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ARTICLE

THEMATIC SECTION
New Findings in Substance Abuse Research

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Gamma-hydroxybutyrate does not maintain self-administration but induces conditioned place preference when injected in the ventral tegmental area

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Abstract

Gamma-hydroxybutyric acid (GHB) is an endogenous brain substance that has diverse neuropharmacological actions, including rewarding properties in different animal species and in humans. As other drugs of abuse, GHB affects the firing of ventral tegmental neurons (VTA) in anaesthetized animals and hyperpolarizes dopaminergic neurons in VTA slices. However, no direct behavioural data on the effects of GHB applied in the VTA or in the target regions of its dopaminergic neurons, e.g. the nucleus accumbens (NAc), are available. Here, we investigated the effects of various doses of intravenous GHB in maintaining self-administration (from 0.001 to 10 mg/kg per infusion), and its ability to induce conditioned place preference (CPP) in rats when given orally (175–350 mg/kg) or injected directly either in the VTA or NAc (from 10 to 300 μg/0.5 μl per side). Our results indicate that while only 0.01 mg/kg per infusion GHB maintained self-administration, although not on every test day, 350 mg/kg GHB given orally induced CPP. CPP was also observed when GHB was injected in the VTA (30–100 μg/0.5 μl per side) but not in the NAc. Together with recent *in-vitro* findings, these results suggest that the rewarding properties of GHB mainly occur via disinhibition of VTA dopaminergic neurons.

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Key words: Conditioned place preference, GHB, nucleus accumbens, self-administration, ventral tegmental area.

Introduction

Gamma-hydroxybutyric acid (GHB) is generated in mammalian brains from the metabolism of gamma-aminobutyric acid (GABA) (Crunelli *et al*. 2006; Maitre, 1997; Wong *et al*. 2004). Although GHB may act as a neurotransmitter (Bernasconi *et al*. 1999; Cash, 1994) or as a source of neuronal GABA (Chambliss & Gibson, 1992), a full understanding of its physiological role(s) remains unclear. GHB has diverse neuropharmacological properties, including activation of both $GABA_B$ receptors (Lingenhoehl *et al*. 1999; Mathivet *et al*. 1997), and putative GHB receptors (Maitre, 1997; but see Crunelli *et al*. 2006).

Exogenously administered GHB elicits sedation, memory loss, euphoria, behavioural disinhibition and sleep (Abanades *et al*. 2007; Carter *et al*. 2006; Laborit, 1960; Miotto *et al*. 2001; Wong *et al*. 2004). GHB has found clinical use in the treatment of alcohol and opiate withdrawal and in promoting long-term abstinence from these drugs (Addolorato *et al*. 1999; Caputo *et al*. 2005; Gallimberti *et al*. 1989; Nava *et al*. 2007), whereas its use as an anaesthetic induction agent has now been discontinued (Kam & Yoong, 1998). More recently, GHB has been prescribed for the treatment of

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narcolepsy and cataplexy (Black & Guilleminault, 2001; Pardi & Black, 2006; Tunnicliff & Raess, 2002).

GHB emerged as a recreational drug in the 1970s and remains one of the commonly used 'club drugs' (Degenhardt *et al*. 2005; Ricaurte & McCann, 2005; Snead & Gibson, 2005; Sumnall *et al*. 2008), with accidental overdoses that occur in recreational nightlife settings accounting for a substantial proportion of the overall hospital emergencies, at least in Europe (EMCDDA, 2008).

To unravel the mechanism underlying the reinforcing properties of GHB, several studies investigated its effects in various species, but the results are inconclusive. Thus, GHB is not intravenously self-administered by monkeys experienced in the self-administration of phencyclidine or methohexital (Beardsley *et al*. 1996; Woolverton *et al*. 1999), but is intravenously self-administered by naive mice (Fattore *et al*. 2001; Martellotta *et al*. 1998), although only data from the first day of self-administration were reported in the latter studies. Orally, GHB is self-administered by mice and rats (Colombo *et al*. 1995; Labouèbe *et al*. 2007), although in the latter species periods of voluntary abstinence were observed and self-administration was often irregular. Oral GHB can induce conditioned place preference (CPP) in rats (Martellotta *et al*. 1997) and mice (Itzhak & Syed, 2002).

The involvement of mesocorticolimbic dopaminergic systems in reward-dependent learning of addictive drugs is well known (Fibiger & Phillips, 1988; Lüscher & Ungless, 2006). Drugs of abuse directly or indirectly activate ascending dopaminergic neurons in the ventral tegmental area (VTA), elevating dopamine levels at their site of termination in the nucleus accumbens (NAc) and prefrontal cortex (Di Chiara & Imperato, 1988; Koob, 1992). GHB too has been shown to target the mesolimbic system *in vivo*, leading to a moderate stimulation of the dopaminergic system (Pistis *et al*. 2005). *In vitro*, low concentration of GHB preferentially inhibits VTA GABAergic neurons, with a resulting disinhibition of dopaminergic neurons, leading to an increased VTA output that would underlie GHB's rewarding properties (Cruz *et al*. 2004; Labouèbe *et al*. 2007).

However, no direct behavioural data are available on the role of the NAc and/or VTA in the rewarding properties of GHB. Thus, here we first investigated its ability to maintain self-administration and to induce CPP in rats, and later assessed whether these effects could be reproduced by GHB injected directly into the VTA or the NAc. Our results demonstrate that GHB, while unable to reliably maintain intravenous self-administration, induces CPP when administered orally or when injected in the VTA but not in the NAc. A preliminary report of some of these results has been published (Watson *et al*. 2007).

Material and methods

Animal procedures were conducted in conformity with institutional guidelines, and national (D.L. 116) and international policies (EEC Directive 86/609; Guide for the Care and Use of Laboratory Animals, USA). Male Wistar rats (Charles River, Italy), weighing 175–200 g, were housed individually in the self-administration studies, or two per cage in the CPP experiments, at constant room temperature ($21 \pm 1\ ^{\circ}C$) and relative humidity (60%) under a 12-h light/dark cycle (lights on 07:30 hours) with food and water available *ad libitum*. Animals were allowed to adapt to the laboratory conditions for at least 1 wk before experiments commenced, and were handled daily during this period.

Self-administration studies

Apparatus

Animals were trained and tested using eight standard rodent operant test chambers (ENV-007, Med Associates Inc., USA) equipped with two retractable levers and three lights, each 2.8 W, 24 V, one in the middle back of the ceiling, and two on the front panel 6 cm above each lever. In four chambers the right-hand lever was designated as the active lever, in the other chambers it was the left-hand one. Each chamber was installed inside a sound-attenuating cubicle, with an exhaust fan mounted on one side. Intravenous infusions were administered via a syringe pump (PHM-100, Med Associates Inc.) located inside the sound-attenuating cubicles. Stimulus lights, pellet dispenser, and syringe pump were controlled by a computer running the Med Associates software.

Experimental procedures

In the first series of experiments, the training and testing was conducted during the light phase of the light/dark cycle, starting at 09:00 hours. To facilitate the acquisition of GHB self-administration, rats were initially trained to press a lever for food pellets on a fixed ratio 2 (FR2) schedule. During this period and throughout these experiments, the animals were placed on a restricted diet (20 g/d rat chow) (Altromin MT, Rieper, Italy), sufficient to maintain body weight and growth. The first session consisted of 30 min of non-contingent delivery of one 45-mg food pellet

(Noyes improved formula A/I, Sandown Scientific, UK) every 30 s. In addition, each lever press delivered one food pellet. From the second 30-min session, food pellets were available on a FR1 schedule with only the active lever being available. From the third session, the FR was increased to 2 and the second inactive lever was introduced. Animals received a minimum of three 30-min food-training sessions under FR2 in which they earned 100 pellets.

At the end of this training (6–7 d), rats were anaesthetized (equithesin 3.0 ml/kg i.p.) and implanted in the right jugular vein with a catheter as previously described (Cervo *et al*. 2003). During the 5-d recovery period, rats received one daily subcutaneous (s.c.) injection of 45 mg/kg ampicillin (Amplital®, Pharmacia, Italy). Catheters were kept patent by daily intravenous infusions of 0.1 ml heparinized (30 U/ml, Opocrin S.p.A., Italy) sterile saline before and after each self-administration session. At the end of the self-administration period, patency was verified by intravenously injecting 0.05 ml of 1.25 mg/ml midazolam maleate (Roche, Switzerland) plus 25 mg/ml ketamine hydrochloride (Sigma-Aldrich, Italy). Animals with patent catheters displayed clear signs of sedation within 3 s (Caine *et al*. 1999). Only data from rats with patent catheter (63/68) were included in the analysis.

GHB self-administration procedure

One week after surgery, rats were allowed to self-administer GHB or vehicle for a 2-h session daily (for 7 d/wk) under a FR2 20-s timeout schedule of reinforcement. The self-administration session started with the introduction of both the active and the inactive levers: two active lever presses resulted in a 6-s infusion of GHB and a stimulus light above the active lever came on for 20 s to signal timeout (i.e. when active lever presses would not result in GHB infusion). Inactive lever presses were recorded but had no programmed consequences. The effects of vehicle and 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 3 and 10 mg/kg per 0.1 ml GHB were studied in nine independent groups of naive rats (each containing 7–8 naive rats). These doses were chosen on the basis of previous studies on GHB intravenous self-administration (Beardsley *et al*. 1996; Fattore *et al*. 2001; Martellotta *et al*. 1998; Woolverton *et al*. 1999). After 14 d GHB self-administration, rats underwent a 2-h extinction session per day, which was identical to the self-administration session except that GHB was replaced with sterile saline. Since no differences in the number of infusions, active and inactive lever presses were observed between groups (see Results section) these extinction sessions were stopped after 7 d.

It has been shown that animals housed under an inverted light/dark schedule are more active in operant cages and that conducting self-administration sessions during the dark phase influences the intake of several drugs (Caine *et al*. 1993; Finlay *et al*. 1989). Therefore, since GHB did not reliably maintain self-administration when available during the light phase of the light/dark cycle in a second experiment, GHB self-administration was evaluated in 18 rats housed under an inverted 12-h light/dark cycle (lights on 19:30 hours). Rats were allowed to adapt to these novel conditions for 3 wk before starting a self-administration procedure. GHB at 0.01 and 0.03 mg/kg per 0.1 ml was tested since these doses were self-administered at a higher rate compared to vehicle when available during the light phase of the light/dark cycle.

CPP

Apparatus

The in-house-made apparatus consisted of four rectangular boxes ($80 \times 40 \times 30$ cm) with three sides made of wood, one long Plexiglas observation wall, and a wooden lid, as previously described (Cervo & Samanin, 1995; Cervo *et al*. 1996, 1997, 2002, 2005). For the conditioning phase, each box was divided into two equal-sized compartments by a sliding wall: one compartment was painted grey, and the other black with vertical white stripes 3 cm wide, whereas the lids were painted to match the respective compartments. The two compartments were fitted with distinctive metal floors: one with a loose mesh and the other with a much closer grid.

For pre-conditioning and test sessions, the partition between the two compartments was raised 12 cm off the floor and a 5×3 cm smooth aluminium platform was inserted between them. Two infrared sensors (IMC-S7801-02, Murata Electronics, Japan), one for each compartment, set to detect any change in the infrared emission due to a moving body, were assembled on the partition with a relevance angle of 20°, so that both signalled the rat in the central zone when it was on the platform or stayed very close to it. The sensors were operated by a computer that recorded the time the animal spent in the different compartments (by activation of a single sensor), and in the central zone (activation of both sensors).

The testing room was closed and ventilated, with dim indirect lighting provided by one 15 W incandescent white bulb positioned 50 cm above the boxes.

A loudspeaker about 1 m above the boxes delivered white noise.

CPP procedure

As previously described (Cervo *et al*. 2002, 2005), on the first day, before any drug treatment, each rat was allowed to explore the apparatus for 15 min, and the time spent in the two compartments was recorded. Rats were then assigned to treatment groups and conditioning compartments, ensuring that all treatments were matched as closely as possible between compartments.

The schedule during the conditioning phase consisted of oral administration of drug or vehicle on alternate days. The interval between conditioning days was no less than 24 h and no more than 72 h, a procedure that does not influence CPP in our experimental conditions (Cervo *et al*. 2002, 2005).

Thus, on odd conditioning days rats were given GHB before being confined to the randomly designated drug side. On the even conditioning days, rats were treated with vehicle and confined to the opposite side. This daily order of exposure to drug and vehicle was counter-balanced for the rats in each group. Control animals received vehicle in both compartments.

On the test day, neither drug nor vehicle was administered. Each rat was placed in the centre of the aluminium platform separating the two compartments, with free access to both sides of the box, and the time spent in each compartment was recorded over a 15-min period. The difference in time spent in the drug- and vehicle-associated compartments in the final test session was taken as a measure of place conditioning.

Evaluation of GHB-induced CPP

To assess whether GHB induced CPP, 30 rats were assigned to three groups of equal size. Two groups received 10 conditioning sessions with oral GHB (175 or 350 mg/kg) every other day (see Martellotta *et al*. 1997) administered 30 min before being confined to the designated drug side for 30 min. On intervening days, animals received vehicle and were confined to the vehicle-designated compartment. The third group received vehicle in both assigned drug- and vehicle-associated compartments every day.

Since the aim of our study was to evaluate the effect of GHB-induced CPP following its injection into localized brain regions, and in view of the unspecific tissue damage that may result from repetitive intracerebral injections, in a second experiment we tried to decrease the number of conditioning sessions required to achieve a significant CPP by oral GHB. To this end, we shortened the time between conditioning sessions, in an attempt to make the association between drug and compartment more stringent. Because GHB has a short half-life (Kaufman & Nelson, 1987), we could reliably perform two conditioning sessions a day, one with the drug and one with the vehicle. Thus, three separate groups of 10 naive rats received 350 mg/kg GHB orally 30 min before being confined to the randomly designated drug side for 30 min. Three different conditioning schedules were examined: 5, 8 and 10 GHB pairings, with rats receiving the vehicle on alternative consecutive pairings. A fourth group of equal size received vehicle in both the randomly assigned drug and vehicle compartment on every session.

GHB-elicited CPP could be confounded by its ability to induce sedation and amnesia (Lobina *et al*. 2001; Miotto *et al*. 2001; Schwartz *et al*. 2000; Varela *et al*. 2004). Therefore, to exclude a possible GHB-induced state-dependence (Overton, 1978; Tzschentke, 2007; Weingartner *et al*. 1978), four groups of rats underwent five conditioning sessions twice daily with oral 350 mg/kg GHB or vehicle as described above, but were tested 30 min after being treated with oral GHB (350 mg/kg) or vehicle.

GHB application in VTA and NAc

GHB was bilaterally injected in the VTA (0, 10, 30, 100, 300 μg/0.5 μl per side) or in the NAc (0, 10, 30, 100 μg/0.5 μl per side) in two groups of 40 and 32 rats, respectively. Animals were deeply anaesthetized with equitesin and placed in a Kopf stereotaxic frame (Kopf, USA). Guide cannulae made of 23-gauge stainless-steel tubing were secured by acrylic dental cement anchored to three stainless-steel screws fixed to the skull. To prevent clogging, 30-gauge stainless steel stylets were placed in the guide cannulae until the animals were given local GHB infusions.

During the 1-wk recovery, rats received an ampicillin injection (45 mg/kg s.c.) daily for the first 5 d and were handled every day. For drug infusion, the stylets were withdrawn and replaced by injection needles (30-gauge stainless-steel tubing) terminating 2 mm below the tip of the guides. Stereotaxic coordinates for the VTA and NAc cannulae were -6.7 mm AP, ± 0.8 mm L, -8.4 mm H, and $+2.2$ mm AP, ± 1.4 mm L, -7.0 mm H, respectively (from bregma) (Paxinos & Watson, 1998). Injections were made using an infusion pump (Harvard Instruments, USA).

After the 15-min preconditioning stage, rats received five conditioning pairings, each consisting of

30-min exposures to two distinct compartments of the CPP chambers. Two conditioning sessions per day were conducted during the light phase of the light/dark cycle: one in the early morning, and one in the afternoon. The injection needle was attached to the pump syringe by polythene tubing filled with either drug or vehicle. The volume of each local infusion was 0.5 μl over 30 s, and the injection needles were left inside the guide cannulae for 1 min before being withdrawn to allow diffusion from the tip and to prevent reflux of the solution.

The rats were then immediately placed into the randomly assigned drug compartment for 30 min. In the afternoon, animals received a sham local injection using an identical procedure as described above, except that the injection cannulae were disconnected from the infusion pump. The injection needle was the same length as the guide cannulae, so the tissue was preserved from mechanical damage. This procedure was utilized instead of a saline infusion to minimize potential damage that might result from repeated local infusions (Baker *et al*. 1998). The drug-designated compartment and the order of treatment (vehicle/drug *vs*. sham local injection) were counter-balanced across groups. On the day following the last conditioning session, animals were tested for CPP. At the end of the experiments, rats were deeply anaesthetized with 200 mg/kg pentobarbital and killed by decapitation. Cannula placements were verified in Cresyl Violet-stained sections, and data from two NAc-implanted and four VTA-implanted rats were discarded because of non-optimal cannula placement.

Drugs

The doses of GHB (CT Laboratories, Italy) are expressed as sodium salt. For self-administration, GHB was dissolved in sterile saline to produce a stock solution of 100 mg/ml. This stock solution was further diluted with sterile saline to produce the appropriate concentration of GHB (mg/kg per 0.1 ml infusion), adjusted for each rat according to body weight. For local injections, GHB was dissolved in sterile saline. Drug solutions were prepared in a laminar airflow cabinet and filtered through a 22-μm syringe filter. For oral administration, GHB was dissolved in sterile water and given by a gavage in a volume of 2 ml/kg. All solutions were freshly prepared immediately before use.

Statistical analysis

The number of infusions, active and inactive lever presses earned during the self-administration and the extinction sessions were analysed by mixed factorial analysis of variance (ANOVA), with GHB doses as between factor and sessions as within factor. *Post-hoc* comparisons were made by Newman–Keuls (NK) test. The mean number of infusions earned during the 14 d GHB self-administration in different light/dark cycle conditions was compared by two-way ANOVA, with light/dark phase and GHB dose as main factors, followed by NK test.

In the CPP experiments, to exclude any unconditioned preference for one or the other side of the apparatus, we compared the time spent by rats in the black/white and grey compartments during the preconditioning session using paired Student's t test. One-way ANOVA followed by Dunnett's test comparing the difference in time spent in the drug-paired and the vehicle-associated compartments in the final test session (i.e. preference for drug-associated side) was used to assess the effects of GHB when administered alone. The two-way ANOVA followed by NK *post-hoc* comparison was used to evaluate differences in preference for drug-associated side when the number of GHB conditioning sessions was varied. The effect of GHB given during the test session on GHB-induced CPP was analysed by two-way ANOVA followed by NK test. The same tests were used to determine any change induced by the different treatments on the time the animals spent in the central zone of the CPP apparatus.

Results

GHB and self-administration

The mixed factorial one-way ANOVA found a significant effect of GHB on the number of infusions and active lever presses but not on the inactive lever presses [infusions: $F_{\mathrm{GHB}}(8,54)=5.5$, $p<0.05$; $F_{\mathrm{sessions}}(13,104)=38.6$, $p<0.05$; $F_{\mathrm{GHB\times sessions}}(104,702)=2.5$, $p>0.05$; active lever presses: $F_{\mathrm{GHB}}(8,54)=4.8$, $p<0.05$; $F_{\mathrm{sessions}}(13,104)=71.7$, $p<0.05$; $F_{\mathrm{GHB\times sessions}}(104,702)=2.2$, $p>0.05$). *Post-hoc* comparisons (NK test) revealed that only 0.01 mg/kg per infusion maintained GHB self-administration, although not on every test day. In particular, a significant increase in the number of infusion (Fig. 1*a*) and active lever presses (data not shown) was found on days 1, 2, 4, 8, 9, 10 and 13 ($p<0.05$ compared to vehicle), but not on days 3, 5, 6, 7, 11, 12 and 14. To visualize the entire dose–response curve, Fig. 1*b* illustrates the mean number of different GHB doses and as infusions during the 14 d self-administration, while the numbers of active and inactive lever

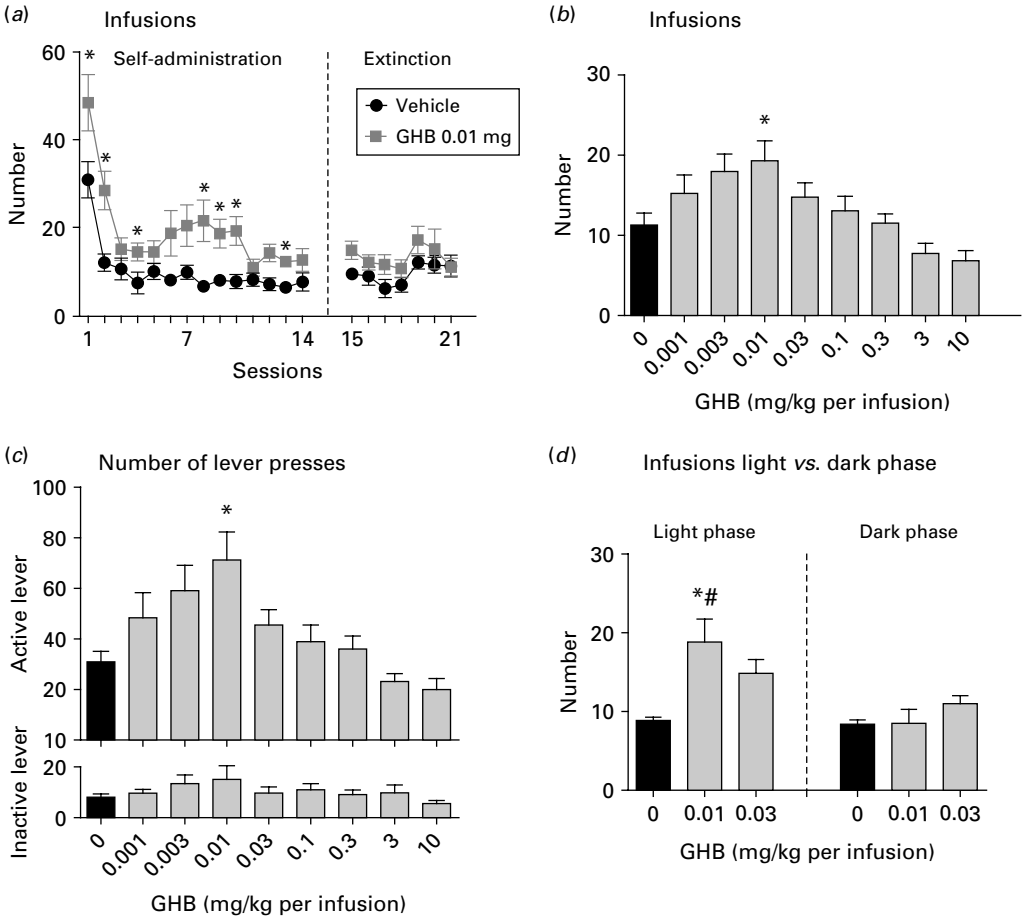

Fig. 1. Gamma-hydroxybutyric acid (GHB) self administration. (*a*) The mean number of 0.01 mg/kg per infusion earned under FR2 20-s timeout in daily 2 h self-administration sessions and during extinction are illustrated. (*b*) The mean (±S.E.M.) number of GHB infusions earned in the 14 d self-administration. For comparison, the mean (±S.E.M.) number of active and inactive lever presses performed in the 14 d self-administration is shown in panel (*c*). (*d*) The mean (±S.E.M.) number of GHB infusions (□) earned during the 14 d self-administration during the light or the dark phases of the light/dark cycle compared to vehicle (■). Data in panel (*a*) were analysed by mixed factorial ANOVA (with GHB dose as between factor and sessions as within factor), whereas data in panel (*d*) were analysed by two-way ANOVA (with light/dark phase and GHB dose as main factors). *Post-hoc* comparisons were made by NK test. (See text for further details.) * $p<0.05$ *vs.* vehicle; # $p<0.05$ *vs.* 0.01 mg dark phase.

presses are depicted in Fig. 1*c*. *Post-hoc* comparisons also revealed that a lower number of 3 and 10 mg/kg per infusion GHB was earned on day 1 (data not shown) ($p<0.05$ compared to vehicle). Moreover, no significant differences in the number of infusions (see Fig. 1*a*) and active and inactive lever presses (data not shown) was found during the extinction sessions.

Figure 1*d* shows the effect of 0.01 and 0.03 mg/kg per infusion GHB when available for self-administration during the dark period of the light/dark cycle. Two-way ANOVA found a significant effect of the light/dark phase [$F(1,30)=12.0$, $p<0.05$] and of GHB [$F(2,30)=4.4$, $p<0.05$]. *Post-hoc* comparisons (NK test) revealed that during the light phase rats earned more infusions of 0.01 but not 0.03 mg/kg per infusion compared to vehicle ($p<0.05$) and compared to rats self-administering the same dose during the dark phase ($p<0.05$). No difference between the number of GHB and vehicle infusions was observed during the dark phase of the light/dark cycle. Moreover, no changes in the number of infusions, and of active or inactive lever presses were observed during the period of extinction (data not shown).

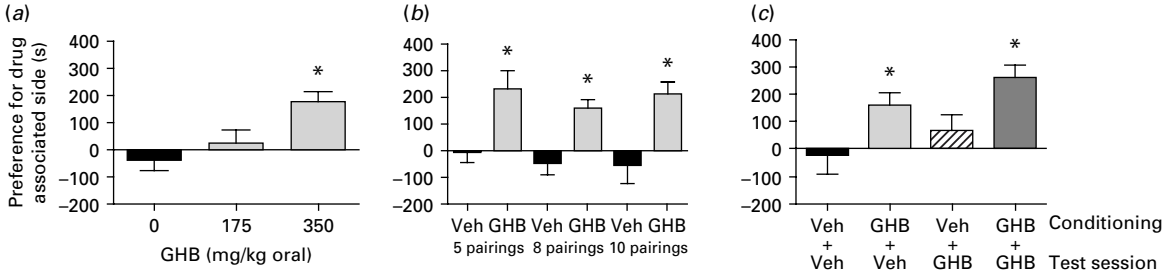

Fig. 2. Gamma-hydroxybutyric acid (GHB)-induced conditioned place preference (CPP). (*a*) Histograms show the mean time (±S.E.M.) spent by 10 rats in the GHB-paired side minus the time in the vehicle-paired side (10 GHB pairings, one every other day). Data were analysed by one-way ANOVA followed by Dunnett's test. (*b*) Comparison of the effect of 5, 8 and 10 GHB daily pairings with 350 mg/kg GHB on CPP. Histograms show the mean time (±S.E.M.) spent by 10 rats in the GHB-paired side minus time in the vehicle (Veh)-paired side. (*c*) Effects of 350 mg/kg GHB or vehicle given 30 min before the measurement of the CPP induced by 350 mg/kg GHB or vehicle (five pairings once daily). Data are the mean time (±S.E.M.) spent by eight rats in the GHB-paired side minus time in the vehicle-paired side. In panels (*b*) and (*c*), data were analysed by two-way ANOVA with number of conditionings and treatments (*b*) or with conditioning and test sessions (*c*) as main factors. *Post-hoc* comparison between the means was done by NK test. * $p<0.05$ *vs.* vehicle-treated group.

Overall, therefore, these data indicate that GHB is unable to reliably maintain intravenous self-administration in naive food-restricted rats, when it is available under a FR2 20-s timeout schedule.

GHB and CPP

No difference was found in the unconditioned preference for grey or black/white compartment (385.3 ± 3.7 s or 390.8 ± 4.1 s, $n=188$, respectively; $p>0.05$, paired Student's t test). Moreover in all experiments, the animals spent from 97.5 ± 7.6 to 146.0 ± 28.4 s in the central zone of the CPP apparatus, and no difference could be detected for the various experimental groups ($p>0.05$, Dunnett's or NK test). Thus, the preference for the GHB-paired side in the different experiments reported below was not significantly influenced by changes in time spent in the central zone.

Figure 2*a* shows the effects of 10 conditioning sessions with 175 and 350 mg/kg GHB given orally. One-way ANOVA indicated a significant effect of treatment [$F(2,27)=6.6$, $p<0.05$]. *Post-hoc* comparisons demonstrated that 350 mg/kg GHB, but not 175 mg/kg, induced a clear CPP, with treated animals spending more time in the drug-associated compartment ($p<0.05$ *vs.* control group, Dunnett's test).

Figure 2*b* shows the place conditioning induced by 5, 8 and 10 daily pairings with 350 mg/kg GHB given orally, with these rats receiving the vehicle on alternative consecutive pairings. A significant effect of treatment [$F(1,54)=34.1$, $p<0.05$] but not of sessions was found. *Post-hoc* analyses revealed that independently from the number of conditioning sessions rats spent more time in the GHB-associated compartment ($p<0.05$ *vs.* respective control group, NK test) for both 5, 8 and 10 pairings.

As shown in Fig. 2*c*, the CPP induced by five pairings with oral 350 mg/kg GHB was found regardless of whether testing was carried out in the drugged or un-drugged state ($p<0.05$ *vs.* respective vehicle-treated group, NK test), thus excluding potential state-dependent effects as an explanation of the GHB-induced CPP [$F_{cond}(1,28)=11.6$, $p<0.05$; $F_{test}(1,28)=3.0$, $p>0.05$; $F_{cond\times test}(1,28)=0.1$, $p>0.05$, two-way ANOVA].

The effect on CPP of GHB locally applied in the VTA or the NAc is reported in Fig. 3. Five injections of GHB into the VTA but not into the NAc induced a significant CPP [$F_{VTA}(3,26)=4.5$, $p<0.05$; $F_{NAc}(3,26)=0.3$; $p>0.05$, one-way ANOVA]. *Post-hoc* comparisons indicated that VTA-injected 30 and 100 μg GHB, but not 10 and 300 μg, induced a significant CPP with treated animals spending more time in the drug-associated compartment ($p<0.05$ compared to vehicle-treated group, Dunnett's test).

Discussion

The main finding of this study is that GHB induces CPP when injected directly into the VTA but not in the NAc, demonstrating that the former but not the latter brain area is likely to represent one of the primary sites of action for the rewarding properties of this drug. Moreover, our study is the first to demonstrate that the GHB-elicited CPP is not influenced by GHB-induced

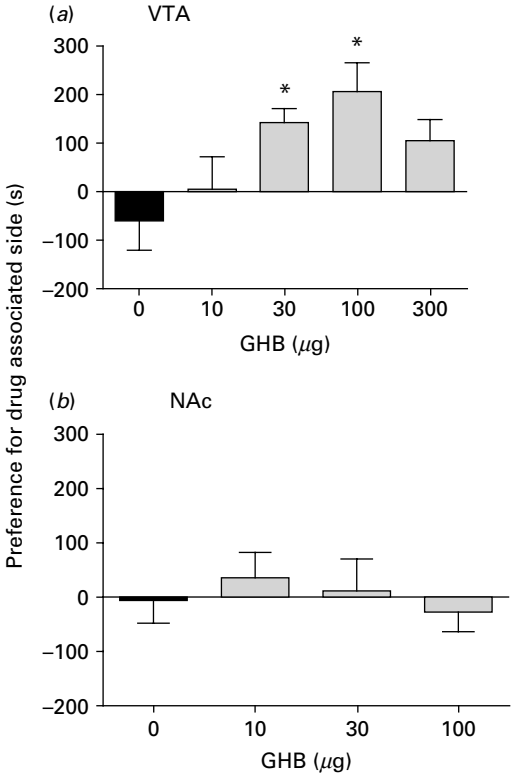

Fig. 3. Gamma-hydroxybutyric acid (GHB) elicits CPP when injected directly in the VTA (*a*) but not in the NAc (*b*). Rats received five pairings with GHB once daily. Histograms represent the mean time (±S.E.M.) spent by 7–8 rats in the GHB-paired side minus the time in the vehicle-paired side. Data were analysed by one-way ANOVA followed by Dunnett's test. * $p < 0.05$ *vs.* vehicle-treated group.

state-dependence, since CPP could be observed in animals that were both in a 'drugged' and 'undrugged' condition.

The dopaminergic VTA neurons project to basal forebrain structures, such as the NAc, amygdala, and frontal and limbic cortex (Camí & Farré, 2003; Gardner & Lowinson, 1993; Maldonado, 2003). Activation of VTA neurons, together with the resulting increase in dopamine output in the innervated structures, has been suggested to mediate the rewarding properties of drugs of abuse (Koob *et al*. 1998; Robbins & Everitt, 1999; Wise & Bozarth, 1987). The remaining VTA neurons are mainly GABAergic and function either as local interneurons to modulate the activity of the dopaminergic cells or as projection neurons providing an inhibitory input to the cortex and the NAc (Carr & Sesack, 2000; Kalivas, 1993). With respect to mechanisms of GHB action, *in-vitro* evidence has demonstrated bi-directional effects of $GABA_B$ receptor activation on the VTA output. Cruz *et al*. (2004), and more recently Labouèbe *et al*. (2007) showed that, owing to differences in distribution of inwardly rectifying K^+ channel subunits, $GABA_B$ receptor agonists hyperpolarize GABAergic neurons with a smaller ED_{50} than dopaminergic neurons. Thus, these authors hypothesized that at doses typically used during its recreational use GHB would preferentially inhibit VTA GABAergic neurons, with a resulting disinhibition of dopaminergic neurons, leading to an increased VTA output that would underlie GHB's rewarding properties. With higher GHB doses leading to higher brain concentrations, the VTA dopaminergic neurons would also be directly hyperpolarized, with a resulting decrease in VTA output, which may explain the beneficial properties of GHB in alcoholics (Addolorato *et al*. 1999; Caputo *et al*. 2005; Gallimberti *et al*. 1989; Nava *et al*. 2007) and our finding of its inability to induce CPP when injected at higher concentrations in this brain region. This scenario is also supported by the finding that intravenous administration of relatively high doses of GHB either depresses or increases firing of VTA dopaminergic neurons with a high or low baseline firing rate, respectively (Pistis *et al*. 2005). Finally, the lack of GHB action in the NAc supports the specificity of the effects we observed when injected in the VTA, and would exclude a direct involvement of the NAc in the rewarding properties of this drug when administered orally or systemically. However, because of the GABAergic projections from the VTA to the cortex (Carr & Sesack, 2000) and the NAc (Van Bockstaele & Pickel, 1995), an indirect involvement of the latter two brain regions to the rewarding action of GHB cannot be excluded (Johnson & North, 1992; Kalivas, 1993).

Another major result of our study is that GHB is only a weak reinforcer when evaluated in intravenous self-administration, both during the light and the dark phases of the light/dark cycle, with only 0.01 mg/kg per infusion GHB being self-administered at higher frequency compared to vehicle, and not at each evaluated day. Moreover, when available at the highest doses (3 and 10 mg/kg per infusion) GHB reduced the number of infusions compared to vehicle, at least during the first day of self-administration, suggesting a potential sedative or aversive activity of this drug, as hypothesized in monkeys (Woolverton *et al*. 1999). The fact that no extinction was observed when GHB was substituted with vehicle (at least in animals that self-administered 0.01 mg/kg per infusion GHB), together with the inability of this drug to be self-administered during the dark phase of the light/dark cycle, support our conclusion of a weak reinforcing property of GHB as evaluated in this behavioural procedure.

Nevertheless, caution should be used in interpreting potentially contradictory results, since it is impossible to demonstrate that a drug will not produce CPP or be self-administered under any condition at different doses in various species. Indeed, although the lack of a consistent self-administration of intravenous GHB in our study agrees with previous results in monkeys (Beardsley *et al*. 1996; Woolverton *et al*. 1999), it has also been reported that GHB is intravenously self-administered by mice (Fattore *et al*. 2001; Martellotta *et al*. 1998). However, the methodology between these studies and our study differs greatly. Whereas we investigated the ability of GHB to maintain self-administration, Martellotta *et al*. (1998) and Fattore *et al*. (2001) studied only acute GHB self-administration, i.e. each mouse underwent only one self-administration session. In our experiments, rats indeed self-administered GHB more than vehicle during the first sessions (at least at 0.01 mg/kg per infusion), but this does not necessarily mean that the compound was maintaining a self-administration behaviour, as indicated by the fact that in the subsequent 13 d there was no overall higher infusion rate of GHB compared to vehicle. Moreover, no extinction was evident after replacing GHB with vehicle. All these findings, therefore, indicate that in rats, under the conditions used in our study, GHB is, at most, a weak reinforcer. However, it cannot be excluded that different conditions, i.e. longer sessions and/or time out, could bring about a more robust self-administration.

In humans, GHB is self-administered orally. Oral self-administration of GHB, in preference to water, has been observed in rats (Colombo *et al*. 1995), although periods of voluntary abstinence were seen during the tests and the self-administration was often irregular. In a recent study, GHB was also orally self-administered by mice (Labouèbe *et al*. 2007), although comparison with other studies is difficult due to the paucity of information on preconditioning and test procedures. Thus, the reasons for the different effects observed when GHB is given orally or made available as intravenous infusion in operant self-administration are at present unclear, although difference in metabolism, speed of brain penetration, cerebral distribution and/or modality of drug availability may account for the reported differences.

In conclusion, our results demonstrate for the first time that GHB injected directly into the VTA can be rewarding in naive rats. Together with recent *in-vitro* findings (Cruz *et al*. 2004; Labouèbe *et al*. 2007), these data suggest that the rewarding properties of GHB mainly occur via disinhibition of VTA dopaminergic neurons.

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Statement of Interest

None.

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