Biochemical Pharmacology, Vol. 58, pp. 1815–1819, 1999.
© 1999 Elsevier Science Inc. All rights reserved.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00265-8

γ-Hydroxybutyrate Receptor Function Studied by the Modulation of Nitric Oxide Synthase Activity in Rat Frontal Cortex Punches

*Christopher D. Cash, Serge Gobaille, Véronique Kemmel, Christian Andriamampandry and Michel Maitre**

INSTITUT DE CHIMIE BIOLOGIQUE AND ER 2072 CNRS, FACULTÉ DE MÉDECINE, 67085 STRASBOURG, FRANCE

ABSTRACT. Previous results have shown that stimulation of the gamma-hydroxybutyrate (GHB) receptor modulates Ca^{2+} channel permeability in cell cultures. In order to confirm this result, we investigated the consequence of GHB receptor stimulation on nitric oxide synthase (NOS) activity in rat brain cortical punches rich in GHB receptors. The stimulation of these receptors by increasing amounts of GHB induced a progressive decrease in NOS activity. However, for GHB doses above 10 μM, this reduction was progressively lost, either after receptor desensitization or after stimulation of an additional class of GHB receptor having lower affinity. The effect of GHB was reproduced by the GHB receptor agonist NCS-356 and blocked by the GHB receptor antagonist NCS-382. The GHB-induced effect on Ca^{2+} movement was additive to those produced by veratrine, indicating that GHB modulates a specific Ca^{2+} conductance, which explains the modification in NOS activity and the increase in cyclic guanosine monophosphate levels previously reported. BIOCHEM PHARMACOL **58**;11: 1815–1819, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. gamma-hydroxybutyrate receptor; GHB; nitric oxide synthase activity; calcium channel; NCS-382

GHB†, which is endogenously present in micromolar quantities in the brain, is derived mainly from the transamination of GABA followed by the reduction of succinic semialdehyde [1]. GHB possesses neuromodulatory functions in brain via high affinity receptors which are characterized by a specific ontogeny, distribution, kinetics, and pharmacology [2]. These receptors, which do not appear to be expressed by glial cells and peripheral tissue, belong to the G-protein-linked receptor family [3]. They are found exclusively in the rostral part of the brain (whole cortex—including frontal cortex—and hippocampus, striatum and olfactory tracts, thalamus, and some dopaminergic nuclei such as A_9, A_{10}, and A_{12}) but also in neuronal cell cultures [4, 5]. Several results have documented that the brain endogenous GHB system exerts a tonic inhibitory control over both the release of dopamine in striatum and frontal cortex and the release of GABA in thalamus and frontal cortex [6, 7]. Other brain regions have not been studied. These effects are most probably mediated by the GHB receptors localized in these brain regions, because most of the neurophysiological and neuropharmacological effects of administered GHB are reduced or blocked by the GHB receptor antagonist NCS-382 (6,7,8,9-tetrahydro-5-*[H]*-benzocycloheptene-5-01-4-ylidene acetic acid) [8]. When administered peripherally, GHB freely penetrates the brain and interferes with the principal elements of the GHB endogenous system. These interferences, particularly at the level of GHB receptors, result in neuropharmacological effects which are used in therapeutics for sleep regulation in narcoleptic patients [9], for the reduction of withdrawal symptoms in alcohol or heroin addiction [10, 11], and for recreational and psychological benefits [12].

At the cellular level, some molecular events following GHB receptor stimulation have been described. *In vivo* and in brain slices, increases in cGMP and in inositol polyphosphate levels have been observed under the influence of pharmacological doses of GHB [13]. GHB administration generally induces neuronal cell membrane hyperpolarizations [14]. Recently, the stimulation of GHB receptors expressed in differentiated NCB-20 cells appeared to be linked to a reduction in Ca^{2+} channel activities [5]. This effect was blocked by NCS-382 but not by the $GABA_B$ antagonist CGP-55845. Taking into account the GHB effect on Ca^{2+} movements and on cGMP levels, we explored a possible modification of NOS activity induced by micromolar concentrations of GHB in small punches of rat frontal cortex incubated under physiological conditions.

* Corresponding author: Michel Maitre, M.D., Ph.D., Institut Chimie Biologique and ER 2072 CNRS, Faculté de Médecine, 11 Rue Humann, 67085 Strasbourg, France. Tel. 03 88 24 34 93; FAX 03 88 24 35 01; E-mail: maitre@neurochem.u-strasbg.fr

† *Abbreviations:* NOS, nitric oxide synthase; GHB, gamma-hydroxybutyrate; GABA, gamma-aminobutyrate; and cGMP, cyclic guanosine monophosphate.

Received 30 November 1998; accepted 18 June 1999.

MATERIALS AND METHODS

Male Wistar rats (300–350 g; Centre de Neurochimie, Strasbourg, France) were rapidly decapitated after stunning and their brains removed and dissected on a cold glass plate. Coronal sections of 750 μm were cut from the frontal cortex (3.7 mm anterior and 5.2 mm posterior to bregma) according to Paxinos and Watson [15]. Punches were taken from the two slices obtained with the aid of a stainless steel hollow needle with an internal diameter of 1 mm. Punches were immediately transferred into 50 mL of Krebs–Henseleit solution of the following composition: (mM) NaCl 120, KCl 2.0, $CaCl_2$ 2.0, $NaHCO_3$ 26, $MgSO_4$ 1.19, KH_2PO_4 1.18, and glucose 11, which had previously been saturated with 95-5% O_2/CO_2. The solution containing the punches was bubbled with 95-5% O_2/CO_2 at 37° for 60 min. Next, a determined number of punches were transferred into basket-shaped sieves. The baskets were then placed in plastic tubes containing 1 mL Krebs–Henseleit with 20 μM L-arginine, 0.1 μCi U-[^{14}C] L-arginine (11.0 GBq/mmol; 296 mCi/mmol), 1 mM L-citrulline, and 10 μM tetrahydrobiopterin with or without the addition of the various test compounds (veratrine, N^{ω}-nitro-L-arginine, gamma-hydroxybutyrate). The tubes were briefly gassed with 95-5% O_2/CO_2 sealed, and then incubated at 37° for time intervals varying from 20–90 min and as a function of protein (1–4 punches per sieve = 60–240 μg protein). An incubation time of 60 min was usually chosen because of the low amount of citrulline produced. At the end of the incubation period, the sieves were removed and carefully drained into the tubes. Bathing liquid from each tube (0.8 mL) was applied to a 1-mL column of BioRad AG 50W-X8, 200–400 mesh, Na^+ form, and the eluates collected directly into scintillation vials together with two 0.8-mL H_2O rinses. Scintillation fluid (15 mL) was added to each vial, which was then vortexed. After 15 hr, the vials were counted for 10 min to measure the [^{14}C] L-citrulline formed during the course of the NOS reaction.

All measurements were performed by reference to the background formation of [^{14}C] L-citrulline in presence of 1 mM N^{ω}-nitro-L-arginine in the incubation medium. N^{ω}-nitro-L-arginine has been reported to be a strong inhibitor of the calcium/calmodulin-dependent constitutive NOS from brain [16]. Results are percentages of the veratrine-induced stimulation of NOS in the same tissue (taken as 100% reference). Statistical analyses of data (N = 9) were assessed by a one-way Anova followed by a Newmann–Keuls test for dose/effect experiments. Differences between two sets of values were evaluated by the Student's *t*-test.

RESULTS

Determination of NOS Activity as a Function of Time and Amount of Protein

Veratrine induces brain tissue depolarizations and nitric oxide synthesis through multiple mechanisms, including activation of *N*-methyl-D-asparate receptors, voltage-sensitive Ca^{2+} channels, and Na^+/Ca^{2+} exchange channels [17]. Under our experimental conditions, the synthesis of nitric oxide induced by 20 μg/mL veratrine was linear for at least 60 min in the presence of two punches of frontal cortex (121 μg protein per tube, Fig. 1). The kinetics were also linear for up to 3 punches in the same tube (i.e. 183 μg protein) incubated for 60 min (Fig. 2). These preliminary studies were used to define the experimental conditions to test the effect of GHB and synthetic analogues on NOS activity.

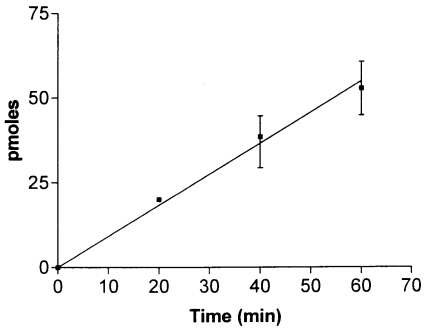

FIG. 1. NOS activity as a function of incubation time. Each sample was incubated in the presence of two punches of the frontal cortex (121 μg protein per tube). Results are expressed in pmol [^{14}C] citrulline formed in the presence of 20 μg/mL veratrine. Backgrounds obtained with 1 mM N^{ω}-nitro-L-arginine were subtracted from all results. Data are given as means ± SEM (N = 9 independent experiments).

GHB Dose–Activity Relationship and NOS Activity

NOS activity was measured in the presence of various concentrations of GHB (2.5 to 100 μM) co-incubated with two punches (121 μg of protein) for 60 min. Results were calculated as percentages of the NOS activity induced by 20 μg/mL veratrine (100% activity = 6 ± 1.2 pmol/min/mg protein) (Fig. 3). Basal levels of [^{14}C] citrulline formation in the presence of 1 mM N^{ω}-nitro-L-arginine were considered as background and always subtracted from the different

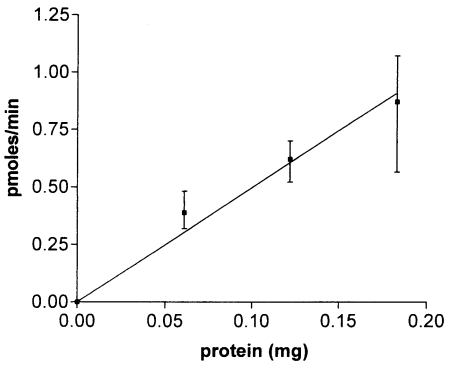

FIG. 2. NOS activity as a function of the amount of tissue. Samples were incubated for 60 min in the presence of either one, two, or three punches (about 60 μg protein per punch). Results (means ± SEM; N = 9) are in pmol [^{14}C] citrulline formed, backgrounds obtained in the presence of 1 mM N^{ω}-nitro-L-arginine always being subtracted.

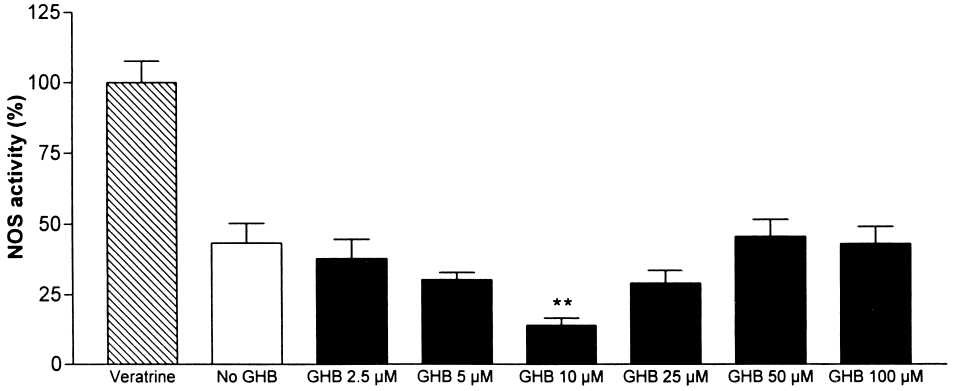

FIG. 3. Effect of GHB on NOS activity. Two punches were incubated for 60 min in the presence of increasing concentrations of GHB (2.5 to 100 µM). Results (means ± SEM, N = 9 independent experiments) are percentages of the control activity obtained with 20 µg/mL veratrine (6 ± 1.2 pmol/min/mg protein). ** = $P < 0.001$ compared to NOS activity measured in the absence of GHB added in the incubation medium.

results. Under these conditions, increasing amounts of GHB from zero to 10 µM induced a progressive reduction in NOS activity (43.2 ± 7% to 13.9 ± 2%). However, when the concentration of GHB in the medium was above 10 µM, a progressive re-increase of NOS activity was seen (28 ± 4% at 25 µM to 45 ± 6% and 43 ± 6% at 50 and 100 µM GHB, respectively). These last two concentrations of GHB induced a NOS activity which was not different from those existing in the punches in the absence of GHB in the incubation medium. Thus, GHB exerts a biphasic effect on NOS activity. Below 10 µM, a reduction was seen. Increasing amounts of GHB above 10 µM induced the progressive disappearance of the GHB effect.

Effects of Synthetic Analogues of GHB

The effects of the GHB receptor agonist NCS-356 (γ-*p*-chlorophenyl-*trans*-hydroxycrotonate) [18] and antagonist NCS-382 [8] were tested under the same conditions in order to characterize the nature of the GHB-induced response pharmacologically. NCS-382 (200 µM) had no effect by itself on NOS activity, but it completely inhibited the decrease in NOS activity induced by a low concentration of GHB (10 µM; 13.9 ± 2.6% in the absence of NCS-382 and 23.9 ± 2.5% in the presence of the antagonist) and also blocked the activating effect of 50 µM GHB (45.5 ± 6.1% in the absence of NCS-382 and 9.5 ± 3.4% in the presence of the antagonist). NCS-356 (50 µM) stimulated NOS, similarly to GHB at the same dosage, and this stimulation was also inhibited by the antagonist NCS-382 (35.3 ± 3.8% to 19.3 ± 3.8%; Fig. 4). Thus, this substance antagonized the GHB-induced inhibition of neuronal NOS observed at low doses of GHB (10 µM), demonstrating that this effect is a specific receptor-mediated effect. At a high dose of GHB (50 µM), it could be suggested that NCS-382 stabilizes the GHB receptor and diminishes its desensitization or, alternatively, that it antagonizes the other class of receptor stimulated only by high doses of GHB.

Additive Effect of Veratrine and GHB on the Stimulation of NOS Activity

When GHB (50 µM) was co-incubated with veratrine (20 µg/mL), a supplementary stimulation of NOS activity was observed compared to those seen with veratrine alone. This additive effect (+27 ± 9%) indicates that GHB modulated the permeability of a calcium channel which was not opened by the action of veratrine. In the presence of the

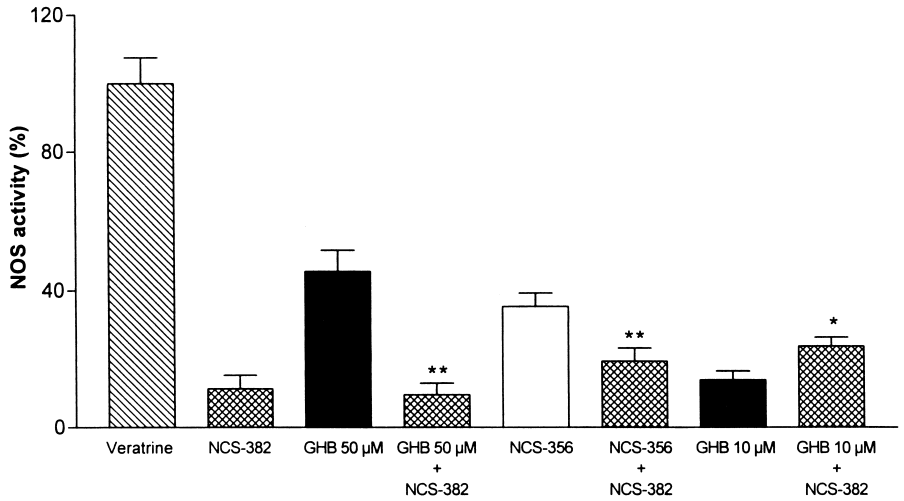

FIG. 4. Effect of synthetic ligands of GHB receptor(s) on NOS activity. Two punches were incubated for 60 min in the presence of: the antagonist NCS-382 (200 µM) alone; the antagonist NCS-382 (200 µM) + GHB (50 µM or 10 µM); the agonist NCS-356 (50 µM) alone; the antagonist NCS-382 (200 µM) + the agonist NCS-356 (50 µM). Results (means ± SEM of 9 independent experiments) are percentages of NOS activity induced by veratrine 20 µg/mL (control activity). * = $P < 0.05$; ** = $P < 0.01$ compared to NOS activity induced by GHB or NCS-356 alone.

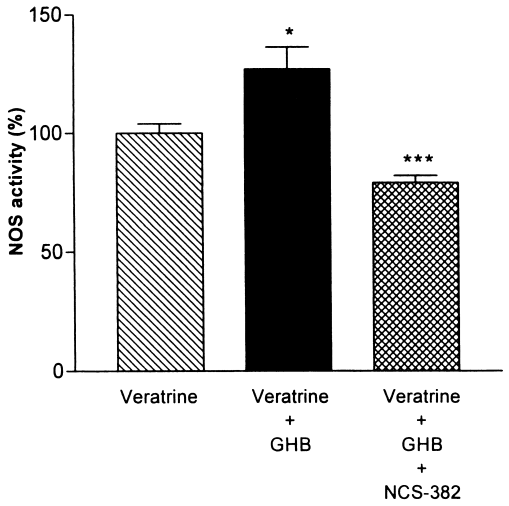

FIG. 5. Effect of GHB on veratrine-induced NOS activity. Two punches were incubated for 60 min in the presence of veratrine alone (20 μg/mL) or of veratrine (20 μg/mL) + GHB 50 μM or of veratrine (20 μg/mL) + GHB (50 μM) + the GHB receptor antagonist NCS-382 (200 μM). Results (means ± SEM of 9 independent experiments) are percentages of control activity (veratrine alone = 100%). * = $P < 0.05$ compared to veratrine alone; *** = $P < 0.0001$ compared to veratrine + GHB.

antagonist NCS-382, the supplementary effect due to GHB disappeared (Fig. 5).

DISCUSSION

GHB is heterogeneously distributed in the rat brain and its maximal concentration is approx. 5–10 μM [19]; however, GHB concentrations increase in the ischemic brain and it is difficult to evaluate the actual GHB levels in the punches of frontal cortex used in this study. Nevertheless, 50–100 μM GHB concentrations are reached in the brain of the animal only after peripheral administration of GHB [20]. Thus, under physiological concentrations of GHB, the present paper describes an inhibition of constitutive NOS by increasing amounts of GHB (up to 10 μM). Neuronal NOS is a Ca^{2+}/calmodulin-dependent enzyme regulated by the steep gradient of Ca^{2+} that occurs in the vicinity of open Ca^{2+} channels [21]. Although constitutive brain NOS is mainly linked to Ca^{2+} influx through the *N*-methyl-D-asparate receptor, it is possible that the GHB receptor also plays a role in this domain. The present study was carried out in order to use neuronal NOS as an indicator of Ca^{2+} movements in the tissue. Interestingly, we confirm the GHB-induced decrease in neuronal Ca^{2+} influx generated by GHB receptor stimulation that has been demonstrated on NCB-20 cells by patch-clamp experiments [5]. These cells are a hybrid between mouse neuroblastoma N18TG2 and Chinese hamster embryonic day-18 brain cells which express many properties characteristic of neurons. When differentiated by dibutyryl cyclic AMP, these cells develop synaptic contacts, and a K^+-evoked, Ca^{2+}-dependent release of GHB takes place. NCB-20 cells also express a homogenous population of high affinity binding sites for [^{3}H] GHB which mediate the GHB-induced modifications in Ca^{2+} conductances, given that this effect was blocked by NCS-382 but not by the $GABA_B$ antagonist CGP 55845. Other electrophysiological results obtained on brain neurons *in vivo* generally support a role for GHB in inducing neuronal cell membrane hyperpolarization [14].

Peripheral administration of GHB in rats and human raises the brain GHB concentration markedly above physiological concentrations. Chronic administration of GHB in rats has been shown to desensitize and down-regulate GHB receptors [22]. Under our experimental conditions (1-hr incubation), concentrations of GHB above 10 μM (25, 50, and 100 μM) induced a progressive re-increase of NOS activity in the punches of frontal cortex. This is probably due to a progressive loss of GHB-induced inhibition of Ca^{2+} influx due to a progressive desensitization of GHB receptors exposed to high GHB concentrations, and NOS activity returns progressively to control values. However, as suggested by the kinetic parameters of GHB binding on rat brain membranes, two populations of GHB receptors exist, one of high affinity (K_{d_1} estimated in the range 30–580 nM) and the other of lower affinity (K_{d_2} in the range of 2 to 16 μM) [2]. High concentrations of GHB in the incubation medium could induce a gradient concentration of GHB in the tissue high enough to stimulate both classes of sites. Under these conditions, the global effect of GHB could be a stimulation of Ca^{2+} influx into the tissue with a stimulation of NOS. Previous results have reported an increase in calcium ion utilization in the substantia nigra, rich in GHB receptors, after local application of GHB at doses above 100 μM [23]. Lower doses have not been tested. In relation to this increase in calcium ion influx and the stimulation of NOS activity at high doses of GHB, an increase in cGMP concentration has been reported in the rat hippocampus *in vivo* following the administration of 400 mg/kg GHB [24]. A possible decrease in cGMP concentrations at low doses of GHB has not been tested and is worthy of investigation.

Thus, several lines of evidence support the notion of a reduction of Ca^{2+} influx in brain tissue after stimulation of GHB receptor, leading to a reduction in NOS activity. This effect could be blocked by the selective GHB receptor antagonist NCS-382. Overstimulation of GHB receptor by high amounts of GHB for 60 min induces the apparent disappearance of this effect. NCS-382 also reduces this second phase either by preventing the desensitization of GHB receptors or by inhibiting the stimulation of Ca^{2+} entry mediated by another class of GHB receptor of lower affinity. The hypothesis that a high dose of GHB could stimulate other kinds of receptors (in particular $GABA_B$ receptors) [25] is unlikely, because the specific synthetic agonist of GHB receptors (NCS-356) reproduces the GHB effect.

It can be concluded that GHB receptor stimulation induces a biphasic effect on calcium ion movements. This could be related to the biphasic effect of GHB on *in vivo* release of dopamine in striatum: low doses induce a decrease

in dopaminergic firing and a reduction of dopamine release, while high doses provoke an extracellular increase of dopamine [26]. As the biphasic modulation of NOS activity via GHB receptor seems unlikely via other mechanisms (e.g. biphasic modulation of NOS phosphorylation) [27], it is proposed that the physiological GHB concentration reduces Ca^{2+} influx and NOS activity. By contrast, the overload of the GHB system by peripheral administration leads to the disappearance of the tonic inhibitory influence of GHB on Ca^{2+} entry, which is followed by an increase in dopamine synthesis and release.

References

1. Rumigny JF, Cash C, Mandel P, Vincendon G and Maitre M, Evidence that a specific succinic semialdehyde reductase is responsible for γ-hydroxybutyrate synthesis in brain tissue slices. *FEBS Lett* **134:** 96–98, 1981.
2. Maitre M, The γ-hydroxybutyrate signalling system in brain: Organization and functional implications. *Prog Neurobiol* **51:** 337–361, 1997.
3. Ratomponirina C, Hodé Y, Hechler V and Maitre M, γ-Hydroxybutyrate receptor binding in rat brain is inhibited by guanyl nucleotides and pertussis toxin. *Neurosci Lett* **189:** 51–53, 1995.
4. Hechler V, Gobaille S and Maitre M, Selective distribution pattern of γ-hydroxybutyrate receptors in the rat forebrain and midbrain as revealed by quantitative autoradiography. *Brain Res* **572:** 345–348, 1992.
5. Kemmel V, Taleb O, Perard A, Andriamampandry C, Siffert JC, Mark J and Maitre M, Neurochemical and electrophysiological evidence for the existence of a functional γ-hydroxybutyrate system in NCB-20 neurons. *Neuroscience* **86:** 989–1000, 1998.
6. Roth RH, Doherty JD and Walters JR. Gamma-hydroxybutyrate: A role in the regulation of central dopaminergic neurons? *Brain Res* **189:** 556–560, 1980.
7. Banerjee PK and Snead OC, Presynaptic gamma-hydroxybutyric acid (GHB) and gamma-aminobutyric $acid_B$ ($GABA_B$) receptor-mediated release of GABA and glutamate (GLU) in rat thalamic ventrobasal nucleus (VB): A possible mechanism for the generation of absence-like seizures induced by GHB. *J Pharmacol Exper Ther* **273:** 1534–1543, 1995.
8. Maitre M, Hechler V, Vayer P, Gobaille S, Cash CD, Schmitt M and Bourguignon JJ, A specific gamma-hydroxybutyrate receptor ligand possesses both antagonistic and anticonvulsant properties. *J Pharmacol Exp Ther* **255:** 657–663, 1990.
9. Mamelak M, Scharf MB and Woods M, Treatment of narcolepsy with gamma-hydroxybutyrate. A review of clinical and sleep laboratory findings. *Sleep* **9:** 285–289, 1986.
10. Gallimberti L, Cibin M, Pagnin P, Sabbion R, Pani PP, Pirastu R, Ferrara SD and Gessa GL, Gamma-hydroxybutyric acid for treatment of opiate withdrawal syndrome. *Neuropsychopharmacology* **9:** 77–81, 1993.
11. Biggio G, Cibin M, Diana M, Fadda F, Ferrara SD, Gallimberti L, Gessa GL, Mereu GP, Rossetti ZL and Serra M, Suppression of voluntary alcohol intake in rats and alcoholics by gamma-hydroxybutyric acid: A non-GABAergic mechanism. *Adv Biochem Psychopharmacol* **47:** 281–288, 1992.
12. Chin MY, Kreutzer RA and Dyer JE, Acute poisoning from gamma-hydroxybutyrate in California. *West J Med* **156:** 380–384, 1992.
13. Vayer Ph and Maitre M, γ-Hydroxybutyrate stimulation of the formation of cyclic GMP and inositol phosphates in rat hippocampal slices. *J Neurochem* **52:** 1382–1387, 1989.
14. Olpe HR and Koella WP, Inhibition of nigral and neocortical cells by γ-hydroxybutyrate: A microiontophoretic investigation. *Eur J Pharmacol* **53:** 359–364, 1979.
15. Paxinos G and Watson C, *The Rat Brain in Stereotaxic Coordinates*. Academic Press, New York, 1986.
16. Furfine ES, Harmon MF, Paith JE and Garvey EP, Selective inhibition of constitutive nitric oxide synthase by L-N^G-nitroarginine. *Biochemistry* **32:** 8512–8517, 1993.
17. Lizasoain I, Knowles RG and Moncada S, Inhibition by lamotrigine of the generation of nitric oxide in rat forebrain slices. *J Neurochem* **64:** 636–642, 1995.
18. Hechler V, Peter P, Gobaille S, Bourguignon JJ, Schmitt M, Ehrhardt JD, Mark J and Maitre M, γ-Hydroxybutyrate ligands possess antidopaminergic and neuroleptic-like activities. *J Pharmacol Exp Ther* **264:** 1406–1414, 1993.
19. Vayer P, Ehrhardt JD, Gobaille S, Mandel P and Maitre M, Gamma-hydroxybutyrate distribution and turnover rates in discrete brain regions of the rat. *Neurochem Int* **12:** 53–59, 1988.
20. Shumate JS and Snead OC, Plasma and central nervous system kinetics of gamma-hydroxybutyrate. *Res Commun Chem Pathol Pharmacol* **25:** 241–256, 1979.
21. Brenman JE and Bredt DS, Synaptic signaling by nitric oxide. *Curr Opin Neurobiol* **7:** 374–378, 1997.
22. Ratomponirina C, Gobaille S, Hodé Y, Kemmel V and Maitre M, Sulpiride, but not haloperidol, up-regulates γ-hydroxybutyrate receptors *in vivo* and in cultured cells. *Eur J Pharmacol* **346:** 331–337, 1998.
23. Harris NC, Webb C and Greenfield SA, The effects of gamma-hydroxybutyrate on the membrane properties of guinea-pig pars compacta neurons in the substantia nigra *in vitro*. *Neuroscience* **31:** 363–370, 1989.
24. Vayer P, Gobaille S, Mandel P and Maitre M, 3′–5′ Cyclic guanosine monophosphate increase in rat brain hippocampus after gamma-hydroxybutyrate administration. Prevention by valproate and naloxone. *Life Sci* **41:** 605–610, 1987.
25. Xie X and Smart TG, γ-Hydroxybutyrate hyperpolarizes hippocampal neurones by activating $GABA_B$ receptors. *Eur J Pharmacol* **212:** 291–294, 1992.
26. Hechler V, Gobaille S, Bourguignon JJ and Maitre M, Extracellular events induced by gamma-hydroxybutyrate in striatum: A microdialysis study. *J Neurochem* **56:** 938–944, 1991.
27. Riccio A, Esposito E and Eboli ML, Modulation by protein kinase C of nitric oxide and cyclic GMP formation in cultured cerebellar granule cells. *Brain Res* **718:** 159–164, 1996.