max}$ of [$^3$H]GHB binding in the rat brain hemispheres by 49% ($p < 0.001$; Table 1 and Fig. 2A, $n = 3$ rats measured in triplicate at each concentration). No significant difference was found between $K_d$ values.

### 3.2. GHB receptor binding after chronic (−)-sulpiride in vivo

Previous studies showed that (−)-sulpiride, like other benzamide neuroleptics, displaced [$^3$H]GHB from its binding sites with low and high $IC_{50}$ values (Maitre et al., 1994). Chronic i.p. treatment of rats with (−)-sulpiride for 5 days significantly increased the $B_{max}$ for [$^3$H]GHB in the brain hemispheres (Table 1 and Fig. 1B), $B_{max}$ values rose by 34% ($p < 0.01$, $n = 3$ rats measured in triplicate at each concentration) in contrast to results obtained with chronic GHB treatments. $K_d$ values were not changed.

When rats were treated with chronically infused (−)-sulpiride (60 μg day$^{-1}$ for 7 days), same results were obtained. Compared to control rats infused with saline, $B_{max}$ values increased by 28% ($p < 0.03$; $n = 3$ rats measured in triplicate at each concentration) but the $K_d$ values showed no significant change (Table 1 and Fig. 2B).

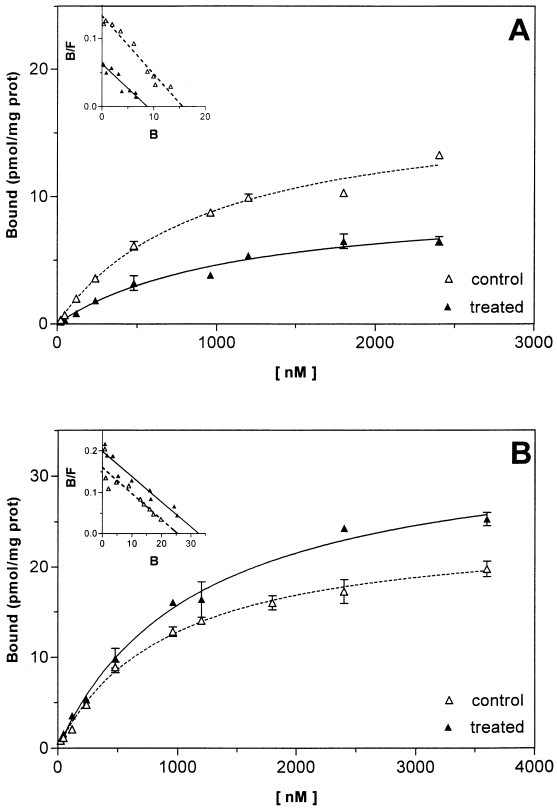


Fig. 1. Chronic GHB (3×500 mg kg$^{-1}$ daily administration i.p. for 5 days (A) and chronic (−)-sulpiride treatment of rats (2×100 mg kg$^{-1}$ daily administration i.p. for 5 days (B). Saturation [$^3$H]GHB binding experiments (non-linear regression lines) and transformation of the data by linear regression (Scatchard plots). In all experiments, $K_d$ of control and treated rats are about 1 μM; $B_{max}$ of control rats are about 9 to 20 pmol mg$^{-1}$ protein. Experiments were carried out on three control rats vs. three rats chronically administered with GHB and on three control rats vs. three rats chronically administered with (−)-sulpiride. Mean ± S.D. are given in Table 1; each experimental point being measured in triplicate. Treated rats must be compared to control rats performed in the same set of experiments.

Table 1

| Experiment | $B_{max}$ in pmol mg$^{-1}$ of proteins (mean ± S.D.) | | % Variation | Statistical comparison |
|---|---|---|---|---|
| | Control | Treated | | |
| 1 | 15.7 ± 3.0 | 7.9 ± 2.6 | −49 | < 0.001 |
| 2 | 19.7 ± 3.1 | 10.8 ± 1.4 | −45 | < 0.02 |
| 3 | 9.1 ± 1.1 | 11.7 ± 0.4 | +28 | < 0.03 |
| 4 | 21.1 ± 0.05 | 28.3 ± 1.2 | +34 | < 0.01 |
| 5 | 16.5 ± 5.2 | 17.6 ± 6.5 | +7 | n.s. |

Effects of chronic GHB, (−)-sulpiride and haloperidol treatments on the expression of GHB receptors in the rat brain hemispheres. Types of experiments: 1 = chronic GHB administered with micropumps; 2 = chronic GHB administered i.p.; 3 = chronic (−)-sulpiride administered with micropumps; 4 = chronic (−)-sulpride administered i.p.; 5 = chronic haloperidol administered i.p. See text for details concerning doses and periods of administration. Results are means ± S.D. of three independent determinations; each experimental point was measured in triplicate. Each set of treated animals has its own set of controls performed exactly in the same experimental conditions.

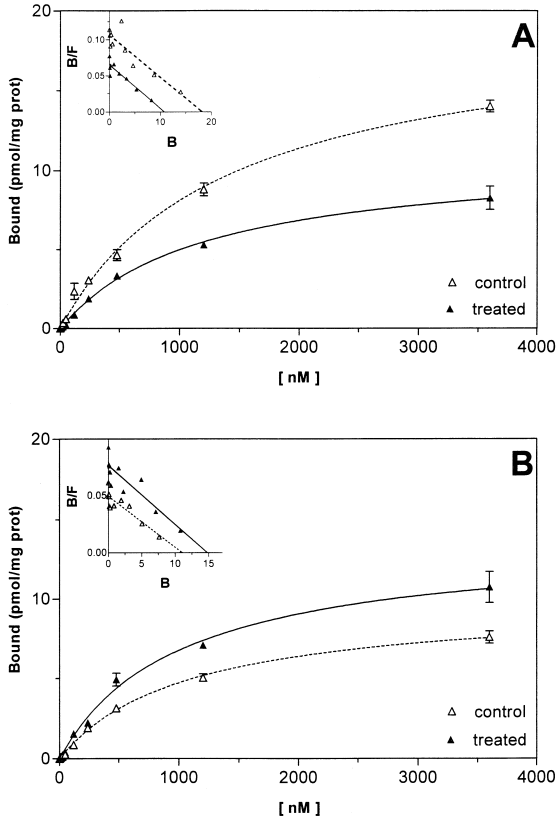


Fig. 2. (A) Chronic GHB treatment (600 $\mu$g 24 h$^{-1}$) for 7 days and (B) chronic (−)-sulpiride treatment (60 $\mu$g 24 h$^{-1}$) for 7 days by brain microinfusion with osmotic pumps. Same representations as in Fig. 1A and Fig. 1B. In all experiments, $K_d$ of control and treated rats are about 1 $\mu$M; $B_{max}$ of control rats are about 9 to 20 pmol mg$^{-1}$ protein. Experiments were carried out on three control rats vs. three rats chronically administered with GHB and on three control rats vs. three rats chronically administered with (−)-sulpiride. Mean ± S.D. are given in Table 1; each experimental point being measured in triplicate. Treated rats must be compared to control rats performed in the same set of experiments.

### 3.3. Chronic haloperidol treatment and GHB receptor binding

In contrast to (−)-sulpiride, haloperidol possessed no in vitro effect on [$^3$H]GHB binding (Maitre et al., 1994). Therefore, chronic haloperidol treatment was chosen as a reference for testing the effect of chronic neuroleptic treatment on GHB receptor binding. Rats were treated with two daily (9:00 AM and 7:00 PM) i.p. injections of haloperidol ($2 \times 2$ mg kg$^{-1}$) for 5 days. Under these conditions, no significant difference between $B_{max}$ and $K_d$ values for [$^3$H]GHB binding was registered for these rats compared to controls treated with saline (Table 1 and Fig. 3).

### 3.4. Effects of (−)-sulpiride and GHB on [$^3$H]GHB binding by NCB-20 cells

NCB-20 cells are a hybrid between mouse neuroblastoma N18TG2 and Chinese hamster 18 days embryonic brain cells which express many properties characteristic of neurons (Minna and Yavelow, 1975; Nirenberg et al., 1983, 1984). Undifferentiated cells bind [$^3$H]GHB in a saturable and reversible manner. In the range of concentration from 10 to 1100 nM, the Scatchard representation of specific binding indicates the presence of a single population of binding sites with a $K_d$ of $250 \pm 44.4$ nM and a $B_{max}$ of $180 \pm 16.2$ fmol mg$^{-1}$ protein. If differentiated by the presence of dibutyryl cyclic AMP, the specific [$^3$H]GHB binding was reduced ($B_{max} = 73.7 \pm 10.9$ fmol mg$^{-1}$ protein) and the $K_d$ increased to $975 \pm 236$ nM after 2 days of treatment (not shown). The effect of chronic treatment of these cells with (−)-sulpiride or GHB increases (40% compared to non-treated cells, $p < 0.001$) or decreases (50% compared to non-treated cells, $p < 0.001$), respectively, the [$^3$H]GHB specific binding capacity measured in the presence of 100 nM radioactive GHB (Fig. 4; three independent experiments in each case). In experiments to determine the reversibility of GHB-induced changes in [$^3$H]GHB binding, cells were treated with 1 mM GHB for 48 h followed by removal of this medium, superficial washing of the cells with GHB-free medium, then incubation for 24 h with the original medium (minus GHB). Under these conditions, recovery of the GHB-induced decrease of $B_{max}$ was complete, with a return to pre-exposure values. Finally, the chronic presence of GABA (1 mM for 48 h) in the cell medium was also tested on GHB binding as a control because GABA has no affinity for the GHB receptor (Benavides et al., 1982). The presence of GABA in the cell medium for 48 h did not

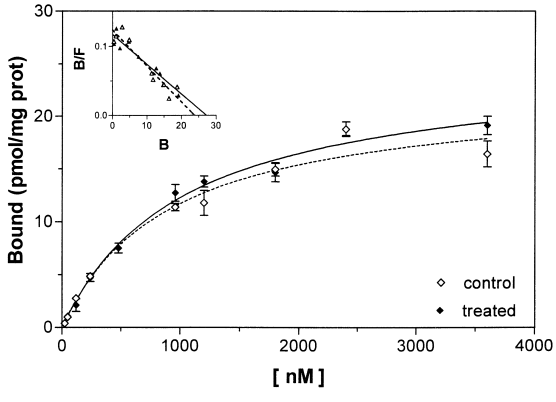


Fig. 3. Chronic haloperidol treatment of rats ($2 \times 2$ mg kg$^{-1}$ for 5 days). In all experiments, $K_d$ of control and treated rats are about 1 $\mu$M; $B_{max}$ of control rats are about 9 to 20 pmol mg$^{-1}$ protein. Experiments were carried out on three control rats vs. three rats chronically administered with haloperidol. Mean ± S.D. are given in Table 1; each experimental point being measured in triplicate. Treated rats must be compared to control rats performed in the same set of experiments.

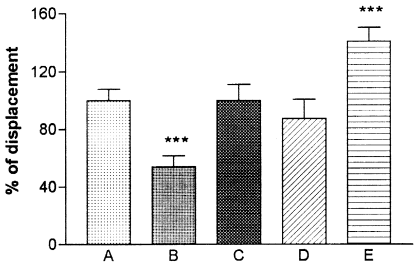


Fig. 4. Modulation of GHB binding sites expression in NCB-20 cells. Ordinate: Percent (%) of GHB specific binding (see kinetic parameters of GHB binding on NCB-20 cells in the text). Control cells (untreated, column A) have been taken as 100% reference. Column B = Cells treated with 1 mM GHB for 48 h, significantly different from control $p < 0.001$. Column C = Cells treated with 1 mM GHB for 48 h and then washed and cultured for 24 h under control conditions, not significantly different from control cells. Column D = Cells treated with 1 mM GABA for 48 h; not significantly different from control cells. Column E = Cells treated with 100 $\mu$M (−)-sulpiride for 48 h; significantly different from control cells, $p < 0.001$. Each result is the mean ± S.D. of three independent determinations and each experimental point has been performed in triplicate.

change the expression of the GHB receptor compared to GHB-treated cells (Fig. 4; three independent experiments in each case).

## 4. Discussion

GHB receptors of the mammalian brain show heterogeneous distribution in the brain, with a maximum in the superficial layers of the cortex and in the $CA_1$ field of the hippocampus. Other brain regions possess intermediate concentrations ($CA_2$ and $CA_3$ fields of the hippocampus, the deeper cortical layers, some regions of the thalamus, the striatum, amygdala and dopaminergic nuclei $A_9$ and $A_{12}$) (Hechler et al., 1987, 1992). This receptor is thought to be stimulated by endogenous concentrations of GHB which can be released in a $Ca^{2+}$-dependent manner (Maitre et al., 1983; Vayer and Maitre, 1988). Second messenger systems and ionic conductances are activated upon GHB receptor activation ($Ca^{2+}$ and $K^+$ movements, cGMP and inositol phosphates increases in some part of the brain) (Vayer et al., 1987a; Vayer and Maitre, 1989; Harris et al., 1989). However, a GHB-induced $GABA_B$ effect has been reported because it was blocked by CGP 35 348 (*P*-[3-aminopropyl]-*P*-diethoxymethylphosphonic acid) (Xie and Smart, 1992). This effect is possibly due to a modification of GABA release after GHB application and/or to a GHB conversion into GABA (Della Pietra et al., 1966; DeFeudis and Collier, 1970; Vayer et al., 1985; Banerjee and Snead, 1995). GHB receptor is probably close to $GABA_B$ receptor from a structural and functional point of view, but it represents a distinct family of receptors by its kinetics, ontogenesis, distribution and pharmacology (Benavides et al., 1982; Hechler et al., 1992; Snead, 1994). Among the ligands which specifically bind to GHB receptor, (−)-sulpiride and some substituted benzamides are of interest because a part of their therapeutic specificities could be due to interactions with GHB receptors (Maitre et al., 1994).

One of the principal findings of the present study was that subchronic treatment of rats with peripheral administration of GHB rapidly down-regulates GHB receptors. GHB administered peripherally in rats penetrates the brain rapidly and for a single dose of 500 mg $kg^{-1}$, the brain GHB concentration is expected to peak at about 1 mM, then decrease during 3–4 h (Shumate and Snead, 1979; Lettieri and Fung, 1979). During the microinfusion with osmotic pumps, the average level of GHB in brain could be estimated to be around 100 $\mu$M. Under both conditions (i.p. administration or intracerebroventricular infusion), brain GHB receptors could be considered saturated by their natural agonist and could exhibit a plastic expression (most probably internalization with down regulation) which confirms the existence of a specific regulatory process at this level. In a neuronal cell line expressing GHB receptors, the down-regulation of these receptors by two days exposure to GHB can be reversed completely after 1 day in a medium without GHB. The presence of GABA, which is not a ligand for the GHB receptor, in these cell culture did not induce an adaptive change of GHB receptor expression.

The demonstration of agonist-induced down-regulation of rat brain GHB receptors leads to the suspicion that similar modifications occur in humans treated with chronic doses of GHB. In narcoleptic patients, GHB is often used at large doses (2 to 4 g each night) for a long period of time (several years) (Mamelak et al., 1986). However, symptoms such as tolerance or addiction have not been reported in this context, although chronic voluntary intake of GHB for psychological benefits has been reported in human (Chin et al., 1992). Succinic semialdehyde dehydrogenase deficiency, a genetic metabolic disease with mental retardation and neurological abnormalities, is accompanied with high and chronic GHB levels (300 to 600 $\mu$M) in the cerebrospinal fluid (CSF) (Rating et al., 1984). Succinic semialdehyde, which cannot be oxidized to succinic acid because of the genetic defect, accumulates as the reductive catabolite (GHB) in brain, serum, urine and CSF. Such chronic high GHB levels most probably down-regulate brain GHB receptors.

(−)-Sulpiride and some other benzamide neuroleptics displace [$^3$H]GHB from its binding sites with $IC_{50}$ values which are in the nanomolar and in the micromolar range, respectively, suggesting interactions of (−)-sulpiride with GHB binding sites of high and low affinities (Maitre et al., 1994). GHB receptors are implicated in the regulation of dopaminergic activities both in the nigrostriatal but also in the mesocorticolimbic pathway (Kelly and Moore, 1978). Thus, at least a part of the effect of (−)-sulpiride on brain dopaminergic activity could be due to its role as a GHBergic system modulator and that could explain some of the therapeutic specificities of (−)-sulpiride compared to those

of other antipsychotics. The results of the present study confirm the interaction of (−)-sulpiride with GHB receptors, either expressed by rat brain or by NCB-20 cells in culture. Haloperidol, which is not a ligand for GHB receptor in vitro, does not modify the expression of GHB receptors after chronic administration in vivo. Whatever the model, (−)-sulpiride up-regulates [$^3$H]GHB binding and this phenomenon could be considered as a compensatory mechanism to functional blockade. Consequently, (−)-sulpiride should be considered as an antagonist at the GHB receptor. GHB receptor ligands, including GHB itself, but also $D_2$ receptor antagonists, possess antidopaminergic activities in several neuropharmacological tests used to predict neuroleptic activities in vivo (Hechler et al., 1993). Thus, if (−)-sulpiride acts partly through GHB receptor stimulation to modify dopaminergic transmission, one might better predict agonistic properties. The classical response of receptors to stimulation by an agonist is down-regulation, however up-regulation of $D_2$ receptors has been reported to be induced by both agonists and antagonists (Starr et al., 1995). This ligand-induced up-regulation of receptors is thought to be produced by a stabilization of receptors by ligands. Thus, the present study confirms the interaction of (−)-sulpiride with GHB receptors in vivo and in cell culture but other approaches are needed to distinguish between agonist or antagonist properties.

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