International Journal of Neuropsychopharmacology (2009), **12**, 1165–1177. Copyright © CINP 2009
doi:10.1017/S1461145709000157

ARTICLE

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Neurotoxic effects induced by gammahydroxybutyric acid (GHB) in male rats

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Abstract

Gammahydroxybutyric acid (GHB) is an endogenous constituent of the central nervous system that has acquired great social relevance for its use as a recreational 'club drug'. GHB, popularly known as 'liquid ecstasy', is addictive when used continuously. Although the symptoms associated with acute intoxication are well known, the effects of prolonged use remain uncertain. We examined in male rats the effect of repeated administration of GHB (10 and 100 mg/kg) on various parameters: neurological damage, working memory and spatial memory, using neurological tests, the Morris water maze and the hole-board test. The results showed that repeated administration of GHB, especially at doses of 10 mg/kg, causes neurological damage, affecting the 'grasping' reflex, as well as alteration in spatial and working memories. Stereological quantification showed that this drug produces a drastic neuronal loss in the CA1 hippocampal region and in the prefrontal cortex, two areas clearly involved in cognitive and neurological functions. No effects were noted after quantification in the periaqueductal grey matter (PAG), a region lacking GHB receptors. Moreover, NCS-382, a putative antagonist of GHB receptor, prevented both neurological damage and working- memory impairment induced by GHB. This suggests that the effects of administration of this compound may be mediated, at least partly, by specific receptors in the nervous system. The results show for the first time that the repeated administration of GHB, especially at very low doses, produces neurotoxic effects. This is very relevant because its abuse, especially by young persons, could produce considerable neurological alterations after prolonged abuse.

Received 7 January 2009; Reviewed 15 January 2009; Revised 18 January 2009; Accepted 20 January 2009; First published online 17 March 2009

Key words: Gammahydroxybutyric acid, neurological damage, working memory, spatial memory, neurotoxicity.

Introduction

Gammahydroxybutyric acid (GHB) is an endogenous constituent of the CNS, where it is believed to act as a neurotransmitter or neuromodulator (Gould *et al*. 2003; Hechler *et al*. 1992, Maitre, 1997). When administered exogenously, GHB is able to cross the blood–brain barrier and produce numerous neuropharmacological effects. GHB has recently received social attention since it has emerged as a major recreational drug, with an increased use, mainly in young people (Degenhardt *et al*. 2005; Sumnall *et al*. 2008). GHB (popularly known as 'G' or 'liquid ecstasy') belongs to the class of substances referred to as 'club drugs' (Hopfer *et al*. 2006; Sicar & Basak, 2004).

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Little is known about the neurophysiological role of GHB in the brain. A large body of evidence suggests that it serves as a relatively specific endogenous regulator of dopaminergic neurons, controlling the release of this neurotransmitter (Cash, 1994; Godbout *et al*. 1995; Howard & Feigenbaum, 1997; Maitre, 1997; Schmidt-Mutter *et al*. 1999). The control of dopamine release could be mediated mainly by activation of GHB receptors in the brain (Bracucci *et al*. 2004; Hechler *et al*. 1992), producing a different action depending on the dose. To date, two different GHB binding sites have been characterized with different affinities: high-affinity ($K_d = 30–580$ nM and $B_{max} = 0.5–1.8$), and low-affinity ($K_d = 2.3–16$ μM and $B_{max} = 11–46$) (Bracucci *et al*. 2004; Hechler *et al*. 1992). Thus, the effects induced by a high dose of GHB are strikingly similar to those produced by drugs inhibiting dopamine release (Navarro & Pedraza, 1996; Navarro *et al*. 1998, 2007; Pedraza *et al*. 2007; Sevack *et al*. 2004). Moreover, we can not rule out the involvement of the $GABA_B$

receptor in this action of GHB on dopamine neurotransmission (Pistis *et al*. 2005). However, GHB has an extremely weak affinity for $GABA_B$ receptors since doses of >200 mg/kg GHB are required to produce CNS effects through the $GABA_B$ receptors (Carter *et al*. 2003, 2006; Crunelli *et al*. 2006). On the contrary, relatively low doses of this compound (5–10 mg/kg) exert an excitatory effect on dopamine release probably involving the high-affinity GHB receptor (Diana *et al*. 1991; Godbout *et al*. 1995; Tremblay *et al*. 1998). It has recently been reported that a single low-dose of GHB induces oxidative stress in the cortex of young rats (Sgaravatti *et al*. 2007). Although acute toxic effects of GHB overdose have been widely described (Raess & Tunnicliff, 2002), no studies have yet examined the possible neurotoxic effects of repeated GHB administration, and the neurobehavioural effects of prolonged GHB exposure thus remain unknown.

Considering these data, the main aim of this study was to examine the existence of a possible neurotoxic effect of GHB administration. Accordingly, we first assessed the effects of repeated administration of two doses of GHB in neurological tests and two spatial memory tasks (hole-board and Morris water maze). Subsequently, the number of neurons in CA1 hippocampal and prefrontal cortex (PFC) regions was estimated, using stereological methods. Both structures are clearly involved in spatial memory (Brasted *et al*. 2003; Yan *et al*. 2007) and have an elevated density of high-affinity GHB receptors (Andriamampandry *et al*. 2003; Kemmel *et al*. 2006; Maitre, 1997).

Materials and methods

Subjects

Adult male Wistar rats (n = 16–35) weighing 275 ± 25 g were used for each behavioural experiment. All rats had free access to food and water. Rats were housed in pairs in a temperature-controlled colony (20 ± 2 °C) on a constant 12-h light/dark cycle (lights on 20:00 hours). All behavioural experiments were made during the dark phase of the light/dark cycle. The experiments were carried out in accordance with the guiding principles for care and use of laboratory animals approved by the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drug treatment and experimental procedure

GHB (Sigma Laboratories, Spain) was diluted in physiological saline immediately before treatment to provide appropriate doses for injection. Rats were randomly assigned to one of three groups: saline (n = 7), 10 mg/kg GHB (n = 7), or 100 mg/kg GHB (n = 6). All treatments were administered once a day for 15 d by an intraperitoneal injection in a volume of 1 ml/kg (see Fig. 1). The GHB doses were chosen on the basis of previous behavioural experiments performed in our laboratory (Navarro *et al*. 2007; Pedraza *et al*. 2007). NCS-382 (10 mg/kg; diluted in 10% DMSO + 90% saline; n = 7), a putative antagonist of GHB receptor (Castelli *et al*. 2004) or vehicle group (DMSO 10% + saline, n = 8) were used in an experiment that attempted to reverse the neurological and behavioural (hole-board test) effects of GHB (10 mg/kg). The dose of NCS-382 (10 mg/kg) was chosen on the basis of previous studies with this compound (Carter *et al*. 2003; Martellotta *et al*. 1998). The interval between injections of NCS-382 and GHB was 15 min. The time of injection was 2 h after the beginning of the dark phase of the light/dark cycle. Behavioural tests were carried out 2 h following GHB injections.

Neurological test

On the first day of treatment, rats were submitted to a neurological test to exclude a possible acute effect of GHB administration on these behaviours. The neurological examination was performed again on the last day of treatment to determine the effects of repeated administration of GHB on these neurological measures.

To test sensory-motor orientation, coordinated limb and neurological functions, the rats were subjected to the battery of tests of Marshall & Teitelbaum (1974), modified by Björklund *et al*. (1980) and extended for some reflexes according to Bures *et al*. (1983). The deficit in each orientation, limb use and neurological test was rated on a three-point scale (0, absent; 1, weak; 2, strong). Performance of animals in some tests has only been categorized as 0 or 2 due to the difficulty for providing intermediate values. Scoring was done blind to the treatment condition by two trained experimenters. (The test and criteria for assessment of the animals that we used are described in the Supplementary information, available online.) The sensorial reflexes were assessed by the following tests: somesthesis, whisker touch, snout probe, olfaction, corneal reflex, auditory startle and head shaking.

Limb reflexes and limb coordination were assessed in the following tests: surface righting reflexes, forelimb suspension, grasping test, equilibrium tests, placing reactions. By the application of this battery of tests it was possible to examine if repeated GHB administration in rats affected a particular brain region,

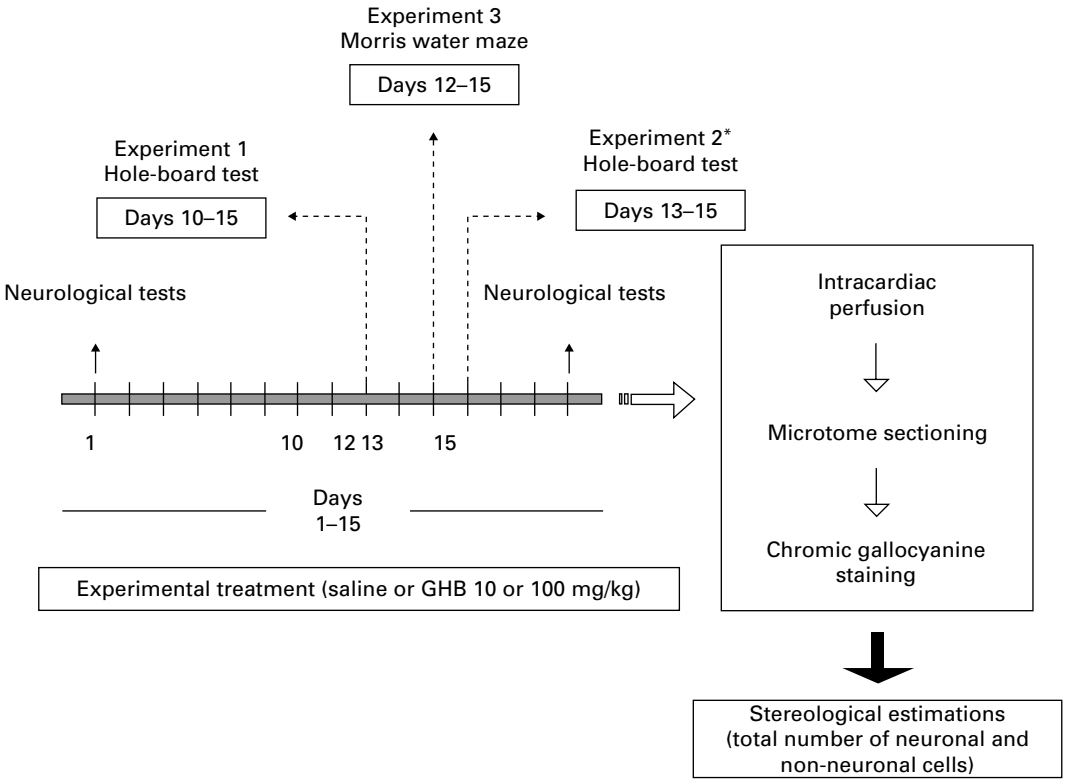

Fig. 1. Schematic representation of the experimental design used in our study representing the days over which the three experiments were developed. The animals were treated with saline or gammahydroxybutyric acid (GHB) (10 or 100 mg/kg) for 15 d. Rats were submitted to a neurological test on the first and last days of treatment. The animals were trained in different tasks. Experiment 1: the animals were trained in the hole-board test on days 10–15 ($n=20$). Experiment 2: the animals were trained in the hole-board test on days 13–15 using a variable inter-trial interval ($n=20$). * NCS-382 (10 mg/kg; diluted in 10% DMSO + 90% saline; $n=7$), a putative antagonist of GHB receptor, was also used in an experiment to attempt to reverse the neurological and behavioural (hole-board test) effects of GHB (10 mg/kg). An additional group was treated with vehicle (DMSO 10%, $n=8$). Experiment 3: in the Morris water maze test, the animals were submitted to the spatial working memory task on days 12–15 ($n=20$). After the completion of the behavioural experiments (day 15), the rats were deeply anaesthetized and perfused transcardially. The brains were removed and processed. Neural and glial cell populations were estimated unilaterally in CA1 and PFC regions using stereological methods in animals evaluated in the first hole-board test.

interfered with a specific function or affected the CNS as a whole (Bures *et al*. 1983).

Behavioural test

Hole-board test

Apparatus. A hole-board maze was used for two behavioural experiments. The maze was surrounded by few auditory and visual cues. The maze is composed of a square arena ($70 \times 70 \times 45$ cm) made of PVC, containing four rows of four equidistant holes (14 cm apart, 3.5 cm diameter, depth 3 cm) in the floor plate (Oades, 1981). Rewards (pieces of biscuit) were placed in these holes. After each trial the box was cleaned with a solution containing neutral soap.

Behavioural procedure. For hole-board testing, each rat was initially deprived of food to 80% of its free-feeding weight and allowed access to water *ad libitum*. During the restriction process, pieces of biscuit were mixed in with the food to accustom animals to the reward that would later be used in the behavioural study.

The test was conducted on six consecutive days with four trials per day. Prior to training, the rats were familiarized with the hole-board maze. During this habituation all holes were baited with an accessible piece of biscuit. After habituation, the animals were subsequently exposed to four daily training trials in two sessions, two trials in the morning and two in the afternoon. The inter-session interval was 3 h, and the

inter-trial interval was 5 min. In the training trials a fixed set of four holes arranged in a symmetrical pattern was baited. This pattern remained constant throughout the experiment. The rats were introduced manually into the maze from one of the four different starting locations (the exit positions differed randomly between trials). A hole visit was scored when the rat introduced its nose in a hole. When the rat obtained all the rewards from the baited holes or after 3 min had elapsed, the rat was manually removed from the maze and returned to its home cage. The latency to obtain the reward and the number of errors were recorded. Likewise, the reference memory and working memory (WM) scores were calculated. The reference memory ratio was defined as the number of visits or revisits to the baited holes divided by the total number of visits and revisits to baited and non-baited holes. WM was expressed as the ratio of the number of food-rewarded visits to the number of visits and revisits to the baited holes (Douma *et al*. 1998).

Considering that animals reach a plateau of performance on the third training day, other groups of rats, treated with saline, 10 or 100 mg/kg GHB, were submitted to 3 d training in the hole-board following the same behavioural procedure described above. However, in this second experiment a variable inter-trial interval was used. The inter-trial interval was 30 s or 5 min that was balanced and randomly allocated over training days.

To determine the effect of this variable inter-trial interval, errors were scored as revisits to holes previously visited within a trial. These errors were divided into revisits to baited holes and visits and revisits to non-baited holes. The number of errors made on the third day of training was recorded and used for data analysis. Similarly, the effects of NCS-382 (10 mg/kg) + GHB (10 mg/kg) were examined for the possiblity of reversing the behavioural hole-board test effects of GHB (10 mg/kg).

Morris water maze

Apparatus

The apparatus consisted of a circular pool with the following dimensions: diameter 150 cm and walls 43 cm high. The pool was filled with water (24–26 °C) and was made opaque with non-toxic white paint. The goal platform (11 cm diameter) could be placed anywhere in the pool at a distance of 30 cm from the pool edge. The platform was submerged to a depth of 2 cm beneath the surface of the water. The pool was divided into four imaginary quadrants (A, B, C, D) and the platform was placed in the centre of the quadrant. The pool was placed in an experiment room furnished with several extra-maze cues and remained immobile in the room throughout the entire experimental period. An automatic video system (Smart, Panlab, Spain) was used to record the animals' movements in the pool.

Behavioural procedures

The day before the learning phase, the animals were released into the circular pool without the goal platform for a 60-s period of free exploration. From the following day onwards, the animals were submitted to the spatial WM task. Over 4 d the animals were submitted to three trials, one acquisition and two retention trials, per day. In the acquisition trial, the animal had to find a submerged platform in order to escape form the water. If the animal did not find the platform in 60 s, the experimenter placed the animal on the platform, where it remained for 15 s before being returned to its cage. Later, the animal was again introduced into the circular pool for the retention trial. The goal quadrant, i.e. the quadrant containing the escape platform, remained constant on the same day for the acquisition and retention trials but varied pseudorandomly over the 4 d of the experiment.

Qualitative analysis of search strategies

The swim path for each trial was plotted using the Smart system (Panlab). A single investigator blinded to the animal treatment assigned a predominant search strategy to each trial using a categorization scheme similar to that described by Brody & Holtzman (2006) and Janus (2004). The use of each search strategy was categorized as a percent of incidences during each trial over the whole experimental period (the two retention trials). Strategies were categorized according to the following criteria: 'spatial strategies', 'systematic but non-spatial strategies' and 'strategies involving repetitive looping paths' (see Brody & Holtzman, 2006; Janus, 2004; and Supplementary information).

Histology

After the behavioural experiments, the rats were deeply anaesthetized (4 ml/kg equitexin) and perfused transcardially with 0.1 M PBS (pH 7.4) and later with 10% formalin in 0.1 M PBS (pH 7.4). The brains were removed and stored in the same fixative solution for 2 wk. The brains were then dehydrated and embedded in paraffin. Coronal sections (20 μm) were obtained with a rotatory microtome (Nahita, Spain), and stained with chromic gallocyanine to identify the

neuronal and glial cells. Each section was numbered according to the rostrocaudal level determined according to the rat brain atlas of Paxinos & Watson (1998).

Neural cell and glial cell populations (small cell and dark cytoplasm) were estimated unilaterally in the dorsal CA1 hippocampal region (CA1) (Paxinos & Watson, 1998), and the PFC (corresponding to Cg1 and prelimbic cortex of Paxinos & Watson, 1998), two regions where there is a high density of high-affinity GHB receptors, using stereological methods.

An Olympus BX51 microscope (Olympus, Denmark) was interfaced with a computer and a colour JVC digital video camera. Neuron cells and non-neuron cells were counted using the CAST-Grid software package (Olympus).

One side of the entire dorsal CA1 hippocampal or PFC regions was defined using a 4× objective. With a random start, the software created counting frames on the images generated by a 100× oil lens within the previously defined PFC and CA1 hippocampal regions. The distance between the sampled fields on each section (x, y steps) was 108.5 and 267 μm, respectively. The counting frame ([a]frame) was 1178 μm^2, giving the second sampling fraction ($f2 =$ x step length × y step length/[a]frame). The sampling volume (dissector) in the z-axis extended 10 μm deep (height of the dissector) after excluding 3 ± 5 μm from the top and bottom of the section. The location of neurons or glial cells within the height of the dissector and inside the frame area was used as the criteria for counting (Janson & Möller, 1993). The total thickness of the sections was also measured, giving the third sampling fraction [$f3 =$(section height)/(dissector height)]. After counting the objects (ΣQ^-) fulfilling the sampling criteria, the total number of neuron cells and non-neuron cells (N_{total}) in the PFC and CA1 hippocampal region was estimated: $N_{total} = \Sigma Q^- \times f1 \times f2 \times f3$. The coefficient of error for each estimation and animal ranged from 0.01 to 0.1. The coefficient of precision ranged from 0.01 to 0.04. The mean volume of neuron cells was estimated by means of the point-sampled intercept method, which is based on Cavalieri's principle (Gundersen *et al*. 1988).

The neuron cells were counted in the dorsomedial periaqueductal grey matter (PAG) (DLMPAG and DMPAG; Paxinos & Watson, 1998), where GHB binding sites are absent. Quantification was by systematically sampling in this region. At least two sections of each region were counted per animal. The number of counting frames used was 120–150 per animal in this region, representing 25% of the whole volume analysed. The resulting densities were averaged in order to obtain the mean (N_v) neuron cells per volume unit (mm^3).

Table 1. Percent values for each treatment of grasping reflex

	Day 1			Day 15		
	Absent	Weak	Strong	Absent	Weak	Strong
Sal	100	0	0	100	0	0
D1	100	0	0	14	38	48
D2	100	0	0	100	0	0
Vehicle	100	0	0	87.5	12.5	0
NCS + D1	100	0	0	100	0	0

Sal, Control group ($n = 18$; no animals showed grasping affectation); D1, GHB (10 mg/kg) ($n = 21$; 3 animals showed any alteration; 8 and 10 animals showed weak or strong deterioration in the grasping reflex, respectively); D2, GHB (100 mg/kg; no animals showed grasping affectation) ($n = 21$); Vehicle (Sal + DMSO 10%) ($n = 8$, 1 animal showed weak alteration); N + D1, NCS-382 (10 mg/kg) + GHB (10 mg/kg) ($n = 8$, no animals showed grasping affectation). The neurological deficit was rated as absent, weak or strong.

Data analysis

Neurological test

The data for sensory-motor tests were analysed using Kruskal–Wallis (and *post-hoc* Mann–Whitney *U* tests). Additionally, the deficits in the grasping test or equilibrium test were presented as a percent of incidences for each treatment [see Table 1 and Supplementary Table S1 (online), respectively].

Hole-board test

Spatial reference memory was analysed using three different analyses for the spatial memory ratio, mean latencies to obtain the reward and errors. The results of this task were analysed using an ANOVA for repeated measures (with three levels: the three training days) and three groups (previously described). When necessary, simple effects and *post-hoc* comparison (HSD) were calculated. The same data analysis was made to analyse the effect of NCS-382 (10 mg/kg) + GHB (10 mg/kg).

Spatial WM

The statistical analysis of the spatial WM ratio was performed in the same way as described above. Data concerning the number of errors were analysed using the Mann–Whitney *U* test to determine the possible effect of different time-intervals between sessions.

Morris water maze

The distances swum by the animals and escape latencies were considered as the dependent variables. Results were analysed by ANOVA for repeated measures. When necessary, simple effects and *post-hoc* comparison (HSD) were calculated. The Kruskal–Wallis test has been utilized to assess the variance of the search strategies over different groups. Subsequently, appropriate paired comparisons were carried out using the Mann–Whitney *U* test. Additionally, the use of direct spatial strategy was presented as a percent of incidences for each treatment (in day 4 and two retention trials).

Histological studies

A one-way ANOVA was applied to the parametric data using Tukey's *post-hoc* test for statistical significance between groups.

Results

Neurological tests

In the neurological examination conducted during the first day of treatment, GHB did not affect neurological reflexes. However, Kruskal–Wallis test showed significant differences over treatment groups ($p < 0.001$) in grasping reflex. Paired comparisons using Mann–Whitney *U* tests revealed that GHB treatment for 15 d (10 mg/kg) resulted in a significant impairment in grasping reflex ($p < 0.001$), in comparison with the control group. GHB (10 mg/kg) consistently affected the grasping reflex in each of the three experiments. Administration of NCS-382 (10 mg/kg) + GHB (10 mg/kg) reversed this effect ($p < 0.005$), compared to animals treated only with GHB (10 mg/kg). No significant differences were found between NCS-382 (10 mg/kg) + GHB (10 mg/kg) and vehicle/saline groups ($p = 0.7$ and $p = 1$, respectively) (see Table 1). In the equilibrium test, some animals presented alterations; however no significant differences were found in groups (Kruskal–Wallis test, $p = 0.389$) (see Supplementary information). No significant differences were obtained in the rest of the neurological reflexes examined.

Hole-board test

Spatial reference memory

No significant differences were found between the three groups in the spatial memory rate ($F_{2,17} = 1.249$, $p = 0.32$) or escape latencies ($F_{2,17} = 1.5$, $p = 0.25$). Animals in all groups increased the spatial memory rate after training ($F_{5,85} = 14.810$, $p < 0.000001$) and reduced the latencies to obtain the reward over training days ($F_{5,85} = 14.90$, $p < 0.00001$).

In the second task, no significant differences were found between the three groups in the spatial memory rate ($F_{2,17} = 0.18$, $p = 0.83$) or the latency to obtain the reward ($F_{2,17} = 1.04$, $p = 0.4$). Animals in all groups increased the spatial memory rate ($F_{2,34} = 23.918$, $p < 0.000001$) and reduced the latency to obtain the reward over training days ($F_{2,34} = 54.980$, $p < 0.000001$). No significant differences in the spatial memory index were found between NCS-382 (10 mg/kg) + GHB (10 mg/kg), vehicle/saline and GHB alone (10 mg/kg) groups ($F_{3,24} = 1.8$, $p = 0.17$). All animals increased the spatial memory index ($F_{2,48} = 7.52$, $p = 0.001$) and reduced the latency to obtain the reward ($F_{2,48} = 14.16$, $p < 0.00001$) over training days.

Spatial WM

Statistical analysis of the spatial WM index revealed significant differences between the groups ($F_{2,17} = 13.66$, $p = 0.00029$). *Post-hoc* comparison showed differences between animals treated with 10 mg/kg GHB and the control group ($p = 0.005$) and between animals treated with 10 mg/kg and 100 mg/kg GHB ($p = 0.05$). Moreover, this index rose in all groups during training ($F_{5,85} = 19.656$, $p < 0.000001$).

Similarly, in the second task, analysis of the spatial WM index revealed significant differences between groups ($F_{2,17} = 4.51$, $p = 0.026$) and over training days ($F_{2,34} = 24.01$, $p < 0.0000001$). All experimental groups increased the spatial WM index with training in the spatial task. *Post-hoc* analysis showed significant differences between animals treated with 10 mg/kg GHB and the control group ($p < 0.05$) (Fig. 2). In relation to the effect of NCS-382 analysis of the spatial WM index revealed significant differences among groups ($F_{3,24} = 5.75$, $p = 0.005$) and over training days ($F_{2,48} = 14.18$; $p < 0.0001$). *Post-hoc* comparison showed that administration of NCS-382 (10 mg/kg) + GHB (10 mg/kg) did not affect WM compared to saline/vehicle groups. However, in the third day of training significant differences ($p = 0.029$) were found between NCS-382 (10 mg/kg) + GHB (10 mg/kg) and rats treated with GHB alone (10 mg/kg) (Fig. 2).

The results obtained on the third training day by recording the data to study the number of errors during sessions revealed that when 5 min elapsed between sessions there was a significant increase in the number of errors in animals treated with 10 mg/kg GHB compared to the control group ($p < 0.05$).

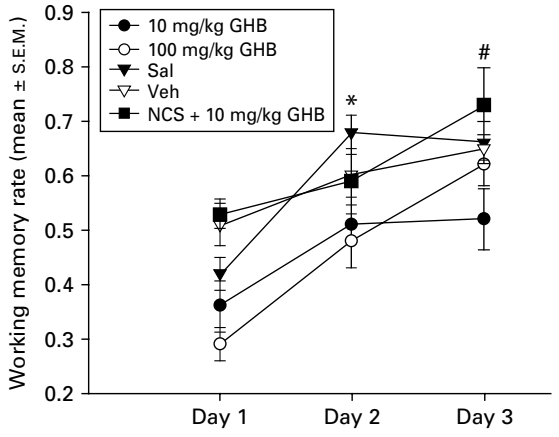

Fig. 2. Graphical representation of the working memory rate in the hole-board test. * Significant differences between animals treated with 10 mg/kg gammahydroxybutyric acid (GHB) or 100 mg/kg GHB and the control group ($p<0.05$). # Significant differences between animals treated with 10 mg/kg GHB and animals treated with NCS (10 mg/kg)+GHB (10 mg/kg) ($p<0.05$). ANOVA for repeated measures and HSD *post-hoc* test has been applied.

Moreover, after an interval of 30 s, the rats treated with 100 mg/kg GHB made a significant number of errors compared to the control group ($p<0.05$). Similarly, animals treated with 10 mg/kg GHB also showed a non-significant increase in the number of errors ($p=0.08$). In addition, when the inter-trial delay was 30 s, the rats treated with 10 mg/kg GHB made more revisits to the rewarded holes that they had visited earlier ($p=0.022$).

Morris water maze

Although the distances swum were greater in the experimental groups, no significant differences were found between groups. In all experimental conditions, escape latencies ($F_{3,51}=13.152$, $p<0.00001$) and distances swum ($F_{3,51}=11.844$, $p<0.0001$) decreased with training.

Analysis of the search strategy used by the animals in the Morris water maze showed no differences between the treatment groups during first three days of training ($p>0.05$). All the groups changed their search strategies over several days of training. In this sense, the qualitative analysis made on swim search strategy revealed that animals first used non-spatial strategies (less efficient strategies) and subsequently, over training days, spatial strategies (more efficient). However, the analysis carried out on the fourth day of training revealed that the control group used a high proportion (50%) of direct spatial strategies (a direct swim path to the location containing the escape platform; Janus, 2004). However, the animals treated with GHB at doses of 10 or 100 mg/kg used this strategy in just 7% or 17%, respectively. Kruskal–Wallis tests revealed the existence of significant differences among groups ($p=0.024$). *Post-hoc* comparisons using Mann–Whitney *U* test showed significant differences between GHB (10 mg/kg) and saline groups ($p=0.016$), as well as between GHB (100 mg/kg) and saline groups ($p=0.05$). No significant differences were found between both doses of GHB ($p=0.549$).

Histology

In the control group the total number of neurons and non-neuronal cells on one side of the dorsal CA1 was $49\,373\pm16\,311$ and 5839 ± 1905, respectively. In the PFC the number of neurons and non-neuronal cells was $140\,597\pm37\,543$ and $58\,990\pm17\,974$, respectively. Repeated administration of GHB significantly altered the number of neurons and non-neuronal cells in both regions. In dorsal CA1 regions, GHB (10 or 100 mg/kg) induced a reduction of 61% or 38% in the number of neurons ($19\,378\pm4977$, $30\,691\pm9637$, respectively; Fig. 3). Significant differences were observed compared to the control group ($p<0.05$) (Fig. 4*a*). Moreover, GHB reduced by 32% (10 mg/kg) or 9% (100 mg/kg) the number of neurons in the PFC ($96\,285\pm36\,033$ and $128\,283\pm25\,916$, respectively). Significant differences were only noted between the animals treated with 10 mg/kg GHB and the control group ($p=0.03$) (Fig. 4*b*). Moreover, GHB administration altered the numbers of non-neuronal cells in both regions studied. The number of non-neuronal cells in CA1 increased significantly by 147% (10 mg/kg) and 20% (100 mg/kg) after GHB treatment ($14\,470\pm4442$ and 7007 ± 1921, respectively) (Fig. 5*a*). In the PFC, although GHB administration increased the non-neuronal cells, a significant increase was only found after 10 mg/kg GHB compared to the control group ($p=0.002$). This treatment increased by 106% the non-neuronal cells in these regions ($121\,947\pm31\,716$). The administration of 100 mg/kg induced an increase of 35% in this cell population ($79\,732\pm6894$) (Fig. 5*b*). No significant differences were found between groups in PAG ($p>0.05$, Fig. 6).

Discussion

The aim of this study was to examine the possible neurotoxic effects following administration of GHB in male rats. Specifically, the neurotoxic effect of

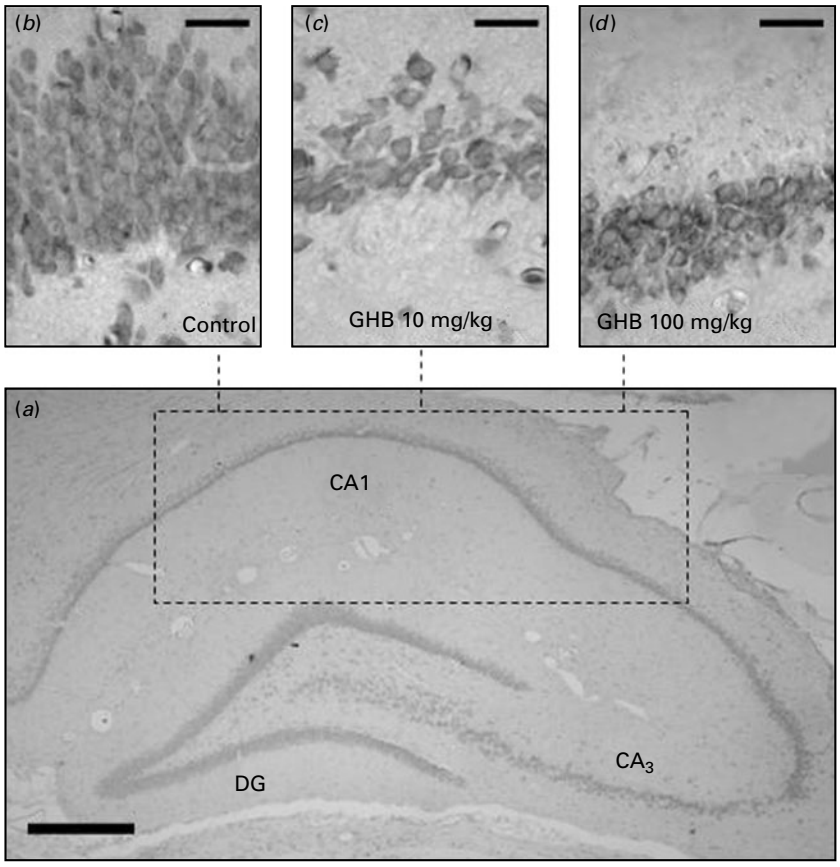

Fig. 3. (*a*) Micrograph of the hippocampus (unilateral). (*b–d*) Micrograph of pyramidal cells of the CA1 region. DG, Dentate gyrus. Scale bar: (*a*) 350 μm; (*b–d*) 50 μm.

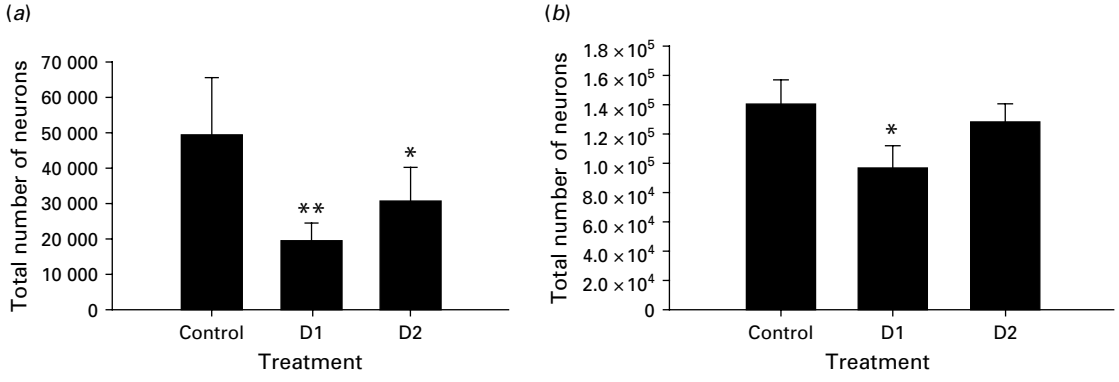

Fig. 4. Effect of gammahydroxybutyric acid (GHB) on the total number of neurons in one side of the rat dorsal CA1 (*a*) or PFC (*b*) after 15 d administration. One-way analysis of variance (ANOVA) and Tukey's *post-hoc* test (mean ± S.E.M., * $p < 0.05$, ** $p < 0.005$). Control, Control group; D1, GHB (10 mg/kg); D2, GHB (100 mg/kg).

subchronic administration of GHB (10 and 100 mg/kg) was assessed in rats. First, we conducted two behavioural tests (hole-board and Morris water maze) and a neurological battery, to serve as the dependent variables of neurotoxic damage following prolonged GHB administration. Subsequently, a histological study was performed to quantify neuronal loss and cell damage resulting from GHB administration.

Concerning the evaluation of neurological reflexes, our results showed deterioration in the grasping reflex

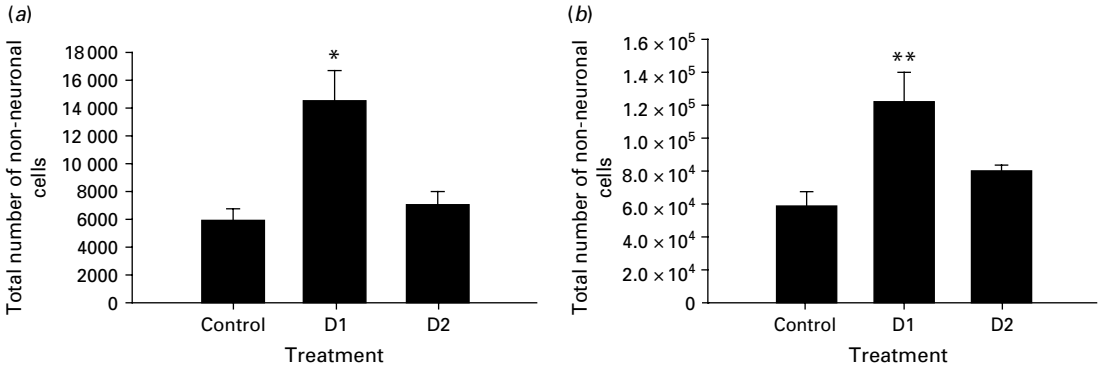

Fig. 5. Effect of gammahydroxybutyric acid (GHB) on the total number of non-neuronal cells in dorsal CA1 (*a*) or PFC (*b*) after 15 d administration. One-way analysis of variance (ANOVA) and Tukey's *post-hoc* test (mean ± S.E.M., * $p < 0.05$, ** $p < 0.005$). Control, Control group; D1, GHB (10 mg/kg); D2, GHB (100 mg/kg).

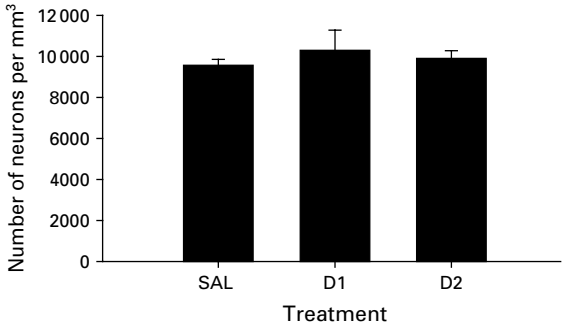

Fig. 6. Number of neurons per mm³ in dorsal periaqueductal grey matter. Sal, Control group; D1, gammahydroxybutyric acid (GHB) (10 mg/kg); D2, GHB (100 mg/kg).

of animals after subchronic administration of GHB (10 mg/kg), an effect that was prevented with NCS-382 (10 mg/kg) pretreatment (Table 1). The neurological study undertaken during the first day of GHB administration failed to show any alterations in this reflex. Consequently, the grasping reflex impairment observed only with repeated GHB administration may result from damage of the brain areas that control the expression of this reflex, i.e. the PFC (Matsumura *et al*. 1996). However, normal reactions were observed in the rest of neurological reflexes examined in our study, mainly showing the absence of impairments, after GHB administration, in the remainder of CNS structures related to these reflexes (Crawley, 1999; Lalonde *et al*. 2005). No neurological effects were observed in animals treated with 100 mg/kg GHB (Table 1).

The spatial memory was examined by using two tests: hole-board and Morris water maze. Regarding the results obtained in spatial memory index with the hole-board test, we observed that subchronic GHB administration did not affect spatial memory. All animals reduced the time required to find the first rewarded hole and increased the spatial memory rate over training days. But the hole-board test as designed may not require spatial strategies to solve it. However, the Morris water maze, was designed following a spatial memory protocol. In fact, analysis of the navigation strategies used on the last training day revealed that GHB disrupted the use of direct spatial strategy. Thus, in the two retention trials on the last day of training, 50% of the control rats exhibited a direct spatial strategy (a direct swim path to the location containing the escape platform, that it is an extremely effective and well-acquired strategy) whereas the animals treated with 10 or 100 mg/kg GHB only exhibited 7% or 17% direct spatial strategy, respectively. The non-direct spatial use of navigation strategies resulted in a less efficient process (Janus, 2004) and could explain why the distances swum were greater in the experimental groups. Several evidences indicate that the hippocampus is essential for spatial strategies (Gallagher & Rapp, 1997; Miettinen *et al*. 1993; Smith *et al*. 2000). Thus, our results are consistent with an alteration in the hippocampus induced by GHB. Additionally, after hippocampal injury the animals may have adopted alternative strategies. In this way, the data obtained by analysis of navigation strategies in the Morris water maze suggest that some relevant information about the requirements of the task was acquired by GHB-treated rats, implying that this improvement relied on repeated training.

In the hole-board task, rats needed to recognize the place where a hole was baited (spatial processing) and also to maintain and utilize the information about which holes they had or had not already visited. The

latter requirement is called WM (Olton & Papas, 1979) since the memory is necessary until rats have consumed all the pieces of biscuit in the maze. The results of our hole-board test suggested that spatial WM was damaged in animals after subchronic GHB administration. The animals treated for longer with GHB had more difficulties retaining and utilizing the information about which holes they had or had not visited. Pretreatment with NCS-382 (10 mg/kg) was effective in preventing this action. Many authors have emphasized the importance of the medial PFC in the WM task. Data suggest that the PFC might be necessary when a rule is applied to a considerable number of trial-unique items (i.e. the 4 different holes per trial in our task) (Floresco *et al*. 1997). However, hippocampal lesions, especially in CA1, a region traditionally considered as an output region of the hippocampus (Amaral & Witter, 1994) should disrupt spatial WM (Davis *et al*. 1987; Whishaw *et al*. 1994). Moreover, in the hole-board test, with a short-term delay (i.e. 30 s) between trials, the GHB-treated animals showed impaired performance. Previous lesion experiments have shown that interaction between the dorsal hippocampus and mPFC is necessary for rats to perform a short-term spatial WM test (Lee & Kesner, 2003*b*).

Animals treated with 10 mg/kg GHB made a significant number of errors after a 5-min inter-trial delay. The 5-min delay was labelled as an intermediate-term delay (Eichenbaum, 2000), because it is longer than a short-term delay (e.g. 30 s), but shorter than a usual long-term (e.g. 24 h) delay. The dorsal CA1 region appears to become a necessary structure for spatial WM after an intermediate-term delay (i.e. 5 min) (Lee & Kesner, 2003*a*). However, the PFC could also be implicated in intermediate-term memory (Touzani *et al*. 2007).

Stereological quantification of the number of neurons in the PFC and CA1 showed a significant neuronal loss in both regions after 10 mg/kg GHB. Although a moderate dose of GHB also resulted in a reduction in the number of neurons in the CA1 and PFC, this neuronal loss was lower than the low-dose group. This effect could be mediated by the specific action of GHB on its receptors in these areas. We found no post-treatment differences in other areas lacking these receptors, i.e. PAG (Andriamampandry *et al*. 2003; Kemmel *et al*. 2006; Maitre, 1997). On the other hand, quantification of the non-neuronal cells, presumably glial cells, in the PFC and CA1 in the group receiving low-dose GHB showed a significant increase in these cell populations. The group of rats treated with 100 mg/kg only showed a significant increase in non-neuronal cells in the PFC. In this way, it has been reported that an increase of non-neuronal cells occurs in response to neuronal injury (Kim *et al*. 2000). Thus, the neuronal loss, together with the increase in glial cells, suggests toxic damage to both regions after subchronic GHB treatment. These results are strongly consistent with neurological and cognitive alterations previously reported after repeated GHB administration.

The neurotoxic effect induced by a very low dose of GHB (10 mg/kg) is in contrast with the neuroprotective actions of high doses of this drug. A protective effect of GHB with high doses (300 mg/kg) could be induced by activation of the $GABA_B$ receptor (Ottani *et al*. 2003; Vergoni *et al*. 2000). However, this dose is 30-fold higher than used in our study. In fact, GHB has an extremely weak $GABA_B$ affinity. Doses of GHB much higher than used in our study are required for activation of the $GABA_B$ receptor (Carter *et al*. 2003, 2006). Nevertheless, NCS-382 (10 mg/kg) prevented the neurological damage (grasping reflex) and the alteration in the WM index induced by GHB (10 mg/kg). Moreover, our results showed absence of cellular impairments after GHB administration in PAG, an area lacking these receptors. These results suggest that neurotoxicity induced by low doses of GHB might be mediated by GHB receptors.

Our data are consistent with previous results obtained by Sgaravatti *et al*. (2007). Thus, it has been observed that acute administration of GHB (10 mg/kg) provoked a significant enhancement of thiobarbituric acid-reactive substance levels and a decrease of total radical-trapping antioxidant potential and total antioxidant reactivity measurements. These results indicate that GHB induces oxidative stress by stimulating lipid peroxidation and decreasing the non-enzymatic antioxidant defences in the cerebral cortex of rats.

One point that deserves further consideration is the finding that the magnitude of effect is different after administration of two doses of GHB, causing different neuronal damage. Administration of 100 mg/kg induced a moderate effect on the integrity of PFC (see Fig. 4*b*) that might be explained because it does not impair the performance of rats in tasks mediated by this region, such as grasping reflex or WM. Although the behavioural alterations induced by this dose are more moderate than those induced by low doses, they appear to coincide with hippocampus damage being observed. The reasons for the different effects of these doses remain to be resolved, but it is interesting to consider that two different GHB receptors have so far been characterized with different affinities and B_{max} (Andriamampandry *et al*. 2003; Hechler *et al*. 1993; Maitre, 1997). The two types of receptor

could have different effectors systems (Kemmel *et al*. 2006) and different locations on synaptic terminals (Andriamampandry *et al*. 2003; Kemmel *et al*. 2006), which would explain the different results obtained for the two doses of GHB.

Although the mechanism accounting for the effects of prolonged GHB administration is presently unknown, a hypothesis through dopamine release modulation can be proposed (Godbout *et al*. 1995; Hechler *et al*. 1993; Pedraza *et al*. 2007). Previous studies have shown that low-dose GHB may result in a marked increase in dopaminergic levels (Diana *et al*. 1991; Godbout *et al*. 1995; Tremblay *et al*. 1998). An important increase in basal dopamine levels could cause dopaminergic neurotoxicity through diverse mechanisms, such as oxidative stress, generation of reactive oxygen (Chiueh *et al*. 2000) or activation of the D_1 dopamine receptor (Chen & Sidhu, 2005). However, other possibilities cannot be excluded. The administration of GHB has been shown to induce a high increase in levels of glutamate (Castelli *et al*. 2003) or GHB, at very low doses, exerts a feedback inhibition on GABA synapses via GHB receptors and reduces GABA release. These could have a role in the neurotoxic effect.

Conclusion

In contrast with the popular belief that GHB is innocuous, the results of the present study suggest that its continued administration, especially at very low doses, has neurotoxic effects. Our study shows for the first time that GHB produces neurological impairment and neuronal damage in the CA1 and PFC. The drastic neuronal death in these regions could explain, at least partly, the spatial and WM problems seen after 15 d of GHB administration. Although the dose used in the present study is very low (10 mg/kg), compared to doses used in humans, these findings are important because abuse of GHB has increased considerably over recent years, especially among young persons, who might develop neuropsychological disorders after prolonged consumption of this recreational drug. However, our results require substantiating by future research examining the mechanisms underlying the neurotoxic effects of GHB, leading to a better understanding of the negative effects associated with the consumption of this recreational drug.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

Acknowledgements

This study was supported by grants from the Fondo de Investigaciones Sanitarias (FIS01/0954) and Consejería de Innovación, Ciencia y Empresa (CTS-195), Spain. We are grateful to Luis Santín and Guadalupe Dávila for their comments on this manuscript and to Vanessa De Castro for her technical assistance.

Statement of Interest

None.

References

Amaral DG, Witter MP (1994). Hippocampal formation. In: Paxinos G (Ed.), *The Rat Nervous System*, 2nd edn (pp. 443–494). San Diego: Academic.

Andriamampandry C, Taleb O, Viry S, Muller C, Paulhumbert J, Gobaille S, Aunis D, Maitre M (2003). Cloning and characterization of a rat brain receptor that binds the endogenous neuromodulator γ-hydroxybutyrate (GHB). *FASEB Journal* **17**, 1691–1693.

Björklund A, Dunnet SB, Stenevi U, Lewuis ME, Iversen SD (1980). Reinervation of the denervate striatum by substantia nigra. *Brain Research* **199**, 307–333.

Bracucci A, Berretta N, Mercuri NB, Francesconi W (2004). Gamma-hydroxybutyrate and ethanol depress spontaneous excitatory postsynaptic currents in dopaminergic neurons of substantia nigra. *Brain Research* **997**, 62–66.

Brasted PJ, Bussey TJ, Murray EA, Wise SP (2003). Role of the hippocampal system in associative learning beyond the spatial domain. *Brain* **126**, 1202–1223.

Brody D, Holtzman D (2006). Morris water maze search strategy analysis in PDAPP mice before and after experimental traumatic brain injury. *Experimental Neurology* **197**, 330–340.

Bures J, Buresova O, Huston JP (1983). *Techniques and Basic Experiments for the Study of Brain and Behaviour*. Amsterdam: Elsevier Science Publishers.

Carter LP, Flores LR, Wu H, Chen W, Unzeitig AW, Coop A, France CP (2003). The role of $GABA_B$ receptors in the discriminative stimulus effects of γ-hydroxybutyrate in rats: time course and antagonism studies. *Journal of Pharmacology and Experimental Therapeutics* **665**, 668–674.

Carter LP, Chen W, Coop A, Koek W, France CP (2006). Discriminative stimulus effects of GHB and $GABA_B$ agonists are differentially attenuated by CGP35348. *European Journal of Pharmacology* **538**, 85–93.

Cash CD (1994). Gamma-hydroxybutyrate: an overview of the pros and cons for it being a neurotransmitter and/or a useful therapeutic agent. *Neuroscience and Biobehavioural Reviews* **18**, 291–304.

Castelli MP, Ferraro L, Mocci I, Carta F, Carai MA, Antonelli T, Tanganelli S, Cignarella G, Gessa GL (2003).

Selective gamma-hydroxybutyric acid receptor ligands increase extracellular glutamate in the hippocampus, but fail to activate G protein and to produce the sedative/hypnotic effect of gamma-hydroxybutyric acid. *Journal of Neurochemistry* **87**, 722–732.

Castelli MP, Pibiri F, Carboni G, Piras AP (2004). A review of pharmacology of NCS-382, a putative antagonist of gamma-hydroxybutyric acid (GHB) receptor. *CNS Drug Reviews* **10**, 243–260.

Chen J, Sidhu A (2005). The role of D1 dopamine receptors and phospho-ERK in mediating cytotoxicity. Commentary. *Neurotoxicology Research* **7**, 179–181.

Chiueh CC, Andoh T, Lai AR, Lai E, Krishna G (2000). Neuroprotective strategies in Parkinson's disease: protection against progressive nigral damage induced by free radicals. *Neurotoxicology Research* **2**, 293–310.

Crawley J (1999). Behavioural phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioural tests. *Brain Research* **835**, 18–26.

Crunelli V, Emri A, Leresche N (2006). Untravelling the brain targets of γ-hydroxybutyric acid. *Current Opinion in Pharmacology* **6**, 44–52.

Davis HP, Baranowski JR, Pulsinelli WA, Volpe BT (1987). Retention of reference memory following ischemic hippocampal damage. *Physiology and Behavior* **39**, 783–786.

Degenhardt L, Copeland J, Dillion P (2005). Recent trends in the use of 'club drugs': an Australian review. *Substance Use & Misuse* **40**, 1241–1256.

Diana M, Mereu G, Mura A, Fadda F, Passino N, Gessa G (1991). Low doses of γ-hydroxybutyric acid stimulate the firing rate of dopaminergic neurons in unanesthetized rats. *Brain Research* **566**, 208–211.

Douma BR, Korte SM, Buwalda B, Fleur SE, Bohus B, Luiten PG (1998). Repeated blockade of mineralocorticoid receptors, but not of glucocorticoid receptors impairs food rewarded spatial learning. *Psychoneuroendocrinology* **23**, 33–44.

Eichenbaum H (2000). A cortical-hippocampal system for declarative memory. *Nature Reviews in Neuroscience* **1**, 41–50.

Floresco SB, Seamans JK, Phillips AG (1997). Selective roles for hippocampal, prefrontal cortical, and ventral striatal circuits in radial-arm maze tasks with or without a delay. *Journal of Neuroscience* **17**, 1880–1890.

Gallagher M, Rapp PR (1997). The use of animal models to study the effects of aging on cognition. *Annals Reviews of Psychology* **48**, 339–370.

Godbout R, Jelenic P, Labrie C, Schmitt M, Bourguignon JJ (1995). Effect of gamma-hydroxybutyrate and its antagonist NCS-382 on spontaneous cell firing in the prefrontal cortex of the rat. *Brain Research* **673**, 157–160.

Gould G, Mehta AK, Franzer A, Ticku MK (2003). Quantitative autoradiographic analysis of the new radioligand [^{3}H](2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[*a*][7]annulen-6–3 ylidene) ethanoic acid ([^{3}H]NCS-382) at γ-hydroxybutyric acid (GHB) binding sites in rat brain. *Brain Research* **979**, 51–56.

Gundersen HJG, Bagger P, Bendsten TF, Evans S, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, *et al.* (1988). The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **96**, 857–881.

Hechler V, Gobaille S, Maitre M (1992). Selective distribution pattern of γ-hydroxybutyrate receptors in the rat forebrain and midbrain as revealed by quantitative autoradiography. *Brain Research* **572**, 3345–3348.

Hechler V, Gobaille S, Bourguignon JJ, Mark J, Maitre M (1993). γ-hydroxybutyrate ligands posses antidopaminergic and neuroleptic-like activities. *Journal of Pharmacology and Experimental Therapeutics* **264**, 1406–1414.

Hopfer C, Mendelson B, Van Leeuwen JM, Kelly S, Hooks S (2006). Club drug use among youths in treatment for substance abuse. *American Journal of Addiction* **15**, 94–99.

Howard SG, Feigenbaum JJ (1997). Effects of γ-hydroxybutyrate on central dopamine release in vivo. *Biochemical Pharmacology* **53**, 103–110.

Janson AM, Möller A (1993). Chronic nicotine treatment counteracts nigral cell loss induced by a partial mesodiencephalic hemitransection: analysis of the total number and mean volume of neurons and glia in substantia nigra of the male rat. *Neuroscience* **57**, 931–941.

Janus C (2004). Search strategies used by APP transgenic mice during navigation in the morris water maze. *Learning and Memory* **11**, 337–346.

Kemmel V, Miehe M, Roussel G, Taleb O, Nail-Boucherie K, Marchand C, Stutz C, Andriamampandry C, Aunis D, Maitre M (2006). Immunohistochemical localization of a GHB receptor-like protein isolated from rat brain. *Journal of Comparative Neurology* **498**, 508–524.

Kim HC, Bing G, Jhoo VK, Ko HK, Kim WK, Suh JH, Kim SJ, Kato K, Hong JS (2000). Changes of hippocampal CuZn-superoxide dismutase after kainate treatment in the rat. *Brain Research* **853**, 215–226.

Lalonde R, Marchetti N, Strazielle C (2005). Primary neurologic screening and motor coordination of *Dst^{dt-J}* mutant mice (*Dystonia musculorum*) with spinocerebellar atrophy. *Physiology & Behavior* **86**, 46–51.

Lee I, Kesner RP (2003*a*). Differential roles of dorsal hippocampal subregions in spatial working memory with short versus intermediate delay. *Behavioral Neuroscience* **117**, 1044–1053.

Lee I, Kesner RP (2003*b*). Time-Dependent relationship between the dorsal hippocampus and the prefrontal cortex in spatial memory. *Journal of Neuroscience* **15**, 1517–1523.

Maitre M (1997). The γ-Hydroxybutyrate signalling system in brain: organization and functional implications. *Progress in Neurobiology* **551**, 337–361.

Marshall F, Teitelbaum P (1974). Further analysis of sensory inattention flowing lateral hypothalamic damage in rats. *Journal of Comparative Physiological Psychology* **86**, 375–395.

Martellotta MC, Cossu G, Fattore L, Gessa GL, Fratta W (1998). Intravenous self-administration of gamma-hydroxybutyric acid in drug-naïve mice. *European Neuropsychopharmacology* **8**, 293–296.

Matsumura M, Kawashima R, Naito E, Satoh K, Takahashi T, Yanagisawa T, Fukuda H (1996). Changes in rCBF during grasping in humans examined by PET. *Neuroreport* **7**, 749–752.

Miettinen R, Sirvio J, Riekkinen Sr. P, Laakso MP, Riekkinen M, Riekkinen P (1993). Neocortical, hippocampal and septal parvalbuminand somatostatin-containing neurons in young and aged rats: correlation with passive avoidance and water maze performance. *Neuroscience* **53**, 367–378.

Navarro JF, Pedraza C (1996). An ethopharmacological assessment of the effects of gammahydroxybutyrate on agonistic interactions in male mice. *Medical Science Research* **24**, 817–819.

Navarro JF, Pedraza C, Martín M, Manzaneque JM, Dávila G, Maldonado E (1998). Tiapride-induced catalepsy is potentiated by gammahydroxybutyric acid administration. *Progress in Neuropsychopharmacology & Biological Psychiatry* **22**, 835–844.

Navarro JF, González F, Pedraza C (2007). Acute and subchronic effects of gammahydroxybutyrate (GHB) on isolation-induced aggression in male mice. *Methods and Findings in Experimental and Clinical Pharmacology* **29**, 379–382.

Oades RD (1981). Types of memory or attention? Impairment after lesions of the hippocampus and limbic ventral tegmentum. *Brain Research Bulletin* **18**, 533–545.

Olton DS, Papas BC (1979). Spatial memory and hippocampal function. *Neuropsychology* **17**, 669–682.

Ottani A, Saltini F, Bartiromo M, Zaffe D, Botticelli AR, Ferrari A, Bertolini A, Genedani S (2003). Effect of γ-hydroxybutyrate in two rat models of focal cerebral damage. *Brain Research* **896**, 181–190.

Paxinos G, Watson C (1998). *The Rat Brain in Stereotaxic Coordinates*, 4th edn. San Diego: Academic Press.

Pedraza C, Dávila G, Martín-López M, Navarro JF (2007). Antiaggressive effects of GHB in male mice: involvement of dopamine D2 receptors. *Progress in Neuropsychopharmacology and Biological Psychiatry* **31**, 337–342.

Pistis M, Muntoni AL, Pillilla G, Perra S, Melis M, Gessa GL (2005). Gamma-Hydroxybutyric acid (GHB) and the mesoaccumbens reward circuit: evidence for GABAB receptor-mediated effects. *Neuroscience* **131**, 465–474.

Raess BU, Tunnicliff G (2002). Abuse potential and toxicology of gamma-hydroxybutyrate. In: Tunnicliff G, Cash CD (Eds), *Gamma-Hydroxybutyrate: Molecular, Functional and Clinical Aspects* (pp. 188–196). London: Taylor and Francis.

Schmidt-Mutter C, Muller C, Zwiller J, Gobaille S, Maitre M (1999). Gamma-hydroxybutyrate and cocaine administration increases mRNA expression of dopamine D1 and D2 receptors in rat brain. *Neuropsychopharmacology* **21**, 662–669.

Sevack RJ, France CP, Koek W (2004). Neuroleptic-like effects of γ-hydroxybutyrate: interactions with haloperidol and dizocilpine. *European Journal of Pharmacology* **483**, 289–293.

Sgaravatti AM, Sgarbi MB, Testa CG, Durigon K, Pederzolli CD, Prestes CC, Wyse AT, Wannmacher CM, Wajner M, Dutra-Filho CS (2007). γ-Hydroxybutyric acid induces oxidative stress in cerebral cortex of young rats. *Neurochemistry International* **50**, 564–570.

Sicar R, Basak A (2004). Adolescent γ-hydroxybutyric acid exposure decreases cortical N-methyl-D-aspartate receptor and impairs spatial learning. *Pharmacology Biochemistry and Behavior* **79**, 701–708.

Smith TD, Adams MM, Gallagher M, Morrison JH, Rapp PR (2000). Circuit-specific alterations in hippocampal synaptophysin immunoreactivity predict spatial learning impairment in aged rats. *Journal of Neuroscience* **20**, 6587–6593.

Sumnall HS, Woolfall K, Edwards S, Cole JC, Beynon CM (2008). Use, function, and subjective experiences of gamma-hydroxybutyrate (GHB). *Drug and Alcohol Dependence* **92**, 286–290.

Touzani K, Puthanveetti SV, Kandel ER (2007). Consolidation of learning strategies during spatial working memory task requires protein synthesis in the prefrontal cortex. *Proceedings of the National Academy of Sciences USA* **104**, 5632–5637.

Tremblay H, Godbout R, Girodias V, Schmitt M, Bourguignon JJ (1998). Effects of gamma-hydroxybutyrate on ventral tegmental unit activity in the rat: considerations on REM sleep control. *Sleep Research Online* **1**, 152–158.

Vergoni VA, Ottani A, Botticelli AR, Zaffe D, Guano L, Loche A, Genedani S, Gessa GL, Bertolini A (2000). Neuroprotective effect of gamma-hydroxybutyrate in transient global cerebral ischemia in the rat. *European Journal of Pharmacology* **397**, 75–84.

Whishaw IQ, Rod MR, Auer RN (1994). Behavioral deficits revealed by multiple tests in rats with ischemic damage limited to half of the CA1 sector of the hippocampus. *Brain Research Bulletin* **34**, 283–289.

Yan XB, Wang SS, Hou HL, Ji R, Zhou JN (2007). Lithium improves the behavioral disorder in rats subjected to transient global cerebral ischemia. *Behavioural Brain Research* **177**, 282–289.