Improvement in γ-hydroxybutyrate-induced contextual fear memory deficit by systemic administration of NCS-382

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Low, nonsedative doses of γ-hydroxybutyric acid (GHB) produce short-term anterograde amnesia in humans and memory impairments in experimental animals. We have previously shown that acute systemic treatment of GHB in adolescent female rats impairs the acquisition, but not the expression, of contextual fear memory while sparing both the acquisition and the expression of auditory cued fear memory. In the brain, GHB binds to specific GHB-binding sites as well as to γ-aminobutyric acid type B (GABA$_B$) receptors. Although many of the behavioral effects of GHB at high doses have been attributed to its effects on the GABA$_B$ receptor, it is unclear which receptor mediates its relatively low-dose memory-impairing effects. The present study examined the ability of the putative GHB receptor antagonist NCS-382 to block the disrupting effects of GHB on fear memory in adolescent rat. Groups of rats received either a single dose of NCS-382 (3–10 mg/kg, intraperitoneally) or vehicle, followed by an injection of either GHB (100 mg/kg, intraperitoneally) or saline. All rats were trained in the fear paradigm, and tested for contextual fear memory and auditory cued fear memory. NCS-382 dose-dependently reversed deficits in the acquisition of contextual fear memory induced by GHB in adolescent rats, with 5 mg/kg of NCS-382 maximally increasing freezing to the context compared with the group administered GHB alone. When animals were tested for cued fear memory, treatment groups did not differ in freezing responses to the tone. These results suggest that low-dose amnesic effects of GHB are mediated by GHB receptors. *NeuroReport* 27:627–631 Copyright © 2016 Wolters Kluwer Health, Inc. All rights reserved.

Introduction

γ-Hydroxybutyric acid (GHB), a short-chain fatty acid found endogenously in the mammalian brain at micromolar concentrations, is considered to act as a neurotransmitter/ neuromodulator [1–3]. When administered exogenously, GHB rapidly crosses the blood–brain barrier to enter the central nervous system (CNS) [4]. In the brain, GHB is converted into γ-aminobutyric acid (GABA) [4].

GHB was synthesized as an anesthetic agent [5], and has been used in the treatment of narcolepsy [6] and alcoholism [7]. Because of its euphorogenic, relaxing, and sedative effects, GHB has been used and abused as a recreational drug ('club drug') mostly by teenagers and young adults [8]. Because of its short half-life (20–50 min), GHB is rapidly eliminated from the body and by the time blood and/or urine samples are collected for examination, the levels of GHB are usually undetectable. Therefore, the actual prevalence of GHB misuse remains underestimated [9]. In humans, low, nonsedative doses of GHB have been reported to produce short-term anterograde amnesia [10,11].

In animals, GHB has been shown to affect learning and memory. Our laboratory has previously reported that, in adolescent rats, the acquisition of spatial reference memory, as investigated in the Morris water maze, was disrupted by the repeated administration of GHB at nonsedative doses [12–14], while having minimal effects in adult rats [14]. Using the fear-conditioning paradigm, we have further shown that in adolescent rat, acute exposure to GHB impaired the acquisition of contextual fear memory, while sparing the expression of contextual fear memory. Both the acquisition and the expression of auditory cued fear memory were unaffected by GHB [15].

In the brain, GHB binds to two populations of binding sites with distinct ontogeny and anatomical distribution patterns, a specific GHB receptor, and the GABA$_B$ receptor [16,17]. GHB binds to the GHB receptor with high affinity and to the GABA$_B$ receptor with low affinity [17]. At low doses (≤ 100 mg/kg intraperitoneally), GHB acts as a specific agonist at the GHB receptor [18], whereas at relatively high doses (> 200 mg/kg), GHB interacts with the GABA$_B$ receptor both directly as a partial receptor agonist [19] and indirectly through GHB-derived GABA [20]. It is unclear which receptor mediates low-dose GHB-induced adolescent amnesia.

The GHB-like compound NCS-382 (6,7,8,9-tetrahydro-5-hydroxy-5H-benzocyclohept-6-ylideneacetic acid) binds specifically to the GHB receptor and shows antagonist properties at the GHB receptor [21]. It has little affinity for the GABA$_B$ receptor [21]. To elucidate the possible role of the GHB receptor in the amnesic effects of low-dose GHB in adolescent animals, the present study was carried out. Whether the putative GHB receptor antagonist NCS-382 could reverse the GHB-induced deficit in contextual fear memory in adolescent female rats was examined.

Materials and methods
Subjects
A total of 129 adolescent (postnatal day 26–30) female Sprague–Dawley rats (Taconic, Germantown, New York, USA) with no previous drug experience served as subjects. Rats were group housed in plastic cages with ad-libitum access to food and water in a temperature-controlled and humidity-controlled animal care facility with a 12 h light/12 h dark cycle. Efforts were made to minimize animal suffering and to reduce the number of animals used. The number of animals used in the study was determined by power analysis. All experimental protocols were approved by the institutional animal review committee and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals (2011).

Drugs
GHB sodium salt (Sigma-Aldrich, St Louis, Missouri, USA) was dissolved in 0.9% sterile saline; saline was used as the vehicle control. NCS-382 (Tocris, Ellisville, Missouri, USA) was dissolved in DMSO (50%) and 0.9% saline (50%). The latter mixture (50% DMSA + 50% saline) was used as an additional vehicle control. All drug and vehicle solutions were injected intraperitoneally in a volume of 1 ml/kg. The dose of GHB (100 mg/kg) was selected on the basis of our previous studies, where this dosage of GHB was found to interfere with the acquisition of contextual fear memory [15] and spatial reference memory [12–14], but did not have any sedative and/or cataleptic effects. The doses of NCS-382 (3, 5, and 10 mg/kg) were chosen on the basis of a preliminary study.

Experimental procedures
Drug administration
Animals were assigned randomly to six treatment groups ($n = 12$–17 per group). On the training day of fear conditioning, each rat received an intraperitoneal injection of one of several doses of NCS-382 (3, 5, or 10 mg/kg) or vehicle 40 min before training. Ten minutes later (30 min before training for fear conditioning), each animal was injected with GHB (100 mg/kg, intraperitoneally) or an equivalent volume of saline. The treatment groups were as follows: vehicle + saline, vehicle + GHB, 3 mg/kg NCS-382 + GHB, 5 mg/kg NCS-382 + GHB, 10 mg/kg NCS-382 + GHB, and 5 mg/kg NCS-382 + saline.

Fear conditioning
The fear conditioning apparatus and protocol used were as described before [15]. Briefly, on the training day, 30 min following GHB or saline injection, each animal was placed in the conditioning chamber. Rats were presented with a continuous tone (2.9 kHz, 80 dB) for 30 s, at the end of which an electric shock (1 mA) was delivered through the floor grid for 2 s and coterminated with the tone. Each rat received two tone-shock pairings. Approximately 24 h after the training session, all animals were tested for contextual fear memory and auditory cued fear memory. The time interval between the two tests was ~1 h, during which time the animal was returned to its home cage. For the test of contextual fear memory, the rat was placed in the same conditioning chamber as had been used for training and observed for freezing behavior in the absence of any shock unconditional stimulus (US) or tone conditional stimulus (CS) for 3 minutes. To test for cued fear memory, the animal was placed in a modified chamber (the chamber was altered with a rectangular partition placed at a diagonal, one of the walls covered with novel texture, a Plexiglas floor cover, and a novel scent) and observed for freezing behavior following the presentation of the tone continuously for 3 min. In both tests, each animal's behavior was scored manually every 10 s on a three-point scale (0: moving; 1: showing head movements only; and 2: not moving except for respiration) by a trained observer to determine whether freezing occurred within each 10 s bin. The scores obtained were summed up to obtain a freezing sum score for contextual as well as cued fear conditioning.

Data analysis
All data were analyzed using one-way analysis of variance, followed by Tukey's post-hoc tests. The level of significance was set at P less than 0.05. Statistical analyses were carried out using the Prism software (GraphPad, La Jolla, California, USA).

Results
As reported earlier, GHB in female adolescent rats significantly reduced contextual fear freezing [15]. There was no significant effect on cued fear freezing.

NCS-382 pretreatment on the GHB-induced inhibition of contextual fear
Figure 1 shows the effects of systemic administration of NCS-382 on the acquisition of contextual fear memory in adolescent female rats treated with GHB (100 mg/kg, intraperitoneally). Analysis of variance showed a significant treatment effect on freezing [$F_{(5,85)} = 3.821$; $P < 0.0037$]. Post-hoc comparisons showed that the group injected with 100 mg/kg of GHB in the absence of NCS-382 froze significantly less than the control group treated with vehicle and saline ($P < 0.05$), indicating that GHB at this dose attenuated the acquisition of contextual fear memory. Animals pretreated with 5 mg/kg ($P < 0.0004$)

Fig. 1
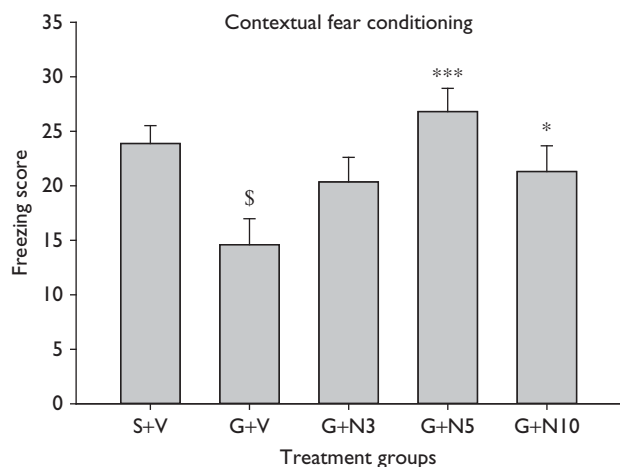
Dose-dependent effects of NCS-382 administration on the acquisition of contextual fear conditioning. Rats were injected with one of three doses of NCS-382 (3, 5, 10 mg/kg, intraperitoneally) or saline 10 min before a single dose of GHB (100 mg/kg, intraperitoneally) treatment. Thirty minutes after GHB or saline administration, rats were trained for fear conditioning. Twenty-four hours later, all rats were tested for contextual fear memory in the same training environment. No foot shock was administered on the test day. A significant treatment effect on freezing [$F_{(5,85)} = 3.821$; $P < 0.0037$] was observed and post-hoc comparisons showed that the group injected with 100 mg/kg of GHB in the absence of NCS-382 (G + V group) froze significantly less than the control group (S + V group; $P < 0.05$). Animals pretreated with 5 mg/kg (G + N5 group; $P < 0.0004$) and 10 mg/kg (G + N10 group; $P < 0.01$) of NCS-382 in combination with GHB showed significantly more freezing to the context compared with the group administered GHB and vehicle (G + V group). Values indicate mean ± SEM with 12–17 rats in each group. $^{\$}P < 0.05$ compared with the saline + vehicle (S + V) group, $^{*}P < 0.05$ compared with the GHB + vehicle (G + V) group, $^{***}P < 0.0004$ compared with the GHB + vehicle (G + V) group. GHB, γ-hydroxybutyric acid.

Table 1 Effect of NCS-382 (5 mg/kg) on contextual and cued freezing summed scores

Fear conditioning	Mean ± SEM	
	Saline + vehicle ($n = 17$)	Saline + NCS-382 ($n = 13$)
Contextual	23.88 ± 1.64	19.69 ± 2.33
Cued	29.59 ± 1.29	28.21 ± 1.33

and 10 mg/kg ($P < 0.01$) NCS-382 in combination with GHB showed significantly more freezing to the context compared with the group administered GHB and vehicle. This suggested that NCS-382 dose-dependently reversed deficits in contextual learning induced by GHB. When injected in the absence of GHB, the maximal effective dose of NCS-382 (5 mg/kg) did not significantly alter the amount of freezing to the context compared with vehicle-treated control rats (Table 1).

NCS-382 pretreatment on GHB-associated acquisition of cued fear

Systemic pretreatment with NCS-382 (3–10 mg/kg) did not significantly alter the amount of freezing elicited by

Fig. 2
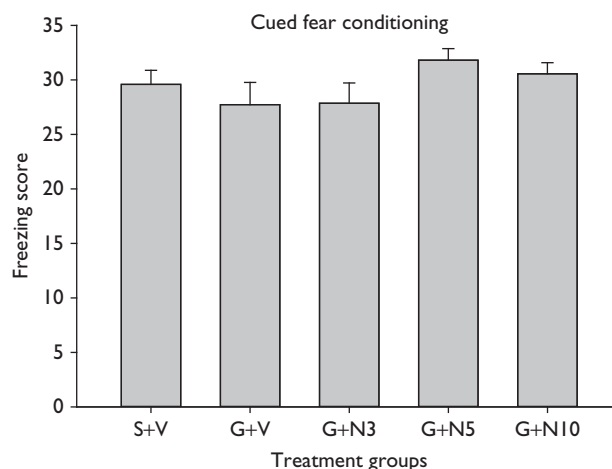
Effects of NCS-382 pretreatment on the acquisition of cued fear memory in GHB-treated and saline-treated rats. There was no significant effect of NCS-382 pretreatment on GHB-induced cued fear memory. Values indicate mean ± SEM; $n = 12$–17 rats per group. GHB, γ-hydroxybutyric acid.

the tone CS in adolescent female rats treated with 100 mg/kg of GHB (Fig. 2). There was no significant treatment effect [$F_{(5,93)} = 1.583$; $P = 0.1725$]. When administered in the absence of GHB, NCS-382 (5 mg/kg) did not affect the acquisition of cued fear (Table 1).

Discussion

The primary aim of the present study was to determine the involvement of specific GHB-binding sites in GHB-mediated memory loss in adolescent rats. The ability of the putative GHB receptor antagonist NCS-382 to reverse the disrupting effects of acute GHB exposure on the acquisition of contextual fear memory was tested. Acute administration of GHB impaired fear conditioning to the training context, while sparing cued fear conditioning to the tone CS. These results confirm our previous findings that acute exposure to a nonsedative dose of GHB in adolescent rats impeded the acquisition of contextual fear memory without affecting that of auditory cued fear memory [15]. Our results further indicate that the GHB antagonist NCS-382 dose dependently antagonized the GHB-induced deficits in the acquisition of contextual fear memory, thus supporting the hypothesis that specific GHB-binding sites mediate the GHB-induced contextual fear memory.

Contextual fear memory acquisition is believed to involve a type of configural learning in which the hippocampus rapidly ties together information on spatial, contextual, and temporal relationships among multimodal sensory stimulus elements of episodic experiences [22]. The hippocampus, an area known to be associated with spatial memory and contextual learning [22], has the highest density of GHB receptors [18]. Although the

amygdala is associated with fear conditioning to discrete sensory cues such as tone, rapid formation of conditioned fear responses to environmental context is mediated by the hippocampus [23]. Our findings suggest that non-sedative doses of GHB preferentially interfere with the mnemonic information processing at the level of the hippocampus. The present results are also in line with our previous findings in adolescent rats that repeated administration of low-dose GHB disrupted the acquisition of spatial reference memory in the Morris water maze [12–14]. Acquisition of spatial memory is also mediated by the hippocampus and involves the general context processing function of the hippocampus. These findings are in accordance with reports in humans that acute exposure to GHB results in anterograde amnesia of episodic memory [10,11].

Several studies have documented the antagonistic properties of NCS-382 at the GHB receptor. NCS-382 has been shown to reduce the sedative and cataleptic effects of an acute exposure to GHB [24]. In a drug self-administration study in mice, NCS-382 completely abolished the reinforcing effects of GHB [25]. In the present study, pretreatment with NCS-382 (3–10 mg/kg, intraperitoneally) produced a dose-dependent reversal of GHB-induced deficits in the acquisition of contextual fear memory, with the peak effect observed at 5 mg/kg. Increasing the dose of NCS-382 did not produce any further improvement. Our present findings showing the capability of NCS-382 to ameliorate the disruptive effects of acute administration of GHB on the acquisition of contextual fear memory, a hippocampus-dependent task, extend our previous findings [15] and indicate that the amnesic effects of GHB at relatively low doses are mediated by GHB receptors in the hippocampus. One study [26] showed that NCS-382 reversed deficits in the hole-board test performance following repeated administration of a low dose of GHB (10 mg/kg). These findings [26] and our present results indicate that the amnesic effects of low doses of GHB (≤ 100 mg/kg) appear to be mediated by the GHB receptor.

It remains unclear how GHB at low doses exerts its amnesic effects. GHB interacts with both the GHB receptor as well as the GABA$_B$ receptor. Endogenous levels of GHB in the rat brain are in the order of 2 μmol/l. The GHB receptor reacts to low concentrations of GHB near this low micromolar physiological range, whereas much higher concentrations (high micromolar to low millimolar) of GHB are required to activate the GABA$_B$ receptor [19]. Electrophysiological studies further support that the differential CNS effects of low and high concentrations of exogenously administered GHB are mediated by the GHB receptor and the GABA$_B$ receptor, respectively. Effects of relatively large doses of GHB (>200 mg/kg) are antagonized by GABA$_B$ receptor antagonists, indicating that these effects are mediated by the GABA$_B$ receptor. Low (nanomolar) concentrations of

GHB have been reported to increase extracellular glutamate levels in the CA1 region, whereas high (millimolar) concentrations decrease their levels. The stimulating effect on glutamate release by low concentrations of GHB has been reported to be attenuated by NCS-382, but not by GABA$_B$ antagonists [21]. Earlier, our laboratory has shown that GHB reduces NMDA receptor levels in the hippocampus [12]. Taken together, these electrophysiological and biochemical findings indicate that the GHB receptor plays an important role in the modulation of synaptic activity and glutamatergic mechanisms. It is possible that the amnesic effects of low doses of GHB may be a result of excitotoxicity caused by enhanced glutamate release.

In the dose range used, NCS-382 produced no significant changes in the amount of freezing to the auditory cue in either the presence or the absence of GHB. The conditioning of fear responses to discrete sensory cues is mediated by the amygdala and apparently does not require the hippocampus [23]. Although the amygdala has GHB receptors [18], our previous [15] and present findings suggest that GHB receptors in the amygdala may not play a major role in cued fear memory.

Conclusion
The present study shows that the putative GHB receptor antagonist NCS-382 in adolescent female rats is capable of dose dependently reversing deficits in the acquisition of contextual fear memory induced by low-dose GHB. This suggests that GHB receptor-mediated mechanisms underlie low-dose GHB-induced memory deficits.

Conflicts of interest
There are no conflicts of interest.

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