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ORIGINAL INVESTIGATION

Residual social, memory and oxytocin-related changes in rats following repeated exposure to γ-hydroxybutyrate (GHB), 3,4-methylenedioxymethamphetamine (MDMA) or their combination

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Abstract
Rationale There has been little investigation of the possible lasting adverse effects of γ-hydroxybutyrate (GHB).
Objectives This study aims to study whether GHB produces residual adverse effects on memory and social behaviour in rats and lasting changes in brain monoamines and oxytocin-related gene expression.
Methods Rats received daily intraperitoneal injections of GHB (500 mg/kg), methylenedioxymethamphetamine (MDMA; 5 mg/kg) or their combination (GHB/MDMA) over ten consecutive days. Locomotor activity and body weight were assessed during the dosing period and withdrawal-related anxiety was assessed 24 h after drug cessation. After a washout of 4 weeks, rats were tested on the emergence, social interaction, and object recognition tasks over a 2-week period. Monoamine levels in cortex and striatum, and hypothalamic oxytocin and oxytocin receptor mRNA, were then assessed.
Results MDMA and GHB/MDMA caused modest sensitization of locomotor activity over time, while sedative effects of GHB diminished with repeated exposure. GHB-treated rats showed reduced social interaction 24 h after the final dose, indicating GHB withdrawal-induced anxiety. All drug-treated groups displayed residual deficits in social interaction and object recognition. No changes in monoamine levels were detected 8 weeks post-drug. However, MDMA pre-exposure increased hypothalamic oxytocin mRNA while GHB pre-exposure upregulated oxytocin receptor mRNA. GHB/MDMA pre-exposure caused intermediate changes in both of these measures.
Conclusions GHB treatment caused residual impairments in memory and social behaviour and increases in anxiety, paralleling the lasting adverse effects of MDMA. Both drugs caused lasting neuroadaptations in brain oxytocin systems and this may be related to the long-term social interaction deficiencies caused by both drugs.

Keywords GHB · MDMA · Oxytocin · Memory · Anxiety · Social behaviour · 5-HT

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Introduction

γ-hydroxybutyrate (GHB) is a popular party drug that causes distinctive prosocial, euphoric and sedative effects in users (Sumnall et al. 2008). GHB exerts many of its functional effects through $GABA_B$ receptors (Kaupmann et al. 2003; Queva et al. 2003; van Nieuwenhuijzen et al. 2009b) but also binds to its own G protein-coupled GHB receptor that is densely expressed in the hippocampus (Andriamampandry et al. 2003). GHB increases 5-hydroxytryptamine (5-HT) turnover and can modulate dopamine (DA) release by binding to $GABA_B$ receptors on midbrain dopaminergic neurons (Cruz et al. 2004). GHB

users sometimes combine the drug with 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy"), which is another frequently used party drug (Lee and Levounis 2008; Uys and Niesink 2005). Some users report that GHB enhances the "high" and prevents negative effects during the "comedown" from MDMA (Uys and Niesink 2005). This effect may reflect an overlapping action of GHB and MDMA on brain 5-HT, DA and neuropeptide systems. However, the interactions between GHB and MDMA are only beginning to be documented at a behavioural and neurochemical level (van Nieuwenhuijzen et al. 2009a; van Nieuwenhuijzen and McGregor 2009).

The evidence for lasting harms arising from party drug use are a matter of substantial current debate and can be usefully investigated using animal models (Green et al. 2008; McGregor et al. 2008; Nutt 2009). Studies from several independent groups indicate that rats briefly exposed to MDMA display long-term adverse effects including increased anxiety, decreased social interaction and impaired object recognition memory (Able et al. 2006; Fone et al. 2002; McGregor et al. 2003b; Morley et al. 2001; Piper et al. 2008). These effects of MDMA in laboratory animals agree with some reports of cognitive deficits and emotional problems in human MDMA users, although these human studies are frequently compromised by the methodological problems arising from the polydrug use that is typical of MDMA using populations (Rogers et al. 2009).

The cognitive deficits observed in laboratory animals following MDMA exposure could reflect a particular vulnerability of the hippocampus to MDMA-related effects (Gouzoulis-Mayfrank et al. 2003), with oxidative stress (Frenzilli et al. 2007) and serotonergic alterations (McGregor et al. 2003a; O'Shea et al. 2006) documented in the hippocampus after MDMA treatment. Interestingly, GHB and its pro-drug 1,4-butanediol can also increase oxidative stress in the cortex and hippocampus (Pedraza et al. 2009; Sgaravatti et al. 2007, 2009) leading to widespread neuronal loss in these regions (Pedraza et al. 2009). In rodent models, GHB caused acute impairment in working memory (Kueh et al. 2008) and spatial learning (Pedraza et al. 2009; Sircar et al. 2008). GHB can also cause acute cognitive disturbance in human users, although this can be difficult to dissociate from the strong sedative effects of the drug (Carter et al. 2006). To date, it is unclear whether there are lasting cognitive deficits arising from GHB use and addressing this issue was a major aim of the present study.

A further aim was to examine whether GHB exposure causes chronic deficits in social behaviour similar to those produced by MDMA. The lasting effects of MDMA on memory and social behaviour can clearly occur in the absence of the characteristic 5-HT depletion that results from high-dose MDMA treatment (Clemens et al. 2007; Fone et al. 2002; McGregor et al. 2003a). This suggests that non-serotonergic mechanisms may be implicated in MDMA-related harms (Baumann et al. 2007; McGregor et al. 2008; Piper et al. 2008). Recent evidence indicates that the acute prosocial effects of MDMA in both rats and humans may involve the release of oxytocin (Dumont et al. 2009; Thompson et al. 2007), a neuropeptide that powerfully regulates mammalian affiliative behaviour (Neumann 2008). There is also presumptive evidence that the acute prosocial effects of GHB (Navarro et al. 2008) involve oxytocinergic mechanisms (Geldenhuys et al. 1968; van Nieuwenhuijzen et al. 2009b). Conversely, the residual adverse effects of MDMA and other drugs on social behaviour have been hypothesised to reflect lasting neuroadaptations in brain oxytocin neurons resulting from acute stimulation of this system (McGregor et al. 2008). In agreement with this hypothesis, long-term changes in brain oxytocin systems have been documented following chronic exposure to alcohol (McMurray et al. 2008; Silva et al. 2002), opiates (You et al. 2000) and cocaine (Kovacs et al. 1998).

The principal aim of the current study was therefore to examine whether GHB causes MDMA-like residual changes in emotion and cognition in laboratory rats, and whether such changes might be associated with alterations in oxytocin (OT) and oxytocin receptor (OTR) mRNA expression in the hypothalamus and/or changes in monoamine levels. The lasting effects of combined MDMA and GHB treatment were also of interest given the current use of this cocktail in drug using populations.

Materials and methods

Animals

The subjects were 48 experimentally naïve male albino Wistar rats (Animal Resource Centre, Perth, Australia) weighing 220–300 g at the start of the experiment. Rats were allocated to one of four treatment conditions (VEH, MDMA, GHB or GHB/MDMA) matched for body weight. The rats were housed in groups of eight in large laboratory cages with raised lids (64×40×22 cm) in a temperature (21°C) controlled colony room maintained on a reverse 12-h light/12-h dark cycle (lights on at 7:00 pm). The four different treatment conditions were equally represented within each home cage of eight rats.

The rats had ad libitum access to tap water and food in their home cages. All testing took place in the dark cycle. All procedures were approved by the University of Sydney Animal Ethics Committee in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

Drugs and doses

GHB was purchased from Sigma (Castle Hill, NSW, Australia). Racemic 3,4-MDMA as the hydrochloride salt was purchased from the Australian Government Analytical Laboratories (Pymble, NSW, Australia). Drugs were dissolved in 0.9% saline and injected intraperitoneally in a volume of 2 ml/kg. Each rat received two injections each day with the treatment conditions as follows: (a) GHB (500 mg/kg)+vehicle, (b) MDMA (5 mg/kg)+vehicle, (c) GHB (500 mg/kg)+MDMA (5 mg/kg), or (d) vehicle+vehicle. The vehicle used for all injections was 0.9% saline (2 ml/kg). Drugs were administered once daily for 10 days. Doses selected were in the moderate range and were based on previous studies showing behavioural effects of these doses (Thompson et al. 2009; van Nieuwenhuijzen et al. 2009b). The MDMA dose regime used here was considered to be too low to produce significant long-term changes in monoamine levels associated with possible neurotoxicity (Baumann et al. 2007; O'Shea et al. 1998).

The drug administration and testing sequence is shown in Table 1.

Locomotor activity

Immediately after injection, rats were placed in small dark holding boxes (45×30×15 cm) with a thick layer of wood shavings as bedding. Twenty minutes later, they were placed in test chambers for 1 h—the 20 min delay being used to ensure that any locomotor effects of drugs would be evident when testing commenced. The test chambers (60×26×36 cm) had black Perspex walls and a black metal grid floor and were fitted with an overhead miniature infrared-sensitive video camera (Jaycar Ltd, Australia, model QC3468) connected to a PC (Dell) running LABVIEW 8.0 software (National Instruments, Australia). Automated video tracking software (Motion Mensura, Cooks Hill, NSW, Australia) measured the distance travelled by the rat over time. The testing room was illuminated with red light (40 W) and ambient temperature was maintained at 28±1°C using fan forced heaters (Sydney, NSW, Australia). This temperature was used to simulate the hot conditions typical of nightclubs in which humans often consume GHB and MDMA.

Withdrawal effects: social interaction

Rats were tested in the social interaction test 24 h after the tenth and final drug injection (see Table 1). The test arena was a black melamine box (50×50×40 cm) illuminated by dim red light with a miniature overhead video camera that transmitted pictures to a remote monitor where an observer, blind to treatment conditions, scored behaviours. Rats were paired with a weight-matched novel conspecific from a different home cage but from the same treatment condition for 10 min (Morley et al. 2001). Each rat was tested twice, with a different conspecific each time with a minimum of 1 h between testing sessions. Scored behaviours included general investigation, anogenital sniffing and rearing (McGregor et al. 2003b).

Long-term effects: social interaction

After a drug-free period of 4 weeks (see Table 1), during which all rats remained in their home cages, and were weighed and handled every 48 h, rats were again tested for social interaction. Each rat was tested twice in an identical manner to that described above.

Long-term effects: the emergence test

On the day following the social interaction test, rats were tested on the emergence test to assess generalised anxiety. The apparatus consisted of a 120×120×45 cm white melamine arena with a 25×40×18 cm black melamine hide box. Testing was conducted as described previously (Morley et al. 2001). The rat was placed in the hide box at the start of the 5-min test period. Scored behaviours included latency to emerge, risk assessment (when the rat

Table 1 Experimental sequence

Experimental day	Weeks post-drug	Treatment or behavioural test
1–10	–	Daily drug treatment and locomotor activity testing
11	–	Social interaction test: test for drug withdrawal-induced anxiety
12–38	0–4	Washout period
39	4	Social interaction test: assess residual social deficits
40	4	Emergence test: assess residual anxiety
44–52	5–6	Habituation to novel object recognition test apparatus and procedure
53	6	Test for novel object recognition memory
67	8	Decapitation to remove brains

extended only its head/nose out of the hide box) and time spent in the open field (defined as when the hind limbs of the rat were outside of the hide box).

Long-term effects: novel object recognition memory

Two weeks after the emergence test (see Table 1), rats were tested on the novel object recognition test as previously described (Quinn et al. 2008). Four habituation sessions were given, one every other day for a total of 8 days, to familiarise rats with the testing arena and procedure prior to testing. Testing was conducted in a circular black plastic arena 75 cm in diameter and 40 cm high. Objects used included flowerpots, glass bottles, flasks and sugar dispensers. All objects were available in triplicate to prevent the use of odour cues; two objects were presented in the first trial (T1) and a third one in the recognition trial (T2). The arena and objects were cleaned with 70% ethanol solution after each rat. All combinations and locations (left or right for novel object) of objects were counterbalanced to reduce potential biases for particular locations or objects. Rats were placed in the arena with two identical objects for 3 min (T1). After 1 h, rats were again placed in the chamber with a copy of the object used in T1 and a novel object. Object exploration was scored when the rat's snout was directed towards, and within 2 cm of, the object. Climbing on or over the object was not recorded as exploration (Ennaceur and Delacour 1988).

Euthanasia and brain dissection

To assess long-term and possibly lasting neural changes after MDMA and/or GHB, the rats were killed after a drug-free period of 8 weeks. Their brains were rapidly removed and manually dissected over dry ice according to the method of Harkin et al. (2001). Samples from prefrontal cortex (PFC) and striatum were stored at −80°C until further analysis. The hypothalamus was placed in RNAlater (Ambion, Sydney, Australia) for 24 h before being stored at −80°C.

Neurochemical analysis

The PFC and striatum were weighed and homogenised in 1,000 and 500 μl, respectively, of 0.2 M perchloric acid containing 0.1% cysteine and 200 nM of internal standard (5-hydroxy-N-methyl-tryptamine) using a glass-Teflon homogeniser and Brinkman polytron. The homogenate was centrifuged for 10 min at 15,000×*g* (4°C) and the pellet discarded. A 10-μl aliquot was analysed for monoamines using high-performance liquid chromatography (HPLC) with electrochemical detection as previously described (McGregor et al. 2003a).

RNA extraction and real-time PCR analysis

Total RNA was extracted from each hypothalamus (N=12) using a PARIS kit (Ambion, Victoria, Australia) following the manufacturer's protocol. RNA quantity and quality were measured with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Eight samples (N=2/group) were rejected due to low RNA yield. RNA from the remaining 40 samples was reverse transcribed using 2 μl of 5×RT-PCR buffer (250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$ at pH 8.3), 0.35 μl 0.1 M DTT, 0.35 μl RNasin RNase inhibitor, 1 μl 10 mM dNTPMix (10 mM each dATP, dGTP, dCTP and dTTP), 0.5 μg of total RNA and RNAse free water making a final volume of 20 μl.

Samples were incubated at 37°C for 1 h, followed by heating at 70°C for 15 min to inactivate the reverse transcriptase then cooling at 4°C for 5 min. cDNA was stored at −20°C until further analysis. The target genes for real time-PCR analyses were OT and OTR. Pre-designed and labelled primer/probe sets (Rn00563503_m1 and Rn00564446_g1, Applied Biosystems, Victoria, Australia) were selected from Applied Biosystems' Assays-on-Demand product line (http://appliedbiosystems.com). Eukaryotic 18S ribosomal RNA (4319413E, Applied Biosystems, Victoria, Australia) was used as an endogenous control.

Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System (SDS, Applied Biosystems, Victoria, Australia) that was set to detect FAM and VIC reporter dyes simultaneously. Reactions of 25 μl volume containing TaqMan Universal Master Mix (2x, 4324018, Applied Biosystems, Victoria, Australia), Gene Expression Assay for the target gene (OT or OTR and 18S, 20×) and 1 μl of cDNA were set up in triplicate. Thermal cycling was initiated by denaturation for 10 min at 95°C, followed by 40 cycles of PCR consisting of heating to 95°C for 15s followed by annealing and extension at 60°C for 1 min.

The SDS software mathematically transforms the raw fluorescence data to establish a comparative relationship between the spectral changes in the passive reference dye and those of the reporter dyes. Based on these comparisons, the software generates cycle time and gene expression analyses compared with a calibrator sample. Results are presented as expression of OT or OTR relative to the expression of 18S rRNA.

Statistical analysis

Body weight differences between groups on specific days (day 1 and day 10 of drug treatment, 1 week and 8 weeks post-drug), were analysed using one-way ANOVA followed by Tukey's post hoc tests.

Locomotor activity during drug treatment was compared across groups using a two-way ANOVA with group as the

between–subjects factor and time (10 days of drug administration) as a repeated measure. This was followed by Tukey's post-hoc tests to identify specific group differences. To assess sensitization/tolerance effects, locomotor activity on day 1 and 10 of drug administration for each treatment group was compared with that in the control group using two-way ANOVA.

Behavioural and HPLC data were analysed using a one-way ANOVA followed by Tukey's post-hoc tests. These data were analysed using SPSS 16 for Macintosh. A significance level of $p<0.05$ was used for all of these analyses.

PCR data analyses were carried out with a relative quantification software tool (REST) using 18S as internal reference gene (Pfaffl et al. 2002). This software compares the level of transcript present in different samples and performs a pairwise fixed reallocation randomization test to determine statistically significant changes in transcript abundance. The number of iterations used for randomization was set at 2000 (Pfaffl et al. 2002). Results were considered significant when $p<0.05$.

Results

Body weight

Body weights in the four treatment groups across the ten consecutive drug treatment days and eight subsequent drug-free weeks are shown in Fig. 1. Groups did not differ on day 1 of treatment but by the tenth and final day of treatment there was a significant main effect of treatment ($F_{(3,44)}=4.87$; $p<0.01$) with post hoc tests showing that the GHB ($p<0.01$), MDMA ($p<0.05$) and GHB/MDMA ($p<0.05$) groups weighing less than the VEHICLE group. One week later, after seven drug-free days, there was still a significant main effect ($F_{(3,44)}=4.59$; $p<0.01$) with post hoc analysis showing that the GHB ($p<0.01$) and MDMA ($p<0.05$), but not the GHB/MDMA ($p=0.1$), groups remained significantly lower in body weight than the VEHICLE group. At the end of the drug-free period, 8 weeks later, there were no longer any significant group differences, although body weight in the GHB group tended to remain lower.

Acute locomotor activity

Analysis of the locomotor activity data across the ten consecutive drug treatments revealed a significant group effect ($F_{(3,44)}=22.13$; $p<0.0001$), with MDMA increasing overall activity ($p<0.0001$) and GHB decreasing ($p<0.01$) activity compared to VEHICLE controls (Fig. 2). Combined administration of GHB and MDMA produced no significant main effect in activity relative to VEHICLE treatment.

To assess tolerance/sensitization to the locomotor activity effects of GHB, MDMA, or GHB/MDMA we compared activity on day 1 to that on day 10 between VEHICLE and each of the three treatment groups. Significant interaction effects with GHB ($F_{(1,22)}=6.98$; $p<0.05$), MDMA ($F_{(1,22)}=5.10$; $p<0.05$) and GHB/MDMA ($F_{(1,22)}=5.79$; $p<0.05$) indicated that there was an increase in locomotor activity over time in these groups relative to VEHICLE treatment (Fig. 2).

Social interaction (acute withdrawal)

The social interaction test was used to assess for any signs of anxiety and withdrawal on the day following the final drug treatment. Results showed a main effect on general investigation ($F_{(3,44)}=4.15$; $p<0.05$), with GHB-treated rats showing reduced time spent in social interaction ($p<0.05$; Fig. 3a) compared to VEHICLE controls, MDMA- and GHB/MDMA-treated animals. Groups did not differ in time spent in anogenital investigation or rearing (Fig. 3a).

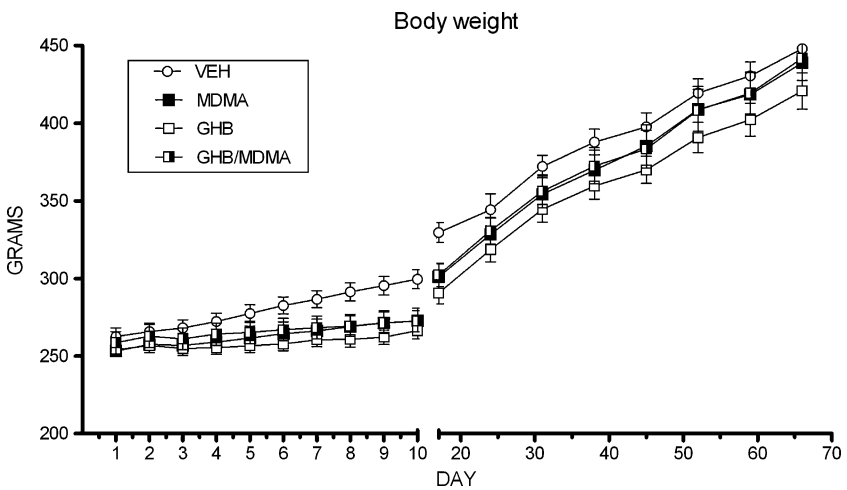

Fig. 1 Mean (SEM) body weight of the VEH (vehicle), MDMA, GHB and GHB/MDMA treatment groups during the 10-day drug treatment phase (body weight measured daily) and over the following eight drug-free weeks (body weight was measured weekly)

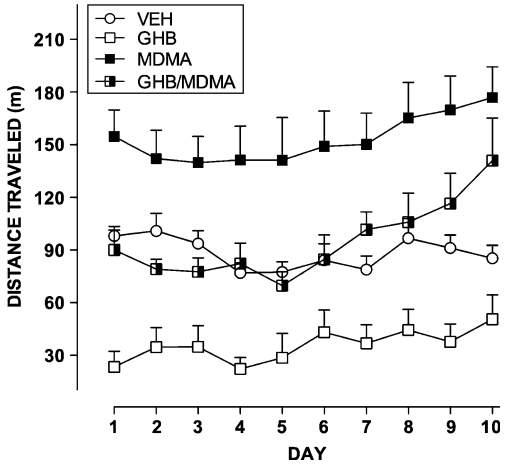

Fig. 2 Mean (SEM) total distance travelled in the 1 h after administration of VEH (vehicle), MDMA (5 mg/kg), GHB (500 mg/kg) or GHB/MDMA (MDMA 5 mg/kg + GHB 500 mg/kg) once daily over ten consecutive days. All drug-treated groups showed an increase in distance travelled over days relative to the vehicle group (N=12 per group, repeated measures ANOVA)

Social interaction (residual effects)

After a drug-free period of 4 weeks, during which the animals were handled and weighed every 48 h, rats were again tested for social interaction to ascertain the presence of residual social deficits (McGregor et al. 2003b). Results showed a significant main effect on general investigation ($F_{(3,44)}$=5.03; p<0.01). Compared to VEHICLE controls, all three groups decreased their time spent in general investigation (MDMA-treated (p<0.01), GHB- and GHB/MDMA-treated animals (p<0.05; Fig. 3b)). There were no group differences in time spent in anogenital investigation or rearing (Fig. 3b).

Emergence test (residual effects)

In the emergence test, there was a treatment main effect on latency to emerge ($F_{(3,40)}$=4.04, p<0.05) and time spent in the open field ($F_{(3,40)}$=4.43, p<0.01) (Table 2). GHB-treated rats took the longest time to emerge (p<0.05), with two out of 11 rats not emerging at all. They spent the least amount of time in the open field (p<0.05; Table 2). There were no differences in risk assessment between treatment groups.

Novel object recognition test (residual effects)

In T1, when rats were exposed to two identical objects, overall exploration time did not differ between groups (mean secs (SEM)=VEH 27.0 (1.9), GHB 25.2 (2.6), MDMA 23.0 (3.4), GHB/MDMA 21.34 (2.6)).

In T2, when both a familiar and a novel object were present, results showed a main effect of group on percentage of time spent exploring the novel object ($F_{(3,44)}$=20.05, p<0.0001), with all drug pre-treatment groups showing reductions in this measure relative to the VEHICLE group (p<0.001; Fig. 4a). There were no group differences in total time spent exploring the objects on T2 (Fig. 4b).

HPLC

One-way ANOVA, followed by post hoc tests, showed that drug pre-treatment did not result in long-term changes in the concentration of dopamine, noradrenaline or 5-HT, or their metabolites in the PFC or the striatum (Table 3).

Real-time PCR

Figure 5a shows the relative expression of OT mRNA in the hypothalamus of the various treatment groups, with increased expression of OT mRNA in the MDMA group compared to VEHICLE controls (p=0.019), while GHB and GHB/MDMA groups were not different compared to VEHICLE controls (p=0.40) and (p=0.24), respectively.

Figure 5b shows the relative expression of OTR mRNA in the hypothalamus for all groups. The GHB group had an increase in OTR mRNA compared to VEHICLE controls (p=0.009) neither MDMA (p=0.91) nor GHB/MDMA (p=0.13) were significantly different from VEHICLE controls.

Discussion

The primary aim of the present study was to assess whether adverse long-term behavioural and neurochemical changes occur in rats after repeated exposure to GHB, or GHB given in combination with MDMA. The lasting adverse effects of MDMA are well characterised in laboratory animals but to our knowledge this is the first study investigating the long-term residual effects of GHB on brain and behaviour. Overall, our results suggest that GHB, and GHB/MDMA combinations, have the capacity to produce MDMA-like deficits in memory and social behaviour.

During acute drug administration, MDMA increased locomotor activity, while GHB produced a decrease in activity. Sedative effects of moderate to high doses of GHB are well documented (van Nieuwenhuijzen et al. 2009b) as is the gradual development of tolerance to such effects with repeated administration in mice (Itzhak and Ali 2002) and rats (Bania et al. 2003). In contrast, MDMA administration resulted in hyperactivity, with some evidence of sensitization effects with repeated administration, as has been previously demonstrated (Colussi-Mas and Schenk 2008; Ludwig et al. 2008). However, overall sensitization effects were small in magnitude and may have been greater had an

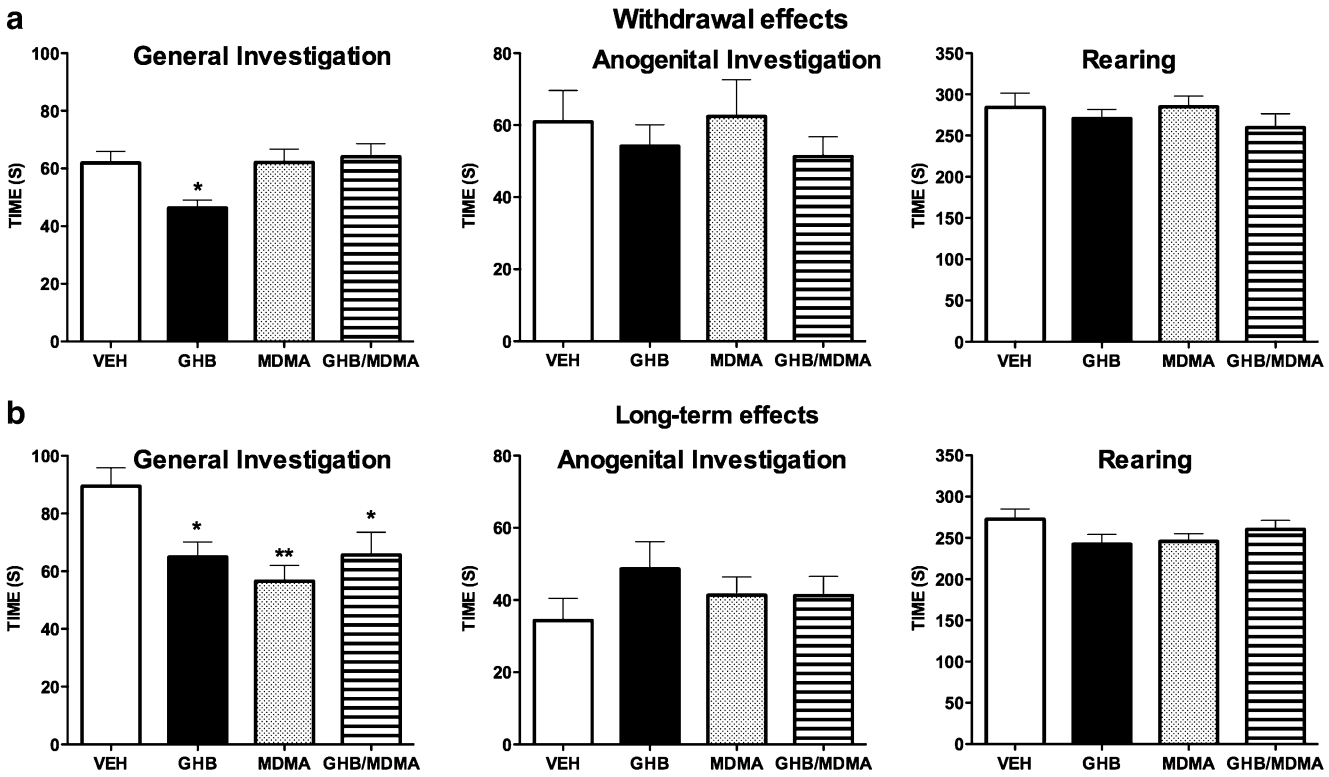

Fig. 3 Results from the social interaction tests. **a** Withdrawal effects were measured 24 h after tenth (final) drug administration. The GHB group spent less time engaged in general investigation, indicating an anxiogenic effect of drug withdrawal. **b** Test for residual changes 4 weeks after the final drug treatment. The three drug pre-treated groups had decreased time spent in general investigation indicating long-term social deficits. Bar graphs represent time (+SEM) in seconds. $N=12$ pairs per condition. One-way ANOVA, $*p<0.05$ and $**p<0.01$ compared to VEHICLE controls (Tukey's post hoc test)

intermittent rather than a daily schedule of drug administration been utilised (Robinson and Berridge 2003; van Nieuwenhuijzen et al. 2009a). Indeed, in our recently published study, in which the same MDMA and MDMA/GHB doses as used here were given weekly, rather than daily, locomotor sensitization effects were of much greater magnitude than those reported here (van Nieuwenhuijzen et al. 2009a).

Rats given a combination of GHB and MDMA exhibited an intermediate level of locomotor activity, consistent with our recently published results involving weekly dosing (van Nieuwenhuijzen et al. 2009a). At first, the activity levels in these rats were indistinguishable from control rats, suggesting that MDMA hyperactivity was opposed by GHB sedation leading to little overall effect on locomotor activity. However with repeated dosing, hyperactivity emerged, a likely consequence of the development of tolerance to GHB and some modest sensitization to MDMA effects. The ability of GHB to delay, but not necessarily prevent, MDMA-induced sensitization, is consistent with our recent findings where weekly exposure to this combination caused progressively greater locomotor stimulation over time with GHB/MDMA rats eventually attaining equivalent levels of locomotor hyperactivity to rats given MDMA alone (van Nieuwenhuijzen et al. 2009a).

Another effect of interest during the drug dosing period was loss of body weight in all three drug treatment groups relative to controls. The ability of MDMA to produce anorexia and body weight loss is well established in rodent models and is thought to involve stimulation of 5-HT_4 and 5-HT_{2C} receptors by the drug in key appetite regulating brain regions (Conductier et al. 2005; Jean et al. 2007). Reports of weight loss with GHB are less common, although popular use of the drug by body builders during the 1990s was predicated on its ability to release growth hormone, leading to increased lean muscular mass and

Table 2 Results from the emergence test

Treatment	Latency	Risk assessment	Open field
VEHICLE	33.1 (8.4)	43.1 (8.2)	142.5 (28.6)
GHB	95.7 (32.0)*	48.5 (6.7)	61.7 (21.8)*
MDMA	47.7 (8.2)	40.7 (5.2)	91.8 (19.3)
GHB/MDMA	21.9 (3.2)	42.5 (7.7)	138.3 (20.4)

Values represent mean (SEM) in seconds

*$P<0.05$ compared to VEHICLE one-way ANOVA followed by Tukey's Post hoc test

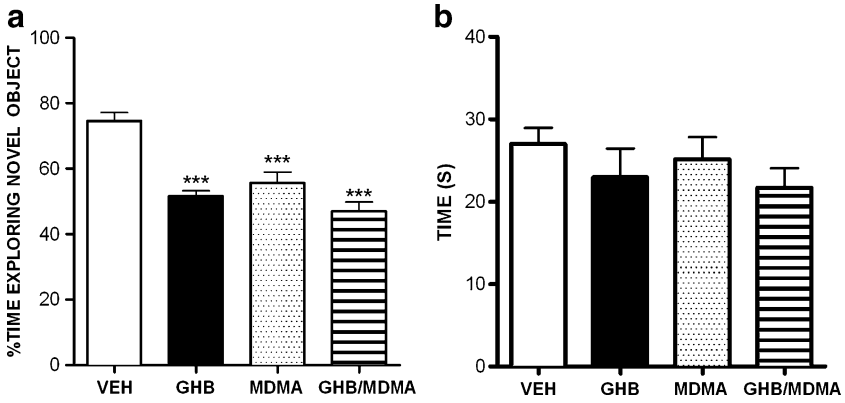

Fig. 4 Results from the novel object recognition test (6 weeks post drug) with a 1 hour delay between T1 (two objects the same) and T2 (one familiar and one novel object). **a** Represents mean (+SEM) of percentage time spent exploring the novel object and **b** Represents mean (+SEM) of total time spent exploring objects on T2 for VEHICLE, GHB, MDMA and GHB/MDMA pre-treated rats. $N=12$/group. One-way ANOVA, $^{***}p<0.001$ compared to VEHICLE controls (Tukey's post hoc test)

diminished body fat (Fuller et al. 2004). Indeed, patients treated chronically with GHB for narcolepsy show loss of body weight over time (Husain et al. 2009) and a recent open-label trial found GHB to be efficacious in promoting appetite control and body weight loss in people suffering from binge eating disorders (McElroy et al. 2010). The present study is, to our knowledge, the first report of chronic GHB-induced body weight loss in rats and it is interesting to note that this effect tended to persist for several weeks following cessation of GHB treatment. This indicates a relatively long lasting change in metabolism caused by the drug, an effect clearly worthy of further investigation.

Table 3 Monoamine levels in the prefrontal cortex and striatum

Region	GHB	MDMA	GHB/MDMA
Prefrontal cortex			
NA	105.1 (5.6)	102.2 (4.5)	103.0 (7.0)
5-HT	101.4 (3.1)	95.5 (2.9)	95.2 (2.5)
5-HIAA	101.0 (3.1)	97.4 (3.7)	94.7 (2.4)
Striatum			
NA	95.9 (4.1)	104.5 (10.4)	96.0 (8.0)
DA	99.8 (4.7)	92.6 (5.6)	93.7 (4.9)
DOPAC	101.4 (4.7)	93.2 (5.5)	97.0 (7.1)
5-HT	100.6 (4.3)	99.7 (5.2)	91.6 (7.3)
5-HIAA	95.0 (4.0)	89.3 (5.5)	91.5 (6.3)

Data represent % of VEHICLE group (mean (SEM))

Absolute mean (SEM) values for VEHICLE group in ng/g tissue for NA, 5-HT, 5-HIAA in prefrontal cortex: 724.7 (27.3), 631.6 (24.6), 239.7 (7.7). Absolute mean (SEM) values for VEHICLE group in ng/g tissue for NA, DA, DOPAC, 5-HT and 5-HIAA in striatum: 754.2 (56.7), 14416.7 (483.3), 850.5 (33.7), 863.1 (57.5), 441.4 (28.0)

NA noradrenaline, *DA* dopamine, *DOPAC* di-hydroxyphenlyacetic acid, *5-HT* 5-hydroxytryptamine, *5-HIAA* 5-hydroxyindolacetic acid

On the day following ten consecutive days of drug exposure, GHB-exposed rats displayed a decrease in time spent in social investigation, suggesting an acute anxiogenic withdrawal-like state in this group. Withdrawal symptoms after repeated administration of GHB have been described in baboons (Weerts et al. 2005), rats (Bania et al. 2003) and humans (LeTourneau et al. 2008) and the present data extend these findings to suggest a withdrawal syndrome after ten daily injections of 500 mg/kg GHB to rats. The increased social anxiety seen after abrupt discontinuation of GHB treatment is reminiscent of that seen in rats undergoing either ethanol (Knapp et al. 2007) or benzodiazepine (File et al. 1991) withdrawal, and consistent with there being some similarity in the neuroadaptations caused by these compounds on brain GABAergic systems. Interestingly, this withdrawal effect was not seen in rats given the MDMA/GHB combination, suggesting an amelioration of acute GHB withdrawal when co-administered with MDMA. It is however important to note that social interaction was not measured during GHB treatment, therefore it remains theoretically possible that the decrease in social interaction seen on day 11 represents a lingering acute effect of the drug.

At 4 weeks following drug exposure, all drug pre-treated rats showed reduced social interaction relative to controls, with present results confirming that MDMA induces long-term residual deficits in social interaction and object recognition in rats (Fone et al. 2002; McGregor et al. 2003b; Morley et al. 2001; Piper et al. 2008). Importantly, the current study shows for the first time analogous residual adverse effects on social interaction with GHB, either given alone or in conjunction with MDMA.

While acute cognitive disruption in rats and humans has been previously observed with GHB (Kueh et al. 2008; Pedraza et al. 2009; Sircar et al. 2008) this is the first report of a lasting residual memory deficit with this drug. The

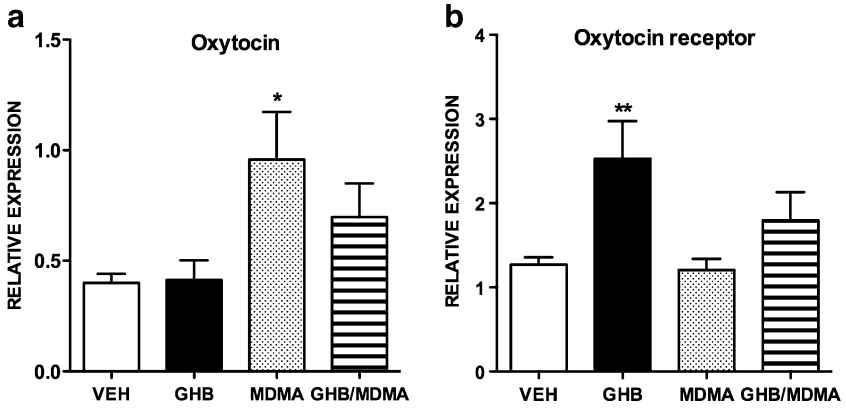

Fig. 5 Real-time quantitative PCR analysis of hypothalamic oxytocin and oxytocin receptor mRNA. Expression was normalised to 18S (endogenous control) and each sample was run in triplicate. Results showed an upregulation in OT mRNA in MDMA treated rats while pre-treatment with GHB resulted in an upregulation of OTR mRNA. Bar graphs represent the relative expression (+SEM) of **a** oxytocin mRNA and **b** oxytocin receptor mRNA in the hypothalamus of groups treated with VEHICLE, GHB, MDMA or GHB/MDMA 8 weeks earlier. N=10/group. *p<0.05 and **p<0.01 compared to VEHICLE controls using REST (see text for further details)

combined administration of GHB and MDMA did not impair memory to a greater extent than either drug alone, although this may well reflect a floor effect, given that investigation of objects by all drug treatment groups approached chance levels. Recent evidence suggests that the hippocampus is particularly vulnerable to oxidative stress caused by the administration of MDMA (Frenzilli et al. 2007) or GHB (Pedraza et al. 2009) with one recent study showing neuronal loss in the hippocampus associated with acute deficits in spatial learning following chronic GHB (Pedraza et al. 2009). The current findings extend the time frame under which deficits in memory are seen after GHB, with a 6-week window between drug administration and memory testing and show that these deficits occur without obvious changes in brain monoamines. Moreover the current study shows that these deficits can occur with repeated administration of relatively low to moderate doses of GHB, MDMA and GHB/MDMA.

The present results also confirm that long-term social deficits after MDMA or GHB occur with these low doses (Fone et al. 2002; McGregor et al. 2003a). GHB also produced residual increases in anxiety in the emergence test, a test of generalised anxiety, similar to effects previously observed with MDMA (McGregor et al. 2003a, 2003b). This suggests that repeated GHB treatment can cause lasting increases in both generalised and social anxiety. There were clear trends in the present study for MDMA treatment to reduce open field time on the emergence test, but this failed to reach significance, perhaps reflecting the relatively low overall anxiety exhibited by rats on this test. Interestingly, rats in the GHB/MDMA condition showed no evidence of increased anxiety on the emergence test suggesting that the drug combination may produce qualitatively different outcomes, in some measures, relative to either drug given alone. While the exact mechanisms underlying this remain unclear, it is interesting to note that our recent analysis of MDMA/GHB treatment effects on hippocampal protein expression indicated that the combination treatment caused several unique proteomic changes relative to either drug given alone (van Nieuwenhuijzen et al. 2010).

Both the social and object recognition deficits occurred in the absence of detectable changes in striatal or cortical monoamines, suggesting that these deficits do not reflect simple depletion of brain 5-HT, DA or noradrenaline. There are two caveats here that are worth noting: firstly, it may have been preferable to measure monoamines in the hippocampus in the present study (rather than the striatum of PFC) given that this is a key region involved in memory. This was not possible as hippocampal tissue was reserved for proteomic analysis (van Nieuwenhuijzen et al. 2010). Secondly, it needs to be acknowledged that brain monoamine analysis was done at a time (8 weeks post-drug) that was considerably later than the time at which social behavioural and memory were assessed (4 and 6 weeks post-drug, respectively). It could be argued then that monoamine depletion may have been present at this earlier stage when testing occurred. However, previous studies give us some confidence that monoamine changes after 5-HT would be similar in striatum/PFC and hippocampus (McGregor et al. 2003a, 2003b), and that brain monoamine levels are relatively stable in the weeks following MDMA treatment, even when 5-HT depletion has occurred following high MDMA doses (Stone et al. 1987).

We present here for the first time evidence to suggest that hypothalamic oxytocin systems may undergo long-term neuroadaptations as a result of MDMA or GHB pre-exposure. The hypothalamus is the principal location of oxytocin-synthesising neurons in the brain, with volume dendritic release of oxytocin influencing a wide variety of

distal brain targets. Neuroadaptations in this system have the capacity to cause profound alterations in emotion, stress resilience and sociability (Neumann 2008) and might therefore have a causal role in the social and emotional deficits caused by abused drugs (McGregor et al. 2008). MDMA treatment, given 8 weeks earlier, increased the relative expression of OT mRNA in the hypothalamus, while GHB increased OTR mRNA expression. GHB/MDMA treatment caused effects that were intermediate to those induced by GHB and MDMA alone, suggesting that each drug moderates the specific changes in oxytocin signalling produced by the other.

The exact causes and consequences of these oxytocinergic changes can only be speculated upon, but might represent compensatory mechanisms following acute MDMA or GHB-induced stimulation of oxytocin release. Consistent with their phasic role in parturition, lactation and parental care, hypothalamic oxytocin systems are highly neuroplastic, and can be rapidly upregulated and remodelled in response to exogenous stimulation (Lipschitz et al. 2005; Theodosis et al. 2004). MDMA directly stimulates the release of oxytocin in humans and rats (Forsling et al. 2002; Thompson et al. 2007; Wolff et al. 2006), while GHB increases uterine contractions in vivo (Geldenhuys et al. 1968), reduces aggression (Navarro et al. 2007) and strongly activates hypothalamic supraoptic and paraventricular nuclei where the magnocellular oxytocin-synthesising neurons are located (van Nieuwenhuijzen et al. 2009b).

With respect to other classes of drugs of abuse, chronic morphine decreases brain OT synthesis (You et al. 2000), chronic ethanol exposure causes degeneration of hypothalamic OT-containing magnocellular neurons (Sivukhina et al. 2006) while repeated cocaine administration depletes hippocampal and hypothalamic OT (Sarnyai et al. 1992). It is similarly possible that MDMA and GHB might deplete hypothalamic OT to cause alterations in OT-related gene expression. However, it is not entirely clear why MDMA would affect OT mRNA while GHB affects OTR mRNA. Follow-up studies will be required to determine this and to document how the mRNA changes seen in the present study relate to tissue levels of OT, and to assess whether delivery of exogenous oxytocin might ameliorate the social and cognitive deficits seen after MDMA and GHB (Lee et al. 2005).

In conclusion, the present study shows that repeated GHB treatment produces similar long-term residual deficits in social behaviour and memory to those seen after MDMA exposure. In addition, acute withdrawal from GHB was associated with decreases in social behaviour and a long-term anxiogenic effect in the emergence test. Social deficits in MDMA and GHB-treated rats are presented here for the first time, as are subsequent neuroadaptations in the hypothalamic oxytocinergic system, opening avenues for future studies that directly test the link between these behavioural and neural observations. Overall, the present results suggest a need for caution in people using MDMA and GHB given the demonstrated potential of both drugs to induce subtle but lasting changes in brain and behaviour.

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