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European Journal of Pharmacology 519 (2005) 246–252
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Drosophila $GABA_B$ receptors are involved in behavioral effects of γ-hydroxybutyric acid (GHB)

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Received 7 April 2005; received in revised form 11 July 2005; accepted 19 July 2005
Available online 29 August 2005

Abstract

γ-hydroxybutyric acid (GHB) can be synthesized in the brain but is also a known drug of abuse. Although putative GHB receptors have been cloned, it has been proposed that, similar to the behavior-impairing effects of ethanol, the in vivo effects of pharmacological GHB may involve metabotropic γ-aminobutyric acid (GABA) $GABA_B$ receptors. We developed a fruitfly (*Drosophila melanogater*) model to investigate the role of these receptors in the behavioral effects of exogenous GHB. Injecting GHB into male flies produced a dose-dependent motor impairment (measured with a computer-assisted automated system), which was greater in ethanol-sensitive *cheapdate* mutants than in wild-type flies. These effects of pharmacological concentrations of GHB require the presence and activation of $GABA_B$ receptors. The evidence for this was obtained by pharmacological antagonism of $GABA_B$ receptors with CGP54626 and by RNA interference (RNAi)-induced knockdown of the $GABA_{B(1)}$ receptor subtype. Both procedures inhibited the behavioral effects of GHB. GHB pretreatment diminished the behavioral response to subsequent GHB injections; i.e., it triggered GHB tolerance, but did not produce ethanol tolerance. On the other hand, ethanol pretreatment produced both ethanol and GHB tolerance. It appears that in spite of many similarities between ethanol and GHB, the primary sites of their action may differ and that recently cloned putative GHB receptors may participate in actions of GHB that are not mediated by $GABA_B$ receptors. These receptors do not have a *Drosophila* orthologue. Whether *Drosophila* express a different GHB receptor should be explored.

Keywords: $GABA_B$ (metabotropic γ-aminobutyric acid) receptor; GHB (γ-hydroxybutyric acid); Ethanol; RNAi (*Drosophila melanogaster*)

1. Introduction

γ-hydroxybutyric acid (GHB) is a naturally occurring metabolite of γ-aminobutyric acid (GABA), found in mammalian tissues including the brain (Roth and Giarman, 1970). Pharmacologically, GHB (sodium oxybate) is considered in the treatment of narcolepsy (Borgen et al., 2004; Tunnicliff and Raess, 2002) and occasionally as an anesthetic (Kleinschmidt et al., 1998). The pharmacological profile of GHB is similar to the profile of ethanol (Poldrugo and Addolorato, 1999). Abuse of GHB, which shares its behavioral effects with a number of classical sedative/hypnotics, is an increasing problem (Nicholson and Balster, 2001; Ricaurte and McCann, 2005). Clinically, there are reports of severe GHB withdrawal symptoms (Craig et al., 2000; Tarabar and Nelson, 2004), and in a rat model, repeated administration of GHB produces both behavioral tolerance and withdrawal (Bania et al., 2003).

It has been proposed that metabotropic GABA receptors, $GABA_B$ receptors, and GHB receptors may mediate the actions of GHB (Andriamampandry et al., 2003; Carter et al., 2003, 2004a,b; Kaupmann et al., 2003). Although a direct binding of GHB to $GABA_B$ receptors has not been conclusively demonstrated (Lingenhoehl et al., 1999; Wu et al., 2004), it appears that to produce its behavioral effects, GHB requires these receptors (Carter et al., 2003, 2004a,b; Kaupmann et al., 2003). In $GABA_{B(1)}$ receptor knockout

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doi:10.1016/j.ejphar.2005.07.016

mice, which lack functional $GABA_B$ receptors, GHB application failed to produce either the behavioral or the biochemical responses seen in wild-type mice (Kaupmann et al., 2003). On the other hand, the binding of a putative GHB antagonist, NCS-382, to the specific [^{3}H]GHB-binding sites was unchanged in $GABA_{B(1)}$ receptor knockout mice, suggesting that the behavioral and biochemical effects of GHB are $GABA_B$ receptor-dependent whereas the nature and signaling properties of the specific [^{3}H]GHB-binding sites remain elusive.

Although $GABA_B$ receptor knockout mice are useful for behavioral studies, they are developmentally altered and their use is complicated by inherited pathologies such as seizures (for a review, see Enna and Bowery, 2004). To circumvent these drawbacks, we developed a *Drosophila* model for the adult $GABA_{B(1)}$ receptor knockdown via the injectable RNA interference (RNAi) method (Dzitoyeva et al., 2003). Recent studies demonstrated that *Drosophila* can be successfully used in neuropharmacological research (Bainton et al., 2000; Manev et al., 2003). Fruit flies possess a physiologically active endogenous GABA system (Leal et al., 2004), express $GABA_B$ receptors (Mezler et al., 2001), and when treated with $GABA_B$ receptor ligands display distinct behavioral responses (Dzitoyeva et al., 2003; Dimitrijevic et al., 2004) and developmental abnormalities (Dzitoyeva et al., in press). Previously, GHB was administered to *Drosophila* either via food (Connolly et al., 1971) or by injection (Satta et al., 2003) and in both conditions GHB impaired their locomotor activity. We observed that similar to mammals, *Drosophila* possess the machinery for GHB synthesis and are capable of metabolizing 1,4-butanediol into GHB in vivo (Satta et al., 2003).

In flies (Dzitoyeva et al., 2003), similar to mice (Zaleski et al., 2001), the cAMP-linked $GABA_B$ receptors participate in the behavior-impairing effects of ethanol. Experiments with mutant flies provided evidence that the cAMP signaling system plays a crucial role in the acute response of fruitflies to ethanol vapor (Moore et al., 1998). These authors found that lack of the *amnesiac* gene, which is thought to encode a peptide that increases levels of cAMP, or a mutation in this gene called *cheapdate* increase sensitivity to ethanol. Here, we investigated whether the *cheapdate* mutation influences behavioral effects of GHB. In this work, we hypothesized that $GABA_B$ receptors participate in the behavioral actions of GHB in flies, and that flies can be used as an in vivo model to investigate the behavioral interactions of GHB and ethanol.

2. Materials and methods

2.1. Drosophila and drug treatments

Canton-S (wild-type) and *cheapdate* flies were cultured at 25 °C, 50–60% humidity, 12 h/12 h light/dark cycle, on yeast, dark corn syrup, and agar. Studies were performed with 5 to 7-day-old males. For injections (Dzitoyeva et al., 2001, 2003), flies were anesthetized by CO_2 (maximally for 5 min). Using custom-beveled glass pipettes (20×40 μm tip diameter) coupled to a cell injector and a micromanipulator we injected a volume of 0.2 μl/fly by a pulse pressure of 300 kPa under a stereo microscope (Dzitoyeva et al., 2003). Drugs were prepared as 10× stock solutions; 0.2 μl were injected per fly. GHB (Sigma, St. Louis, Missouri, USA), [*S*-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl] amino]-2-hydroxypropyl](cyclohexylmthyl) phosphinic acid hydrochloride (CGP54626; Tocris, Ellisville, Missouri, USA), 5,7,8,9-tetrahydro-5-hydroxy-6H-benzocyclohepten-6-ylideneacetic acid sodium–potassium salt (NCS-382; Sigma, St. Louis, Missouri, USA) and ethanol (Sigma, St. Louis, Missouri, USA) were dissolved/diluted with Ringer solution (NaCl, KCl, $CaCl_2$; 7.5, 0.35, 0.21 g/l; pH 7.6–7.8; sterile-filtered). The stock solutions of CGP54626 and NCS-382 were prepared in dimethylsulfoxide (DMSO; Sigma, St. Louis, Missouri, USA); the DMSO concentration in final solutions was 1%. All control flies were injected with the corresponding vehicle. When using multiple drug treatments, drugs were administered with different pipettes and in the indicated time interval (1–4 h).

2.2. Behavioral assay

A *Drosophila* Activity Monitoring System (Trikinetics, Waltham, Massachusetts, USA) coupled to a computer was used to record the locomotor activity of individual flies. In previous studies, we established methods for evaluating the drug-induced changes in locomotor activity, immobility duration, and the number of periods of immobility (Dzitoyeva et al., 2003; Satta et al., 2003; Dimitrijevic et al., 2004). To quantify these parameters, the system was modified, i.e., the space in each individual recording tube was restricted to a length of 8 mm in the center of the photo beam. Flies were placed in the recording tubes within 2 min of injection and the sampling time was set at 1-min intervals. After flies recovered from the injection, they gradually resumed locomotor activity. The *locomotor activity* of flies was calculated as the average number of movements/min recorded and quantified for a 30-min period starting at the time of recovery from the acute immobility induced by vehicle/drug administration. The calculated average 1-min activity of vehicle-treated controls was used to determine the threshold for recovery from injection. Thus, the first 1-min interval after the injection in which a fly reaches this threshold, i.e., the number of "average" moves/min, was used as the time of recovery and to calculate the *duration of immobility*. *Periods of immobility* (i.e., 1-min periods with 0 movements) during the 30-min observation periods following the recovery were also calculated. Typically, 6–10 flies per experimental group were used and experiments were repeated 2–3 times. Statistical analysis was performed by Dunnett's test or Student's *t*-test; $P<0.05$ was taken as significant.

2.3. Gas chromatography/mass spectrometry (GC/MS) assay of GHB

This assay was performed as described elsewhere (Satta et al., 2003). Briefly, groups of five flies were homogenized in 200 μl of 100 mM phosphate buffer pH 6; d6-GHB (2,2,3,3,4,4-d6; Cambridge Isotope Laboratories, Andover, Massachusetts, USA) was added (2.5 ng/μl) to each sample as the internal standard. Each sample was applied onto 1 ml of a AG1X8 formate ion exchange column preconditioned with successive additions of 10

Table 1
The sequences of the sense DNA oligonucleotides corresponding to the $GABA_{B(1)}$ and *Fs(1)Yb* genes used for the in vitro transcription reaction

$GABA_{B(1)}$+T7 promoter (a)	5′-taatacgactcactatattatttgtgtgcatgtgcacaa-3′
$GABA_{B(1)}$+T7 promoter (b)	5′-taatacgactcactatagctgttggcaggatgcagcacg-3′
Fs(1)Yb+T7 promoter	5′-taatacgactcactatattttctgcagtgggaataactt-3′

ml 20% ethanol in 0.5 M formic acid and 10 ml distilled water. GHB was eluted with the successive addition of 4 ml 20% ethanol in 0.5 M formic acid, dried under vacuum, and the residue was dissolved in 200 μl dimethylformamide. After two extractions with 1 ml of hexane, the dimethylformamide layer was dried by evaporation under nitrogen. The samples were derivatized with 100 μl ethyl acetate and 100 μl BSTFA [N,O,-bis(trimethylsilyl)trifluoroacetamide] with 1% trimethylchlorosilane for 10 min at 60 °C, and analyzed on a GC/MS system equipped with a HP-5 column (30 m × 0.25 mm × 0.25 μm). Two μl samples were injected manually. The temperature of the injection port was 250 °C. The oven was initially held at 70 °C for 4 min; thereafter, it was programmed to increase at a rate of 8 °C/min to 100 °C and at a rate of 25 °C/min to 175 °C. The instrument was operated in the selected ion monitoring mode (SIM); the ions 233 and 239 were used for the quantitation of GHB and d6-GHB, respectively. The retention time for GHB and d6-GHB was approximately 9.1 min. The calibration curve ranged from 0.5 to 5 ng/μl GHB; the detection limit for the standard prepared in water was 0.25 ng/μl.

2.4. Synthesis of double-stranded RNA (dsRNA) and RNAi

We targeted two 22-nucleotide long regions; a) 329–350, and b) 1402–1423, of the *Drosophila* $GABA_{B(1)}$ gene. As a control dsRNA, we used a 22nt of the *Fs(1)Yb* gene. 39-mer DNA oligonucleotides with an attached T7 RNA polymerase promoter sequence were synthesized (Integrated DNA Technology, Inc., Coralville, Iowa, USA); these oligonucleotides represented both sense and antisense strands. The sequences of the sense DNA oligonucleotides corresponding to the $GABA_{B(1)}$ and *Fs(1)Yb* genes used for the in vitro transcription reaction are shown in Table 1. Only one match was found in the *Drosophila* genome database pattern search for the $GABA_{B(1)}$ and *Fs(1)Yb* gene, respectively. Equal amounts of oligonucleotides were annealed to form a double-stranded template by heating at 80–85 °C for 5 min and cooling on ice. The in vitro transcription reaction (30 μl volume) for the synthesis of the 22 nt RNA run of transcripts contained 0.1 μg of a

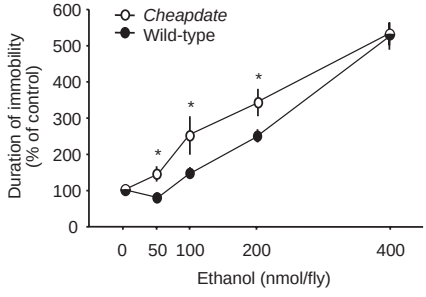

Fig. 1. Increased sensitivity of *cheapdate* flies to injected ethanol. Injections of ethanol caused a dose-dependent prolongation of the immobility observed immediately after injection in both *cheapdate* and wild-type flies. However, the dose response to ethanol-induced prolongation of immobility time in *cheapdate* flies was shifted to the left, indicating their greater sensitivity to ethanol. Results (mean ± S.E.M.; $n = 14–16$/time point) are expressed as a percentage of corresponding immobility of vehicle-treated controls ["0" ethanol; immobility (min) wild-type = 7.2 ± 1.5; *cheapdate* 6.9 ± 0.5]. *$P < 0.05$ compared to corresponding wild-type values.

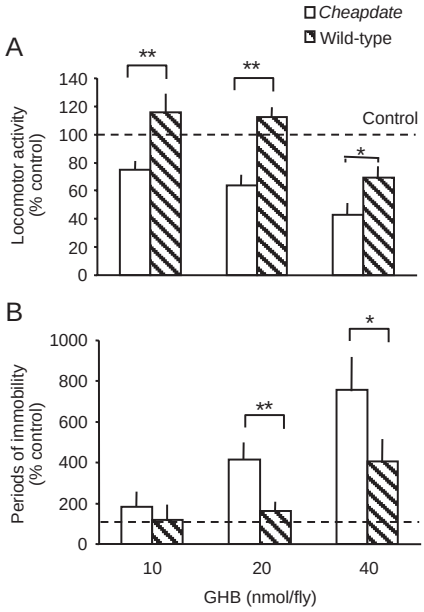

Fig. 2. Increased sensitivity of *cheapdate* flies to behavioral effects of GHB. GHB (doses as indicated) was administered intra-abdominally. Results (mean ± S.E.M.; $n = 15–24$) are expressed as a percentage of the corresponding vehicle-treated controls [Locomotor activity (movement/min): wild-type = 8.2 ± 2.2; *cheapdate* = 7.7 ± 0.7. Periods of immobility (number of immobile periods in 30 min): wild-type = 3.2 ± 2.0; *cheapdate* = 2.9 ± 1.3.]. Locomotor activity (A) and periods of immobility (B) were measured in a 30-min period starting from the time of recovery (Material and methods). In *cheapdate* flies, locomotor activity was decreased and periods of immobility were increased at lower doses of GHB compared to wild-type flies. A dose of GHB 40 nmol/fly resulted in impaired behavior in both strains of flies (*$P < 0.05$; **$P < 0.001$).

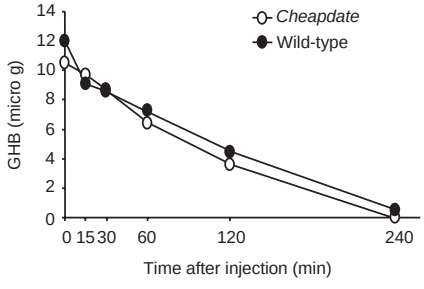

Fig. 3. The elimination rates of injected GHB are comparable in *cheapdate* and wild-type flies. GHB (40 nmol/fly; 5 flies/time point) was injected intra-abdominally, flies were homogenized at different times post-injection (as indicated), and samples were processed for GC/MS assays of GHB. Results are expressed as μg GHB/sample (obtained from 5 flies). Note a similar time-dependent GHB decrease in *cheapdate* and wild-type flies; GHB was barely detectable 4 h post-injection in both strains.

Table 2
Effects of $GABA_B$ receptor antagonist CGP54626 and GHB antagonist NCS-382 on the behavioral effects of GHB

Treatment	Locomotor activity (movements/min)	Periods of immobility (periods/30 min)
Vehicle+Vehicle	8.7±0.9	4.4±1.8
Vehicle+GHB	4.8±0.5*	10.1±1.4*
CGP54626 (0.2 nmol/fly) +GHB	8.0±1.0	4.6±1.2
Vehicle+Vehicle	8.1±1.1	2.5±0.9
Vehicle+GHB	4.6±0.7*	7.7±2.8*
NCS-382 (0.2 nmol/fly) +GHB	4.7±0.8*	8.2±2.7*
NCS-382 (1.0 nmol/fly) +GHB	4.2±0.7*	9.8±1.9*

Wild-type flies were injected with CGP54626 or NCS- 382 1 h prior to GHB (40 nmol/fly). Results are expressed as mean±S.E.M.; *$P<0.01$ compared to the corresponding vehicle+vehicle group.

template, 500 μM each CTP, GTP, ATP, and UTP, 1× transcription buffer (Tris–HCl, pH 7.5, 10 mM dithiothreitol, 1% bovine serum albumin), 20 U of RNAse inhibitor, and 50 U T7 RNA polymerase (Gibco BRL, Invitrogen, Carlsbad, California, USA). The reactions were carried out at 37 °C for 1 h. The RNA molecules were annealed together in heat denaturing conditions (65–70 °C for 5 min) and placed on ice. The quality of both RNA and DNA oligonucleotides was confirmed on a 4% NuSive agarose gel (Sigma, St. Louis, Missouri, USA). Flies were injected with 0.2 μl from a stock (100 ng/μl) of $GABA_{B(1)}$ a+b or Fs(1)Yb dsRNAs. They were used for experiments 3 days after the injection; i.e., when a significant reduction of the endogenous $GABA_{B(1)}$ mRNA content was documented in $GABA_{B(1)}$ RNAi flies (Dzitoyeva et al., 2003).

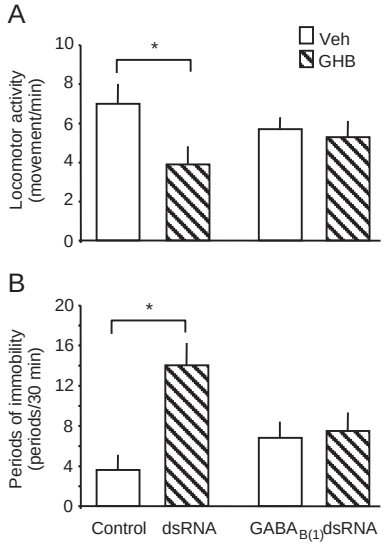

Fig. 4. $GABA_{B(1)}$ receptor RNAi inhibits the behavioral effects of GHB. $GABA_{B(1)}$ dsRNA but not control dsRNA (Table 1) injections into adult wild-type flies prevented the decrease in locomotor activity (A) and the increase of immobility periods (B) induced by GHB (40 nmol/fly). Results are expressed as mean±S.E.M.; *$P<0.01$ compared to the corresponding vehicle-treated group ($n=16$).

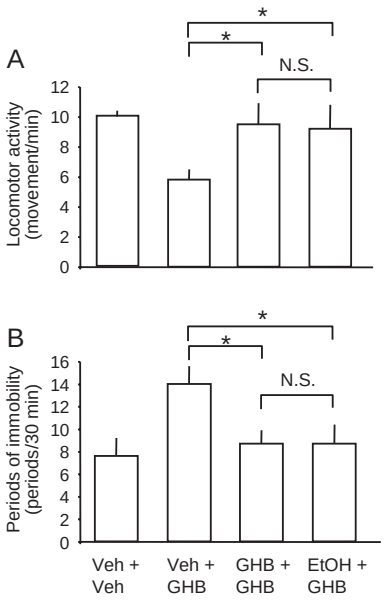

Fig. 5. Previous administration of GHB or ethanol diminishes behavioral responses to subsequent GHB injections. Vehicle, GHB (40 nmol/fly), and ethanol (200 nmol/fly) were injected into wild-type flies 4 h prior to subsequent injections of vehicle or GHB. Thereafter locomotor activity (A) and increase of immobility periods (B) were measured. Results are expressed as mean±S.E.M.; *$P<0.05$ ($n=11–12$).

3. Results

3.1. GHB impairs locomotor activity of flies: cheapdate mutation increases sensitivity to GHB

Both in wild-type and *cheapdate* flies, ethanol injections induced the dose-dependent prolongation of immobility typically observed immediately after the injection. However, the dose–response curve for ethanol-induced prolongation of immobility was

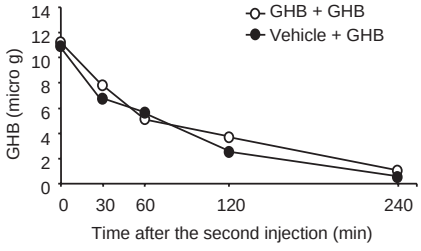

Fig. 6. Previous administration of GHB does not alter the elimination rate of subsequently injected GHB. GHB (40 nmol/fly; 5 flies/time point) or vehicle were injected intra-abdominally. The flies received two injections (GHB +GHB or vehicle+GHB), 4 h apart, and were homogenized at different times after the second injection (as indicated). Samples were processed for a GC/MS assay of GHB. Results are expressed as μg GHB/sample (obtained from 5 flies). Note a similar time-dependent GHB decrease in GHB+GHB and vehicle+GHB groups.

shifted to the left in *cheapdate* vs. wild-type flies (Fig. 1). The effects of GHB injections were qualitatively different from the effects of an ethanol injection. Although GHB slightly prolonged immobility immediately after injection, this action was neither significant nor dose-dependent (not shown). Instead, once GHB-injected flies recovered from the injection their locomotor activity level was lower and interrupted by periods of immobility (Fig. 2). These effects of GHB were observed in both *cheapdate* and wild-type flies, but *cheapdates* were more sensitive. In wild-type flies, behavioral impairment consistently occurred after 40 nmol/fly GHB whereas in *cheapdate*s, even 10 nmol/fly was effective (Fig. 2). We measured whether the elimination of injected GHB differs between *cheapdate* and wild-type flies. Using our recently developed GC/MS methodology for GHB measurement, we found no difference in GHB elimination in these two fly strains (Fig. 3).

3.2. $GABA_B$ antagonism and $GABA_{B(1)}$ RNAi reduce the behavioral effects of GHB

Pretreating flies with 0.2 nmol/fly of the $GABA_B$ receptor antagonist CGP54626 inhibits the behavioral effects of 40 nmol/fly GHB (Table 2). On the other hand, pretreatment with the GHB receptor antagonist NCS-382 did not affect the behavioral consequences of GHB (Table 2). We also used our previously established RNAi method for knocking down $GABA_{B(1)}$ receptors in adult wild-type flies. GHB treatment was introduced 3 days after dsRNA injections. In control-dsRNA-injected flies, GHB produced the typical behavioral impairments that were absent in $GABA_{B(1)}$-dsRNA-injected flies (Fig. 4).

3.3. Repeated administration of GHB produces behavioral tolerance

We found that the previous exposure of flies to GHB reduces the behavior-impairing actions of a second GHB injection administered 4 h later (Fig. 5). In contrast, the previous exposure to GHB did not alter GHB elimination (Fig. 6).

3.4. Behavioral interactions between GHB and ethanol

Interestingly, the behavior-impairing effects of GHB were attenuated not only by previous exposure to GHB but also by previous exposure to ethanol (Fig. 5). When flies were first treated with ethanol and their response to subsequent ethanol administration was examined, we observed diminished behavior impairing activity following the second ethanol injection (i.e., a rapid ethanol tolerance). However, pretreatment with GHB did not alter the response of flies to subsequent ethanol injection (Fig. 7).

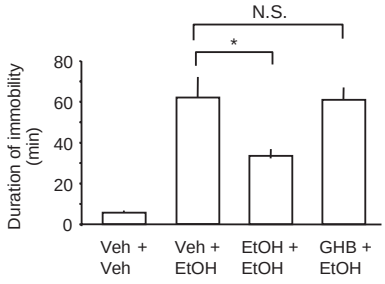

Fig. 7. Previous administration of ethanol but not GHB diminishes prolongation of immobility due to subsequent ethanol injection. Vehicle, ethanol (200 nmol/fly), and GHB (40 nmol/fly) were injected into wild-type flies 4 h prior to subsequent injection of vehicle or ethanol. The time to recovery (i.e., duration of immobility due to ethanol) was measured. Results are expressed as mean±S.E.M.; *$P<0.01$ ($n=16$).

4. Discussion

In this work, we demonstrated that pharmacological concentrations of GHB produce behavioral effects in adult *Drosophila* which require the presence and activation of $GABA_B$ receptors. The evidence for this was obtained by pharmacological antagonism of $GABA_B$ receptors and by RNAi-induced knockdown of $GABA_{B(1)}$ receptor subtypes. Both procedures were capable of inhibiting the behavioral effects of GHB. Our findings with in vivo experiments in *Drosophila* are consistent with observations from in vivo experiments with $GABA_{B(1)}$ receptor knockout mice (Kaupmann et al., 2003), and are somewhat at odds with the recent notion that GHB does not bind $GABA_B$ receptors in vitro in $GABA_B$ receptor-expressing HEK 293 cells (Wu et al., 2004). However, similar experiments in COS cells found that GHB is a weak agonist of recombinant $GABA_B$ receptors (Lingenhoehl et al., 1999). It is possible that the cell type-specific environment could contribute to recombinant metabotropic receptor functionality (Gabellini et al., 1994). Furthermore, the specific GHB binding in the brain of $GABA_{B(1)}$ receptor knockout mice was significantly lower than in wild-type mice (Wu et al., 2004), suggesting that a component of in vivo GHB binding, and possibly its pharmacological actions may involve $GABA_B$ receptors. On the other hand, the functional role of putative GHB receptors, particularly with respect to behavior, is unclear. We found that a GHB receptor antagonist, NCS-382, had no effect on the behavioral GHB actions in *Drosophila.* Others reported that NCS-382 did not block the behavioral actions of GHB in rats whereas these actions in rats were inhibited by the $GABA_B$ receptor antagonist CGP 35348 (Carter et al., 2003, 2004a,b).

Adult RNAi via dsRNA injection into adult insects is a useful tool for investigating the loss-of-function phenotypes that circumvent developmental alterations (Dzitoyeva et al., 2001; 2003; Blandin et al., 2002; Amdam et al., 2003; Goto et al., 2003; Farooqui et al., 2004). We found an inhibition of the behavioral effects of GHB with injectable $GABA_{B(1)}$ RNAi. These results, along with the data obtained with CGP 54626 inhibition of GHB effects, suggest that functional $GABA_B$ receptors are needed to produce behavioral GHB activity in *Drosophila*.

$GABA_B$ receptors, which mediate some of ethanol's behavioral effects in mice (Zaleski et al., 2001) and *Drosophila* (Dzitoyeva et al., 2003), are linked to complex transduction mechanisms and involve negative coupling to the cAMP pathway (Couve et al., 2004; Knight and Bowery, 1996). Regulation of the cAMP pathway is critical for

modifying the sensitivity of *Drosophil*a to ethanol. Thus, *cheapdate* mutants, which are characterized by defective cAMP signaling, are sensitive to ethanol (Moore et al., 1998). Using ethanol injections, we confirmed the increased sensitivity of *cheapdate* compared to wild-type flies in a dose range from 50–200 nmol ethanol/fly. The highest ethanol dose used (400 nmol/fly) was not toxic and prolonged immobility in a manner that was no longer sensitive to *cheapdate*-dependent mechanisms. Interestingly, we found that *cheapdate* mutants are also more sensitive to the behavior-impairing actions of GHB. The increased sensitivity to GHB does not appear to be caused by alterations in GHB metabolism. We found that both *cheapdate* and wild-type flies eliminate the injected GHB equally well. Although the exact mechanism for the increased ethanol sensitivity of *cheapdate* is not clear, Moore et al. (1998) found that this enhanced ethanol sensitivity can be reversed by treatment with agents that increase cAMP levels or protein kinase A (PKA) activity. Conversely, genetic or pharmacological reductions in PKA activity resulted in increased sensitivity to ethanol, providing functional evidence for the involvement of the cAMP signal transduction pathway in the behavioral response to impairing levels of ethanol. Our data extend this notion to the behavior-impairing effects of GHB. Considering the involvement of $GABA_B$ receptors in the behavioral effects of GHB, we investigated whether the content of $GABA_{B(1)}$ mRNA is altered in *cheapdate* and found no difference between these and wild-type flies (data not shown). It is possible that the cAMP-linked functioning of $GABA_B$ receptors is altered by the *cheapdate* mutation.

Rapid tolerance to repeated ethanol exposures was observed under various regimens of ethanol delivery to a number of different species including *Drosophila* (Scholz et al., 2000; Ghezzi et al., 2004). Repeated administration of GHB to rats leads to diminished GHB intoxication; i.e., tolerance (Bania et al., 2003). It has been proposed that the development of tolerance to ethanol (Zaleski et al., 2001; Dzitoyeva et al., 2003) and GHB (Eckermann et al., 2004) may involve $GABA_B$ receptors. Although our data in *Drosophila* indicate that $GABA_B$ receptors participate in the acute behavioral effects of GHB and ethanol, it does not appear that a single mechanism; e.g., a direct action of GHB on these receptors, is responsible for GHB-induced GHB tolerance and ethanol crosstolerance. Namely, although GHB pretreatment produced GHB tolerance it did not produce ethanol tolerance. On the other hand, ethanol pretreatment was able to produce both ethanol and GHB tolerance. Thus, it appears that in spite of many similarities between ethanol and GHB, the primary sites of their action may differ.

We can only speculate about the mechanisms regulated by $GABA_B$ receptors that modify the behavioral responses of *Drosophila* to ethanol (Dzitoyeva et al., 2003) and GHB (this study). The behavior-impairing effects of ethanol in flies can be modified by genetic manipulations that impair the function of insulin-producing cells or of the insulin-receptor signaling pathway (Corl et al., 2005). Interestingly, $GABA_B$ receptors contribute to the modulation of glucose-stimulated insulin secretion in rat pancreatic beta cells (Brice et al., 2002). It should be investigated whether an interaction of $GABA_B$ receptors with *Drosophila* insulin signaling plays any role in modifying the behavioral effects of ethanol and/or GHB.

In conclusion, our *Drosophila* model, similar to recent experiments with $GABA_{B(1)}$ receptor knockout mice (Kaupmann et al., 2003), indicates that $GABA_B$ receptors rather than NCS-382-sensitive GHB receptors mediate the acute locomotor-impairing effects of GHB. Although $GABA_B$ receptors also participate in the behavioral actions of ethanol, they do not appear to be involved in all of the behavioral interactions between GHB and ethanol, for example, in crosstolerance. A recently cloned GHB receptor (Andriamampandry et al., 2003) does not have a *Drosophila* orthologue. Whether *Drosophila* express a different GHB receptor should be explored.

Acknowledgments

This work was supported by the National Institute on Drug Abuse Grant R03DA14811 and a grant from the Alcoholic Beverage Medical Research Foundation (ABMRF). We thank U. Heberlein for the *cheapdate* fly stock.

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