

## ORIGINAL INVESTIGATION

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## In vivo and in vitro hyperbaric studies in mice suggest novel sites of action for ethanol

Received: 15 December 1997 / Final version: 23 July 1998

**Abstract** The present study uses increased atmospheric pressure as an ethanol antagonist to test the hypothesis that allosteric coupling pathways in the GABA<sub>A</sub> receptor complex represent initial sites of action for ethanol. This was accomplished using behavioral and in vitro measures to determine the effects of pressure on ethanol and other GABAergic drugs in C57BL/6 and LS mice. Behaviorally, exposure to 12 times normal atmospheric pressure (ATA) of a helium-oxygen gas mixture (heliox) antagonized loss of righting reflex (LORR) induced by the allosteric modulators ethanol and pentobarbital, but did not antagonize LORR induced by the direct GABA agonist 4,5,6,7-tetrahydroisoxazolo-pyridin-3-ol (THIP). Similarly, exposure to 12 ATA heliox antagonized the anti-convulsant effects versus isoniazid of ethanol, diazepam and pentobarbital. Biochemically, exposure to 12 ATA heliox antagonized potentiation of GABA-activated <sup>36</sup>Cl<sup>-</sup> uptake by ethanol, flunitrazepam and pentobarbital in LS mouse brain preparations, but did not alter GABA-activated <sup>36</sup>Cl<sup>-</sup> uptake per se. In contrast to its antagonist effect versus other allosteric modulators, pressure did not antagonize these behavioral or in vitro effects induced by the neuroactive steroid, 3 $\alpha$ -hydroxy-5 $\beta$ -pregnan-20-one (3 $\alpha$ ,5 $\beta$ -P). These findings add to evidence that pressure directly and selectively antagonizes drug effects mediated through allosteric coupling pathways. The results fit

predictions, and thus support the hypothesis that allosteric coupling pathways in GABA<sub>A</sub> receptors represent initial sites of action for ethanol. Collectively, the results suggest that there may be common physicochemical and underlying structural characteristics that define ethanol sensitive regions of receptor proteins and/or their associated membranes that can be identified by pressure within (e.g., GABA<sub>A</sub>) and possibly across (e.g., GABA<sub>A</sub>, NMDA, 5HT<sub>3</sub>) receptors.

**Key words** Hyperbaric exposure · Helium-oxygen gas mixture ·  $\gamma$ -Aminobutyric acid (GABA) · *N*-Methyl-D-aspartate (NMDA) · 4,5,6,7-Tetrahydroisoxazolo-pyridin-3-ol (THIP) · 3 $\alpha$ -Hydroxy-5 $\beta$ -pregnan-20-one (3 $\alpha$ , 5 $\beta$ -P) · Loss of righting reflex (LORR) · Anticonvulsant effect · GABA-activated chloride ion uptake · Central nervous system · Benzodiazepines · Barbiturates · Neuroactive steroid · C57BL/6 mice · Ethanol antagonist · GABA<sub>A</sub> receptor complex · Ligand-gated ion channels · Allosteric coupling hypothesis

### Introduction

Theories of the molecular and cellular mechanisms causing ethanol's behavioral effects have changed markedly over the past 10 years. In contrast to the non-specific mechanism proposed by "classical" membrane theories (Seeman 1972; Janoff et al. 1981; Lyon et al. 1981), growing evidence indicates that ethanol and other intoxicant anesthetics act directly on a limited number of neuron-specific proteins and/or their associated membrane lipid microenvironments (Deitrich et al. 1989; Harris et al. 1992; Jones and Harrison 1993; Longoni et al. 1993; Franks and Lieb 1994; Mihic et al. 1997). Despite these advances, the precise molecular sites of ethanol's initial action on brain cells are not yet clear (Franks and Lieb 1997). Part of the difficulty in identifying these sites lies in the physicochemical nature of ethanol's mechanism of action, and resultant lack of high

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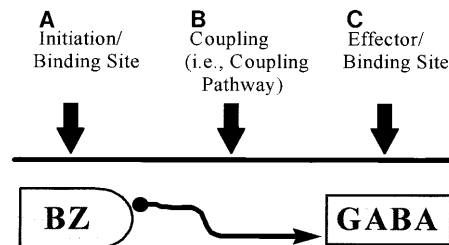
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affinity and pharmacological specificity that limit the number of tools available (Deitrich et al. 1989; Little 1996).

Our approach has been to use low level hyperbaric exposure (i.e., increased atmospheric pressure) as an ethanol antagonist. This work has shown that exposure to 12 times normal atmospheric pressures (12 ATA) of a helium-oxygen gas mixture (heliox) antagonizes several acute behavioral effects of ethanol, including loss of righting reflex (LORR) (Alkana and Malcolm 1981, 1982a,b; Malcolm and Alkana 1982), depression (Syapin et al. 1988; Bejanian et al. 1993) and low dose stimulation (unpublished results) of locomotor activity, depression of aggressive behaviors (Alkana et al. 1991a) and anticonvulsant effects (Davies et al. 1994). Further, exposure to 12 ATA heliox precipitated and exacerbated withdrawal in mice made physically dependent on ethanol (Alkana et al. 1985b), and attenuated the development of ethanol tolerance and physical dependence (Alkana et al. 1987).

Presently, the mechanism and site(s) of ethanol antagonism by low level hyperbaric exposure are not known. In contrast to suggestions about the mechanism of high pressure (100–300 ATA) reversal of anesthesia (Brauer et al. 1979; Halsey 1982; Kendig 1984; Wann and MacDonald 1988; Franks and Lieb 1994; Little 1996), considerable evidence indicates that the antagonistic effects of 12 ATA heliox versus ethanol-induced behaviors cannot be attributed to pressure-induced increases in generalized central nervous system (CNS) excitability (Alkana et al. 1991b; Davies et al. 1994; Syapin et al. 1996). Further studies have also eliminated pressure- or helium-induced changes in body temperature (Malcolm and Alkana 1982), oxygen partial pressure (Alkana and Malcolm 1982b), and ethanol absorption, distribution and elimination (Alkana and Malcolm 1982a,b) as mechanisms for the antagonism. At the biochemical level, exposure to 12 ATA heliox antagonized ethanol potentiation (25–100 mM) of GABA-activated chloride ion uptake in an isolated mouse brain membrane preparation, but did not significantly alter the responsiveness of the preparation to GABA (10–100  $\mu$ M), bicuculline or picrotoxin (Davies and Alkana 1998). Collectively, these studies provide a solid body of evidence that the mechanism by which exposure to 12 ATA heliox antagonizes ethanol cannot be attributed to indirect causes, including changes in physiological function or pharmacokinetics, that could offset ethanol's effects. Therefore, like a traditional pharmacological antagonist, the mechanism of hyperbaric ethanol antagonism appears to be direct.

Moreover, hyperbaric ethanol antagonism at the behavioral level has other characteristics similar to those of a competitive, pharmacological receptor antagonist (Bejanian et al. 1993). Exposure to 12 ATA heliox causes a parallel shift to the right in the ethanol dose-response curve (Bejanian et al. 1993); the antagonism is surmountable by increasing the ethanol dose (Bejanian et al. 1993); and the degree of antagonism is pressure dependent (Alkana and Malcolm 1981). In addition, recent evidence suggests that the antagonism is selective for alco-



**Fig. 1A–C** Coupling cascade. There are three components to allosteric coupling: **A** binding to the initiation site; **B** coupling and **C** downstream changes in the coupled receptor/effector

hol-like drugs. That is, exposure to 12 ATA heliox antagonized several of the behavioral effects induced by anesthetic or related drugs (ethanol and *n*-propanol) (Alkana et al. 1995). In contrast, exposure to 12 ATA heliox did not antagonize locomotor activation induced by morphine (Alkana et al. 1995), or seizures induced by convulsants such as picrotoxin, *dl*-allylglycine and isoniazid (INH) (Alkana et al. 1991b; Davies et al. 1994; Syapin et al. 1996). Thus, the pattern of the antagonism indicates that exposure to 12 ATA heliox directly and selectively antagonizes the effects of drugs like ethanol, which are believed to act via membrane perturbation or allosteric modulation of functional proteins (Miller 1985; Deitrich et al. 1989; Korpi 1994), but does not antagonize the effects of drugs that act via high affinity receptor binding (morphine, picrotoxin, GABA) or modification of enzymatic synthesis reactions (INH and *dl*-allylglycine).

Recent work extended investigation of the selectivity of pressure antagonism to the benzodiazepine, diazepam. Diazepam has a pharmacological profile similar to ethanol in that both diazepam and ethanol cause similar behavioral effects (sedating, anxiolytic, anticonvulsant and amnesic) and both potentiate GABAergic transmission (Ticku and Kulkarni 1988; Harris and Allan 1989a; Ticku 1990; Delorey and Olsen 1992; Korpi 1994). Further, like ethanol, diazepam mediates enhancement of GABA<sub>A</sub> receptor function via allosteric modulation. However, unlike ethanol, diazepam initiates its allosteric modulation via high affinity binding to a specific recognition site on the GABA<sub>A</sub> receptor complex (Ticku 1990; Sieghart 1992). That is, binding by diazepam to the benzodiazepine site is necessary, but not sufficient, for diazepam to alter GABA<sub>A</sub> receptor function. Rather, this binding induces a conformational change in the receptor protein that enhances GABA's effect on the receptor (Macdonald and Olsen 1994; McCauley et al. 1994; Cooper et al. 1996). This process of allosteric coupling, in which binding to one site alters another site on the same receptor complex (Fig. 1), plays an essential role in diazepam's mechanism of action. Therefore, diazepam's mechanism of action represents a combination of the mechanisms for drugs such as ethanol, which are sensitive to antagonism by 12 ATA heliox, and drugs such as morphine, which are not.

The hyperbaric studies found that exposure to 12 ATA heliox significantly antagonized diazepam's locomotor

and anticonvulsant effects (Davies et al. 1996). Further, the pharmacological characteristics of the antagonism (direct, surmountable, rightward dose-response shift) closely matched those seen in previous studies for hyperbaric antagonism of ethanol and could not be attributed to indirect mechanisms including changes in diazepam's pharmacokinetics (Davies et al. 1996). The sensitivity of diazepam to pressure antagonism, taken in context with the relative selectivity of pressure antagonism, suggests that the critical factor which determines whether a drug is sensitive to pressure antagonism is the manner in which its primary effects on receptor function are mediated (i.e., whether allosteric modulation is involved), not on whether the drug initiates its action via receptor binding. In addition, these findings suggest a common feature (sensitivity to low level hyperbaric antagonism) for the allosteric modulation underlying ethanol's and diazepam's effects.

The mechanism for the selectivity of pressure antagonism, in contrast to the mechanism of selectivity for traditional pharmacological antagonists, cannot be based on structure-related competition at receptor binding sites, since pressure does not have a chemical structure. Rather, the selectivity of pressure antagonism presumably is based on pressure's ability to block or offset common physicochemical changes underlying allosteric modulation of receptor function. Since physicochemical changes reflect the underlying chemical structure, the selective antagonism by pressure of ethanol and diazepam suggests a functional and structural link between ethanol's site(s) of action and the allosteric coupling pathways that transduce binding events on the GABA<sub>A</sub> receptor complex.

Therefore, the link identified by pressure, between effector mechanisms for ethanol and diazepam, suggests possible sites of action for ethanol. The rationale for this conclusion is developed in detail elsewhere (Davies et al. 1996). Briefly, these studies reveal that binding to the allosterically modulated benzodiazepine recognition sites on the GABA<sub>A</sub> receptor causes small conformational changes in the receptor protein which induce ethanol-like changes in GABA<sub>A</sub> receptor function and in animal behavior that are sensitive to pressure antagonism. Taken in context with the growing body of evidence indicating that several ligand-gated ion channels (e.g., GABA<sub>A</sub>, NMDA, 5HT<sub>3</sub>) may be primary sites of ethanol action (Deitrich et al. 1989; Alcohol and Health 1993; Franks and Lieb 1994), these findings on GABA<sub>A</sub> receptors suggest that there may be something relatively unique about some ligand-gated ion channels – their allosteric coupling pathways – that make them sensitive to ethanol. This logic also could hold for the sub-population of allosterically modulated voltage-gated calcium channels which have shown ethanol sensitivity (Deitrich et al. 1989; Alcohol and Health 1993; Franks and Lieb 1994). This hypothesis suggests the intriguing possibility that there may be common physicochemical and underlying structural characteristics that define ethanol sensitive regions within a single (e.g., GABA<sub>A</sub>) and between different (e.g.,

GABA<sub>A</sub> and NMDA) receptors. This hypothesis does not exclude other possible sites or mechanisms of ethanol action, but offers a framework for explaining why some allosterically modulated ion channels are sensitive to ethanol.

The present study further tested the hypothesis that allosteric coupling pathways in the GABA<sub>A</sub> receptor complex represent initial sites of action for ethanol. This was accomplished by investigating predictions regarding the selectivity of hyperbaric antagonism versus drugs that are believed to induce their behavioral effects primarily by altering GABA<sub>A</sub> receptor function via mechanisms that either involve, or do not involve, allosteric coupling. To this end we tested the effects of 12 ATA heliox on: 1) ethanol; 2) drugs believed to induce behavioral effects through allosteric coupling initiated by binding to different allosteric modulatory sites on the GABA<sub>A</sub> receptor complex, including the benzodiazepine (diazepam, flunitrazepam), barbiturate (pentobarbital) and neuroactive steroid (3 $\alpha$ -hydroxy-5 $\beta$ -pregnan-20-one; 3 $\alpha$ ,5 $\beta$ -P) sites and 3) drugs believed to act via binding to sites on the GABA<sub>A</sub> receptor complex that do not involve allosteric coupling exemplified by the site for GABA (GABA, 4,5,6,7-tetrahydroisoxazolo-pyridin-3-ol; THIP). The effects of hyperbaric exposure on these drugs was measured behaviorally using loss of righting reflex (LORR) and anticonvulsant efficacy, and biochemically using GABA-activated <sup>36</sup>Cl<sup>-</sup> uptake in mouse brain membranes. Several measures were used to provide insight into the generality of the findings across different behavioral and dose ranges and to test whether behavioral findings would extend to biochemical measurements of GABA<sub>A</sub> receptor function in an isolated in vitro system.

## Materials and methods

Study 1: effects of 12 ATA heliox on ethanol, pentobarbital, 3 $\alpha$ ,5 $\beta$ -P and THIP induced loss of righting reflex (LORR) in mice

### *Experiment 1: ethanol*

Adult, drug naive male C57BL/6 J mice (mean weight $\pm$ SE of 23.1 $\pm$ 0.3 g) obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) were housed four per cage on a 12-h light:dark cycle (0700 hours on) in a room maintained at 22 $\pm$ 1°C for at least 1 week before testing. Food (Hardland Rodent Laboratory Chow) and water were freely available until experimentation was initiated. Previous work has shown that exposure to 12 ATA heliox antagonized ethanol-induced LORR in C57 mice (Alkana and Malcolm 1981, 1982a,b; Malcolm and Alkana 1982; Alkana et al. 1992). Therefore, this experiment was conducted as a positive control for the other drugs to be tested.

*Experimental design.* On test day, experimental animals were tail marked and weighed between 0800 and 0830 hours and returned to their cages. At the start of the experiment (1300–1330 hours), each mouse was injected IP with ethanol. After losing its righting reflex, the mouse was placed on its back in a V-shaped cardboard trough, within a tubular Plexiglas container. Groups of four individually housed mice were placed into a temperature controlled hyperbaric chamber and exposed to one of two atmospheric conditions (1 ATA air or 12 ATA heliox) according to a between-subjects counterbalanced design, as previous described (Alkana et al.

1992). Following experimentation, mice were killed by injecting an anesthetic dose of 20% ethanol followed by cervical dislocation.

**Drug treatment.** Ethanol obtained from Spectrum Chemical Mfg Corp. (Gardena, Calif., USA; 95% USP) was diluted to a final concentration of 20% w/v and administered by IP injection at a dose of 3.6 g/kg body weight at a volume of 0.1 ml/20 g body weight. The dose of ethanol used was selected to induce a LORR duration of approximately 70 min.

**Hyperbaric treatment.** Animals were placed in 18-l cylindrical stainless steel hyperbaric chambers and exposed to 12 ATA heliox or to the control atmospheric condition of 1 ATA air using pre-mixed, certified compressed gases (SoCal Airgas, Los Angeles, Calif., USA) following protocols described elsewhere (Alkana et al. 1992). The chamber temperature was adjusted to 33.5°C for air exposed animals and to 34.5°C for heliox groups with an automated heating/cooling system (Syapin et al. 1988). Previous studies demonstrated that these ambient temperatures offset the hypothermic effects of helium and ethanol (Malcolm and Alkana 1982), thereby minimizing the potential for confounded results due to differences in body temperature (Alkana et al. 1985a). A 1 ATA heliox control was not used in this experiment because previous studies clearly demonstrated that exposure to 1 ATA heliox did not significantly affect ethanol-induced LORR (Alkana and Malcolm 1982a; Malcolm and Alkana 1982).

**Loss of righting reflex (LORR) duration.** LORR duration was defined as the time between the moment following ethanol injection when a mouse placed on its back in a V-shaped cardboard tray lost its ability to right itself to the time at which the mouse turned itself back over and touched all four paws to the tray. The procedures utilized have been previously described (Alkana and Malcolm 1980, 1982a,b; Malcolm and Alkana 1982; Alkana et al. 1992). Two mice regained their righting reflex before the pressurization procedure was completed (one mouse from each atmospheric condition group) and were eliminated from the statistical analyses.

#### *Experiment 2: pentobarbital*

Adult, drug naive male C57BL/6 J mice (mean weight $\pm$ SE of 24.1 $\pm$ 0.2 g) were tested using procedures similar to those of experiment 1, except for the following modifications: 1) body temperature measurements were taken prior to the injection of drug and upon removal from the chamber as described below; 2) immediately after its baseline temperature measurement, each mouse was injected IP with 60 mg/kg pentobarbital. Pentobarbital (Sigma Chemical Co., St Louis, Mo., USA) was dissolved in 4 ml saline to make a 9 mg/ml solution (prepared fresh daily) at a volume of 0.1 ml/20 g body weight; 3) groups of two individually housed mice were placed into a temperature controlled, hyperbaric chamber; 4) mice were exposed to one of three atmospheric conditions (1 ATA air, 1 ATA heliox or 12 ATA heliox). All three atmospheric conditions were tested simultaneously and the atmospheric conditions were rotated between chambers from day to day. The time of individual return of righting reflex of each mouse in the chamber was noted. Two mice regained their righting reflex before the pressurization procedure was completed (12 ATA heliox group) and were eliminated from the statistical analyses.

**Temperature measurement.** Body temperatures were monitored to insure that temperature conditions established for ethanol were appropriate for other GABAergic drugs. Baseline rectal-colonic temperatures were measured with a digital thermometer (model BAT-12, Physitemp, Clifton, N.J., USA) using a glycerol-lubricated metal miniprobe (model RET-3; Physitemp) inserted 1.9 cm into the rectum (Alkana et al. 1985a). Body temperatures were measured in 1 ATA air and 1 ATA heliox groups (as soon as removed from the chamber).

#### *Experiment 3: 3 $\alpha$ ,5 $\beta$ -P*

Adult, drug naive male C57BL/6 J mice (mean weight $\pm$ SE of 25.3 $\pm$ 0.35 g) were tested using procedures similar to those of experiment 2, except for the following modifications: Immediately after its baseline temperature measurement, each mouse was injected IP with 45 mg/kg 3 $\alpha$ ,5 $\beta$ -P. 3 $\alpha$ ,5 $\beta$ -P (Diosynth Inc., Chicago, Ill., USA) suspended in 3 ml solution of 20% 2-hydroxypropyl- $\beta$ -cyclodextrin ( $\beta$ -cyclodextrin, Research Biochemicals International, Natick, Mass., USA) to make a 6.77 mg/ml solution (prepared fresh daily) at a volume of 0.1 ml/15 g body weight. 3 $\alpha$ ,5 $\beta$ -P is a very potent GABA-agonist neuroactive steroid which is relatively specific for GABA<sub>A</sub> receptors.

#### *Experiment 4: THIP*

Adult, drug naive male C57BL/6 J mice (mean weight $\pm$ SE of 26.1 $\pm$ 0.4 g) were tested using procedures similar to those of experiment 2, except for the following modifications. Immediately after its baseline temperature measurement, each mouse was injected IP with 55 mg/kg THIP (Research Biochemicals). THIP was suspended in 4 ml saline to make a 8.25 mg/ml solution (prepared fresh daily) at a volume of 0.1 ml/20 g body weight. THIP is a GABA agonist that passes the blood-brain barrier. One mouse regained its righting reflex before the pressurization procedure was completed (1 ATA heliox group) and was eliminated from the statistical analyses.

Study 2: effects of 12 ATA Heliox on ethanol-, diazepam-, pentobarbital- and 3 $\alpha$ ,5 $\beta$ -P-induced anticonvulsant effect in mice

#### *Experiment 1: ethanol*

Adult, drug naive male LS mice (mean weight $\pm$ SE of 27.1 $\pm$ 0.6 g) obtained from the Institute for Behavioral Genetics (University of Colorado, Boulder Colorado, USA) were utilized in this experiment and were housed and cared for as described in study 1. We used LS mice to extend investigation to a second genotype and to match the genotype used in our previous in vitro studies which found that pressure antagonizes ethanol potentiation of GABA<sub>A</sub> receptor function (Davies and Alkana 1998).

**Experimental design.** On test day, experimental animals were tail marked and weighed between 0800 and 0830 hours and returned to their cages. Experiments began between 1300 and 1330 hours. The consequence of exposure to 12 ATA heliox on ethanol's anticonvulsant effects versus the convulsant, isoniazid (INH), were investigated using a between-subjects counterbalanced design following previously described protocols (Davies et al. 1994). Briefly, each mouse was injected with either saline (vehicle) or ethanol, followed immediately by injection of the INH. Within 4 min after the injection of INH, the animal was exposed to one of the three atmospheric conditions (1 ATA air, 1 ATA heliox, or 12 ATA heliox) for 120 min after INH injection.

**Drug treatment.** INH (40 mg/ml) obtained from Sigma was dissolved in normal saline before intramuscular injection (IM) at 200 mg/kg and was administered at a volume of 0.1 ml/20 g body weight. Ethanol (20% w/v in normal saline from USP 95%) was administered IP at a dose of 2.0 g/kg body weight. The vehicle control solution consisted of normal saline.

**Hyperbaric treatment.** Animals were exposed to 12 ATA heliox or to control atmospheric conditions of 1 ATA air or 1 ATA heliox in 18-l cylindrical stainless steel hyperbaric chambers as described in study 1 with the following modifications: For these studies, chamber temperature was maintained at 25°C during exposure to 1 ATA air and at 30°C during exposure to 1 or 12 ATA heliox. These temperatures were selected to offset the hypothermic effects of helium (Syapin et al. 1988).

*Anticonvulsant effect.* The effects of pressure on anticonvulsant drug effects were measured using previously described techniques (Davies et al. 1994, 1996). Briefly, behavior was videotaped from 15 to 120 min after the last drug injection. The behavior of each mouse was scored from the video tape by a "blinded" observer. The scoring method has been described previously (Davies et al. 1994, 1996). Animals that did not demonstrate myoclonus were assigned a latency score of 120 min.

#### *Experiment 2: diazepam*

Adult, drug naive male LS mice (mean weight $\pm$ SE of 23.3 $\pm$ 0.4 g) were tested using procedures similar to those of experiment 1, except for the following: INH (50 mg/ml) was dissolved in normal saline before IM injection at 250 mg/kg body weight. Diazepam obtained from Sigma was suspended in 3 ml solution of 20% 2-hydroxypropyl- $\beta$ -cyclodextrin, ( $\beta$ -cyclodextrin, Research Biochemicals International) to make a 3.0 mg/ml solution and was injected at a dose of 2 mg/kg body weight at a volume of 0.1 ml/15 g body weight. The vehicle control solution was prepared from 20%  $\beta$ -cyclodextrin dissolved in distilled water. This experiment extended previous diazepam studies conducted in C57 mice (Davies et al. 1996).

#### *Experiment 3: pentobarbital*

Adult, drug naive male LS mice (mean weight $\pm$ SE of 27.1 $\pm$ 0.3 g) were tested using procedures similar to those of experiment 2, except for the following: Pentobarbital obtained from Sigma was dissolved in normal saline to make a 6.25 mg/ml solution and was injected at a dose of 40 mg/kg body weight at a volume of 0.1 ml/15 g body weight. Since atmospheric condition had previously been demonstrated not to affect time to latency in saline controls, we minimized the number of animals tested in this group.

#### *Experiment 4: 3 $\alpha$ ,5 $\beta$ -P*

Adult, drug naive male LS mice (mean weight $\pm$ SE of 22.9 $\pm$ 0.3 g) were tested using procedures similar to those of experiment 2 except for the following: 3 $\alpha$ ,5 $\beta$ -P obtained from Diosynth Inc. (Chicago, Ill., USA), was suspended in 3 ml solution of 20% 2-hydroxypropyl- $\beta$ -cyclodextrin, to make a 5.25 mg/ml solution and was injected at a dose of 35 mg/kg body weight at a volume of 0.1 ml/15 g body weight.

Study 3: effects of low level hyperbaric exposure on ethanol, flunitrazepam, pentobarbital and 3 $\alpha$ ,5 $\beta$ -P potentiation of GABA-activated  $^{36}\text{Cl}^-$  uptake in mouse brain microsacs

#### *Experiment 1: ethanol*

This study used an isolated sub-cellular membrane preparation (microsacs) prepared from adult male LS mouse brains. The microsac preparation consists of pre- and postsynaptic vesicles which retain functional coupling between GABA, benzodiazepine, barbiturate and neuroactive steroid sites, and the chloride channel of the GABA<sub>A</sub> receptor complex (Daly et al. 1980; Harris and Allan 1985; Morrow and Paul 1988; Morrow et al. 1990). The mice were housed and cared for as described in study 1. We recently showed that exposure to 12 ATA heliox antagonizes ethanol potentiation on GABA-activated  $^{36}\text{Cl}^-$  uptake in this preparation (Davies and Alkana 1998). This experiment was included to replicate and extend previous in vitro work to test the effects of 1 ATA heliox on ethanol potentiation of GABA-activated  $^{36}\text{Cl}^-$  uptake.

*Experimental design.* Experimental animals were brought into the laboratory at least 2 h prior to experimentation. Mice were decapitated and their brains (less cerebellum) were removed and a brain

homogenate consisting of membrane vesicles (microsacs) was prepared. The microsacs were combined with ethanol and other GABA<sub>A</sub> ligands and exposed to experimental atmospheric conditions (1 ATA air, 1 ATA and 12 ATA heliox) in temperature controlled, hyperbaric chambers. Experiments were designed to minimize variability in the chloride assay procedure and to ensure that order effects within an experiment did not bias the results. At the end of experimentation, the reaction was terminated and the chambers were decompressed, followed by rapid filtration and washing of the assay preparation. Radioactivity associated with tissue retained on the filters was determined using liquid spectrophotometry.

*Microsac preparation.* The method for preparing the microsacs was modified from those of Allan and Harris (1986) and has been described in detail elsewhere (Davies and Alkana 1998). Briefly, two mice per preparation were killed by decapitation (McQuilkin and Harris 1990) and their brains were removed on ice. The cerebellum and white matter were dissected and discarded and the remaining portion of the brain was homogenized in 20 ml ice-cold 10 mM TRIS-HEPES buffer (145 mM NaCl, 5 mM KCL, 1 mM MgCl<sub>2</sub>, 10 mM *d*-glucose, 1 mM CaCl<sub>2</sub>, pH=7.5 -  $\times$ 1 TRIS), centrifuged at 1000 *g* for 15 min at 4°C. The pellet was washed with 20 ml ice-cold buffer and again centrifuged at 1000 *g* for 15 min at 4°C. The final pellet was suspended in 7.5–8.5 ml ice-cold  $\times$ 1 TRIS buffer which yielded a protein concentration of 3–4 mg/ml, as determined later by the method of Lowry (Lowry et al. 1951). The microsac preparations were utilized within 2 h of preparation to minimize the loss of ethanol sensitivity (McQuilkin and Harris 1990).

*$^{36}\text{Cl}^-$  uptake and hyperbaric chamber.* We utilized a chloride flux filtration assay, developed by Harris and Allan (1989b), modified for use under hyperbaric conditions as described in detail elsewhere (Davies and Alkana 1998). Briefly, 200  $\mu$ l aliquots of microsacs and 200  $\mu$ l aliquots of  $^{36}\text{Cl}^-$  assay buffer [10  $\mu$ M GABA,  $^{36}\text{Cl}^-$  (1.6  $\mu$ Ci/ml) $\pm$ ethanol (25 mM)] were pre-incubated at 35°C for 10 min.  $^{36}\text{Cl}^-$  uptake was initiated by combining the microsacs and  $^{36}\text{Cl}^-$  assay buffer in a 75 ml hyperbaric chamber followed by immediate exposure to 1 ATA air, 1 ATA heliox or 12 ATA heliox using premixed, certified compressed gases. After 7 s, the reaction was terminated by addition of 4 ml ice-cold  $\times$ 1 TRIS assay buffer containing 100  $\mu$ M picrotoxin, followed immediately by decompression of the hyperbaric chamber (15 s decompression time) and rapid filtration of the assay preparation through a Whatman GF/C filter. The filters were washed twice with 4 ml ice-cold  $\times$ 1 TRIS- $\times$ 100 picrotoxin buffer and placed into 7 ml scintillation vials. Aliquots of 5 ml Safety-Solve counting cocktail (Research Products International, Mt Prospect, Ill., USA) was added to each vial and radioactivity associated with the tissue retained on the filters was determined.

#### *Experiment 2: flunitrazepam*

Microsacs were prepared from adult, drug naive male LS mice and treated using procedures similar to experiment 1, with the following modifications: 1) flunitrazepam (10  $\mu$ M) was tested rather than ethanol; 2) 1 ATA heliox was not tested in this experiment.

#### *Experiment 3: pentobarbital*

Microsacs were prepared from adult, drug naive male LS mice and treated using procedures similar to experiment 2, with the following modifications: 1) pentobarbital (50  $\mu$ M) was tested in both the presence and absence of 10  $\mu$ M GABA.

#### *Experiment 4: 3 $\alpha$ ,5 $\beta$ -P*

Microsacs were prepared from adult, drug naive male LS mice and treated using procedures similar to experiment 2, with the follow-

ing modifications:  $3\alpha,5\beta$ -P (1  $\mu$ M) was tested in both the presence and absence of 10  $\mu$ M GABA.

**Drugs and chemicals.** Drugs were prepared daily. Ethanol (Spectrum Chemical; 95% USP),  $\gamma$ -amino-*n*-butyric acid (GABA; ICN Biomedicals, Inc., Aurora, Ohio, USA) and pentobarbital were dissolved in 10 mM TRIS-HEPES buffer (145 mM NaCl, 5 mM KCL, 1 mM  $MgCl_2$ , 10 mM *d*-glucose, 1 mM  $CaCl_2$ , pH=7.5 –  $\times$ 1 TRIS) buffer to their final concentrations. Flunitrazepam (Sigma) and  $3\alpha,5\beta$ -P (Diosynth) were dissolved in dimethyl sulfoxide (DMSO) up to a maximum of 0.5%.  $^{36}Cl^-$  (specific activity 13.98 mCi/g Cl) was purchased from Dupont-New England Nuclear (Boston, Mass., USA). All other drugs and reagents used were analytical grade and were supplied by Sigma.

Data analyses for studies 1–3

The data were analyzed using Prism (GraphPad Software, San Diego, Calif., USA).

#### Study 1

ANOVA was utilized to compare the effects of atmospheric condition on drug-induced LORR duration. When warranted by a significant effect of atmospheric condition, post-hoc comparisons were conducted using unpaired *t*-test.

#### Study 2

ANOVA was utilized to determine the effects of drug and atmospheric condition on latency to INH-induced myoclonus. When warranted, post hoc analyses were conducted using unpaired *t*-test.

#### Study 3

The amount of  $^{36}Cl^-$  bound to the Whatman GF/C filters in the absence of membranes (no-tissue blank) was subtracted from all values. GABA-activated  $^{36}Cl^-$  uptake (NET uptake) was defined as

the amount of  $^{36}Cl^-$  taken up while GABA was present in the medium (total uptake) minus the amount of chloride taken up when agonist was not present (non-specific uptake). Drug potentiation (ethanol, flunitrazepam, pentobarbital,  $3\alpha,5\beta$ -P) of GABA activated  $^{36}Cl^-$  uptake (Allosteric NET uptake) was defined as NET uptake in the presence of drug minus NET uptake in the absence of drug. Data were analyzed as either NET or allosteric NET uptake. ANOVA was utilized to determine the effects of drug and atmospheric condition on GABA-activated  $^{36}Cl^-$  uptake. When warranted, post hoc analyses were conducted using unpaired *t*-test.

Data presented in the text and figures are expressed as mean $\pm$ SE and the number of mice tested are given in parentheses unless otherwise noted. The level of statistical significance was set at  $P < 0.05$  for all analyses.

## Results

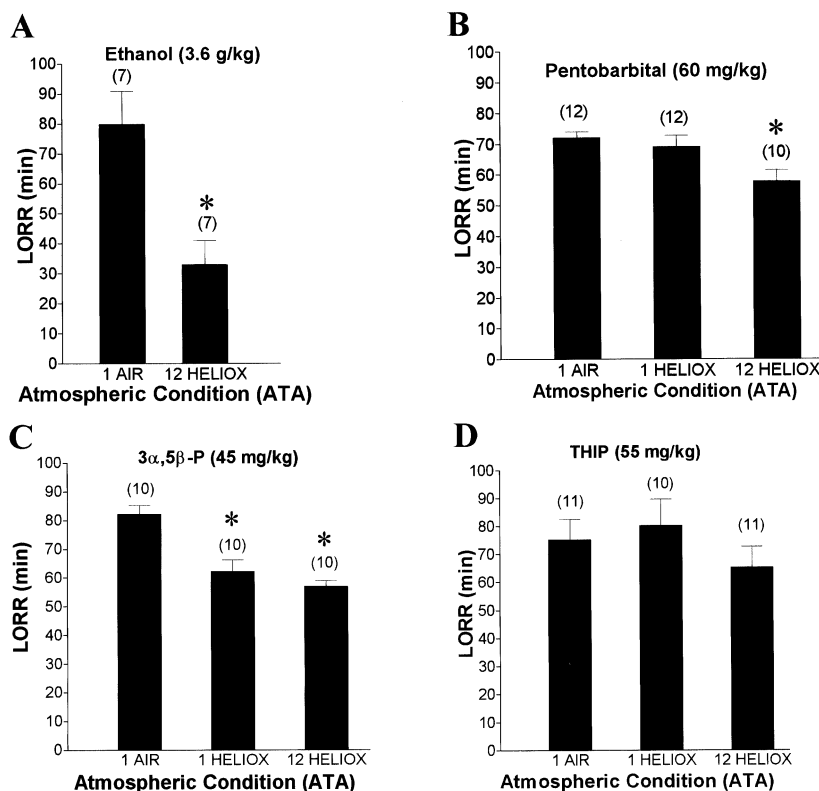
### Study 1

The effects of 12 ATA heliox on ethanol, pentobarbital,  $3\alpha,5\beta$ -P and THIP induced LORR in C57 mice are depicted in Fig. 2.

#### Ethanol

Unpaired *t*-test indicated that exposure to 12 ATA heliox significantly antagonized ethanol-induced LORR (Fig. 2A).

**Fig. 2A–D** Effects of hyperbaric exposure on **A** ethanol, **B** pentobarbital, **C**  $3\alpha,5\beta$ -P and **D** THIP induced loss of righting reflex (LORR) in C57 mice. The bars show the mean $\pm$ SE LORR duration in mice given a hypnotic dose of drug. The number of animals per group is given in parentheses (\* $P < 0.05$  as compared to respective 1 ATA air control)

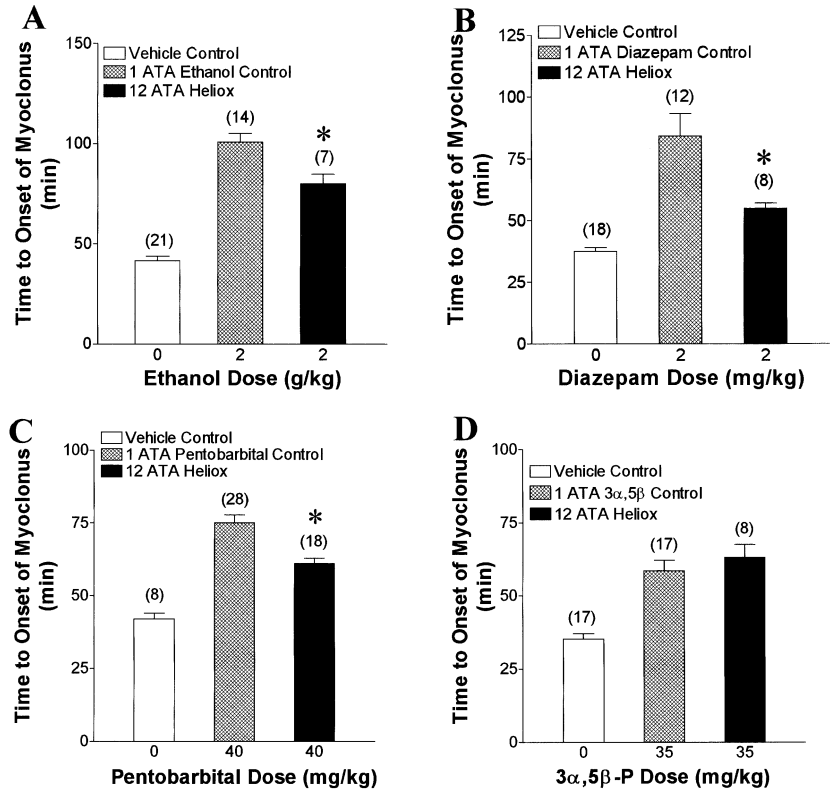


**Table 1** Rectal temperature at baseline and righting reflex (RORR). Rectal temperatures taken at return of RORR confirmed that the hypothermic effects of pentobarbital, 3 $\alpha$ ,5 $\beta$ -P, THIP and helium

were offset in that body temperatures were held within  $\pm 1^\circ\text{C}$  of air controls. Shown are the mean $\pm$ SE rectal temperatures at baseline and RORR

Drug	1 ATA air		1 ATA heliox	
	Base temp. ( $^\circ\text{C}$ )	RORR temp. ( $^\circ\text{C}$ )	Base temp. ( $^\circ\text{C}$ )	RORR temp. ( $^\circ\text{C}$ )
Pentobarbital	37.0 $\pm$ 0.3	38.0 $\pm$ 0.1	37.6 $\pm$ 0.1	38.0 $\pm$ 0.1
3 $\alpha$ ,5 $\beta$ -P	38.1 $\pm$ 0.2	37.5 $\pm$ 0.5	37.7 $\pm$ 0.2	37.7 $\pm$ 0.2
THIP	37.7 $\pm$ 0.2	36.9 $\pm$ 0.2	37.1 $\pm$ 0.1	36.2 $\pm$ 0.2

**Fig. 3A–D** Effects of hyperbaric exposure on **A** ethanol, **B** diazepam, **C** pentobarbital and **D** 3 $\alpha$ ,5 $\beta$ -P induced anti-convulsant effect versus INH in LS mice. The bars show the mean $\pm$ SE latency to onset of INH-induced myoclonus in mice receiving injections of drug or saline before exposure to control or experimental atmospheric conditions. Exposure to 12 ATA heliox significantly decreased ethanol's (**A**), diazepam's (**B**), pentobarbital's (**C**), but not 3 $\alpha$ ,5 $\beta$ -P's (**D**) anti-convulsant effect as measured by time to onset of INH-induced myoclonic seizures. The number of animals per group is shown in parentheses (\* $P$ <0.05 as compared to respective 1 ATA drug control group)



### Pentobarbital

ANOVA revealed significant differences between pentobarbital-induced LORR in mice exposed to 1 ATA air, 1 ATA heliox and 12 ATA heliox ( $F_{2,33}=5.525$ ,  $P<0.01$ ) (Fig. 2B). Post hoc analysis indicated that pentobarbital-induced hypnotic effects were significantly reduced by exposure to 12 ATA heliox and that exposure to 1 ATA heliox did not significantly alter pentobarbital's hypnotic effects.

### 3 $\alpha$ ,5 $\beta$ -P

ANOVA revealed significant differences between 3 $\alpha$ ,5 $\beta$ -P-induced LORR in mice exposed to 1 ATA air, 1 ATA heliox and 12 ATA heliox ( $F_{2,29}=17.626$ ,  $P<0.01$ ) (Fig. 2C). Post hoc analysis indicated that 3 $\alpha$ ,5 $\beta$ -P induced hypnotic effects were significantly antagonized by exposure to 1 and 12 ATA heliox as compared to 1 ATA air. However, there were no statistically significant differ-

ences between the effects of 1 ATA and 12 ATA heliox on 3 $\alpha$ ,5 $\beta$ -P-induced LORR.

### THIP

ANOVA did not reveal significant differences between THIP-induced LORR in mice exposed to 1 ATA air, 1 ATA heliox or 12 ATA heliox (Fig. 2D).

Rectal temperatures taken at RORR confirmed that the hypothermic effects of pentobarbital, 3 $\alpha$ ,5 $\beta$ -P, THIP and helium were offset in that body temperatures were held within  $\pm 1^\circ\text{C}$  of air controls by the experimental conditions (Table 1).

### Study 2

The effects of 12 ATA heliox on ethanol, diazepam, pentobarbital and 3 $\alpha$ ,5 $\beta$ -P induced anticonvulsant effect in LS mice are depicted in Fig. 3. One-way ANOVAs deter-

mined that there were no significant differences in latency to myoclonus in either vehicle (1 ATA air, 1 ATA and 12 ATA heliox) or drug (1 ATA air and 1 ATA heliox) control groups, respectively, for experiments 1–4 in this study. Hence, exposure to 1 ATA heliox did not significantly affect latency to myoclonus in vehicle- or drug-injected mice and exposure to 12 ATA heliox did not significantly affect seizure latency in mice injected with vehicle. Consequently, the individual vehicle (1 ATA air, 1 ATA and 12 ATA heliox) and drug (1 ATA air and 1 ATA heliox) groups were collapsed into their respective control groups (vehicle control; drug control) prior to further analysis.

### Ethanol

One-way ANOVA revealed a significant treatment effect ( $F_{2,41}=82.25$ ,  $P<0.0001$ ) (Fig. 3A). Post hoc analysis indicated that exposure to 12 ATA heliox significantly antagonized ethanol's (2 g/kg) anticonvulsant effect, as demonstrated by a significant reduction in latency to onset of myoclonus induced by 200 mg/kg of INH compared to mice exposed to drug control conditions (collapsed 1 ATA air and 1 ATA heliox).

### Diazepam

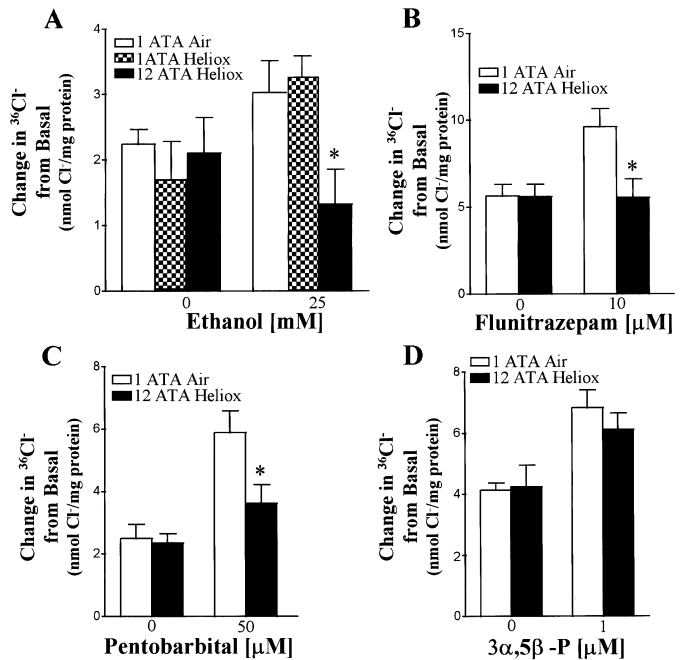
One-way ANOVA revealed a significant treatment effect ( $F_{2,35}=23.12$ ,  $P<0.0001$ ) (Fig. 3B). Post hoc analysis indicated that exposure to 12 ATA heliox significantly antagonized diazepam's (2 mg/kg) anticonvulsant effect, as demonstrated by a significant reduction in latency to onset of myoclonus induced by 250 mg/kg INH compared to mice exposed to drug control conditions.

### Pentobarbital

One-way ANOVA revealed a significant treatment effect ( $F_{2,51}=26.66$ ,  $P<0.002$ ) (Fig. 3C). Post hoc analysis indicated that exposure to 12 ATA heliox significantly antagonized pentobarbital's (40 mg/kg) anticonvulsant effect as demonstrated by a significant reduction in latency to onset of myoclonus induced by 250 mg/kg INH compared to mice exposed to drug control conditions.

### 3 $\alpha$ ,5 $\beta$ -P

One-way ANOVA revealed a significant treatment effect ( $F_{2,39}=23.14$ ,  $P<0.0001$ ) (Fig. 3D). Post hoc analysis indicated that 3 $\alpha$ ,5 $\beta$ -P (35 mg/kg) significantly increased the latency to onset of myoclonus induced by 250 mg/kg INH, as compared to vehicle treated mice. Exposure to 12 ATA heliox did not significantly antagonize the anticonvulsant effect of 3 $\alpha$ ,5 $\beta$ -P.



**Fig. 4A–D** Effects of hyperbaric exposure on **A** ethanol, **B** flunitrazepam, **C** pentobarbital and **D** 3 $\alpha$ ,5 $\beta$ -P induced potentiation of 10  $\mu\text{M}$  GABA-activated  $^{36}\text{Cl}^-$  uptake in LS mouse brain microsacs. The ordinate represents net  $^{36}\text{Cl}^-$  uptake in nmol/mg protein per 7 s. The abscissa represents the concentration of drug per liter. Exposure to 12 ATA heliox significantly decreased ethanol's (A), flunitrazepam's (B), pentobarbital's (C), but not 3 $\alpha$ ,5 $\beta$ -P's (D) potentiating effect of GABA-activated  $^{36}\text{Cl}^-$  uptake. Each point represents mean  $\pm$  SE,  $n=4-9$ , in triplicate (\* $P<0.05$  as compared to respective 1 ATA Air control)

### Study 3

The effects of low level hyperbaric exposure on ethanol, flunitrazepam, pentobarbital and 3 $\alpha$ ,5 $\beta$ -P potentiation of GABA-activated  $^{36}\text{Cl}^-$  uptake in LS mouse brain microsacs are depicted in Fig. 4.

### Ethanol

One-way ANOVA determined that there were no significant differences in  $^{36}\text{Cl}^-$  uptake in either GABA (1 ATA air, 1 ATA and 12 ATA heliox) or ethanol (1 ATA air and 1 ATA heliox) control groups (Fig. 4A). Consequently, the individual GABA (1 ATA air, 1 ATA and 12 ATA heliox) and ethanol (1 ATA air and 1 ATA heliox) control groups were collapsed into their respective control groups (GABA control; ethanol control) prior to further analysis. One-way ANOVA comparing the GABA control, ethanol control and ethanol pressure groups revealed a significant effect of treatment ( $F_{2,39}=6.59$ ,  $P<0.003$ ). Post hoc analysis indicated that exposure to 12 ATA heliox significantly antagonized ethanol (25 mM) potentiation of GABA (10  $\mu\text{M}$ )-activated  $^{36}\text{Cl}^-$  uptake as compared to 1 ATA ethanol control. Exposure to 1 ATA heliox did not significantly affect GABA-activated  $^{36}\text{Cl}^-$  uptake or ethanol's potentiation of GABA-activated  $^{36}\text{Cl}^-$

uptake and exposure to 12 ATA heliox did not significantly alter GABA-activated  $^{36}\text{Cl}^-$  uptake as compared to 1 ATA air.

### *Flunitrazepam*

Two-way ANOVA revealed significant main effects of flunitrazepam ( $F_{1,16}=4.83$ ,  $P<0.05$ ) and atmospheric condition ( $F_{1,16}=5.41$ ,  $P<0.05$ ) (Fig. 4B), with a significant interaction between flunitrazepam and atmospheric condition ( $F_{1,16}=5.12$ ,  $P<0.05$ ) on GABA-activated  $^{36}\text{Cl}^-$  uptake. Post hoc analysis revealed that exposure to 12 ATA heliox significantly antagonized flunitrazepam (10  $\mu\text{M}$ ) potentiation of GABA (10  $\mu\text{M}$ )-activated  $^{36}\text{Cl}^-$  uptake as compared to 1 ATA air. Exposure to 12 ATA heliox did not significantly affect GABA-activated  $^{36}\text{Cl}^-$  uptake as compared to 1 ATA air.

### *Pentobarbital*

Two-way ANOVA revealed significant main effects of pentobarbital ( $F_{2,48}=49.9$ ,  $P<0.01$ ) (Fig. 4C) and atmospheric condition ( $F_{1,48}=4.38$ ,  $P<0.050$ ), with a significant interaction between pentobarbital and atmospheric condition ( $F_{2,48}=3.43$ ,  $P<0.05$ ) on GABA activated  $^{36}\text{Cl}^-$  uptake. Post hoc analysis revealed that exposure to 12 ATA heliox significantly antagonized pentobarbital (50  $\mu\text{M}$ ) potentiation of GABA (10  $\mu\text{M}$ )-activated  $^{36}\text{Cl}^-$  uptake as compared to 1 ATA air. Exposure to 12 ATA heliox significantly antagonized pentobarbital's potentiation of GABA activated  $^{36}\text{Cl}^-$  uptake. Exposure to 12 ATA heliox did not significantly affect GABA activated  $^{36}\text{Cl}^-$  uptake as compared to 1 ATA air. Pentobarbital (50  $\mu\text{M}$ ) did not significantly affect  $^{36}\text{Cl}^-$  uptake in the absence of GABA (data not shown).

### *3 $\alpha$ ,5 $\beta$ -P*

Two-way ANOVA revealed a significant main effect of 3 $\alpha$ ,5 $\beta$ -P ( $F_{2,18}=65.19$ ,  $P<0.01$ ) (Fig. 4D), but not of atmospheric condition on GABA activated  $^{36}\text{Cl}^-$  uptake and no significant interaction between drug and atmospheric condition. Post hoc analysis revealed that exposure to 12 ATA heliox did not significantly alter 3 $\alpha$ ,5 $\beta$ -P (1  $\mu\text{M}$ ) potentiation of GABA (10  $\mu\text{M}$ )-activated  $^{36}\text{Cl}^-$  uptake as compared to 1 ATA air. 3 $\alpha$ ,5 $\beta$ -P (1  $\mu\text{M}$ ) did not significantly affect  $^{36}\text{Cl}^-$  uptake in the absence of GABA (data not shown).

## **Discussion**

The present experiments tested the hypothesis that allosteric coupling pathways in the GABA<sub>A</sub> receptor complex represent initial sites of action for ethanol. If ethanol acts on allosteric coupling pathways in GABA<sub>A</sub> re-

ceptors, then exposure to a direct ethanol antagonist (pressure) should antagonize the functional effects of ethanol and other allosteric modulatory drugs whose effects on GABA<sub>A</sub> receptors are mediated via allosteric coupling pathways with similar characteristics. Selective pressure antagonism among allosteric modulators would identify a subset of coupling pathways that represent likely sites of action for ethanol. Further, if pressure selectively acts on drug effects mediated through allosteric coupling pathways, then pressure should not antagonize the functional effects of ligands that directly alter GABA<sub>A</sub> receptor function via mechanisms that do not involve allosteric modulation. The findings of the present study, taken with other related work (see Introduction), agree with these predictions. Exposure to 12 ATA heliox antagonized the LORR, anticonvulsant effects and in vitro potentiation of GABA<sub>A</sub> receptor function induced by drugs that act via allosteric modulation including ethanol, pentobarbital, diazepam and flunitrazepam. On the other hand, exposure to 12 ATA heliox did not significantly alter the effects on these measures of drugs that change GABA<sub>A</sub> receptor function by mechanisms that do not involve allosteric coupling, including GABA and THIP. These findings replicate and extend previous behavioral and biochemical studies with ethanol, diazepam and other drugs (see Introduction) and add further support for the hypothesis that allosteric coupling pathways in the GABA<sub>A</sub> receptor complex represent initial sites of action for ethanol (Davies et al. 1996).

In contrast to its antagonistic effects on ethanol, pentobarbital and diazepam, exposure to 12 ATA heliox did not antagonize the anticonvulsant effects or the potentiation of GABA<sub>A</sub> receptor function in vitro by the allosteric modulator 3 $\alpha$ ,5 $\beta$ -P. Exposure to 12 ATA heliox antagonized LORR induced by 3 $\alpha$ ,5 $\beta$ -P, but not to a greater extent than did exposure to 1 ATA heliox, suggesting that the reduction in 3 $\alpha$ ,5 $\beta$ -P-induced LORR in the 12 ATA heliox group was due to an unexpected action of helium rather than pressure. Further study is necessary to determine the importance of this singular result with helium. Regardless, the collective behavioral and in vitro findings with 3 $\alpha$ ,5 $\beta$ -P suggest that the coupling process mediating allosteric modulation of GABA<sub>A</sub> receptor between the recognition sites for this neuroactive steroid and the GABA effector may be resistant to pressure antagonism, compared to the allosteric coupling mediating the effects of benzodiazepines and barbiturates. This finding, suggesting that neuroactive steroids may react differently to pressure, adds to evidence supporting the notion of heterogeneity between coupling for neuroactive steroids versus coupling for other allosteric modulators of GABA<sub>A</sub> receptor function (i.e., benzodiazepines and barbiturates) (Gee et al. 1988; Puia et al. 1990; Sieghart 1992, 1995; Devaud et al. 1995).

The differences in response to pressure antagonism among allosteric modulators has important implications for ethanol's sites of action. As discussed in the introduction, the selectivity of pressure antagonism presumably is based on pressure's ability to block or offset com-

mon physicochemical changes underlying allosteric modulation of receptor function by ethanol and other drugs. Since these physicochemical changes reflect the underlying chemical structure, similarities between the effects of pressure on ethanol and other allosteric modulators suggest a functional and structural link between ethanol's site(s) of action and the allosteric coupling pathways that transduce binding events for these allosteric modulators. Therefore, the ability of pressure to antagonize diazepam, flunitrazepam and pentobarbital suggests that the allosteric coupling pathways for these ligands represent likely sites of action for ethanol. On the other hand, although more complete concentration-response studies are necessary, the failure of pressure to antagonize  $3\alpha,5\beta$ -P suggests that the coupling pathway for this neuroactive steroid differs from those for diazepam, flunitrazepam and pentobarbital and may not represent a likely site of action for ethanol.

The behavioral results in the present study add to earlier findings indicating that the mechanism of pressure antagonism is direct and cannot be explained by pressure- or helium-induced generalized CNS excitatory effects or to alterations in oxygen partial pressure (Alkana and Malcolm 1982b), drug pharmacokinetics or changes in body temperature (Alkana and Malcolm 1982a,b; Alkana et al. 1991b; Davies et al. 1994, 1996; Syapin et al. 1996). First, pressure antagonized behavioral effects of ethanol, diazepam and pentobarbital, but did not antagonize the effects of THIP or  $3\alpha,5\beta$ -P. This demonstration that pressure antagonism did not generalize across these drugs that act via stimulation of GABA<sub>A</sub> receptor function indicates that pressure antagonism cannot be explained by a simple physiological response like increased general CNS excitability induced by compression, or by stress induced by exposure to hyperbaric conditions after compression that would counteract the effects of GABA<sub>A</sub> receptor stimulation. Second, exposure to 1 ATA heliox did not significantly alter the behavioral effects of ethanol, diazepam and or pentobarbital indicating that the antagonism induced by exposure to 12 ATA heliox did not reflect an action of helium per se. Third, baseline temperatures and temperatures taken at RORR were not significantly different. Thus, taken in context with previous studies (Finn et al. 1990), antagonism by pressure of drug induced behaviors cannot be attributed to drug or helium-induced changes in body temperature. Collectively, these behavioral results provide consistent evidence that exposure to 12 ATA heliox directly antagonizes allosteric modulators of GABA<sub>A</sub> receptor function.

The present in vitro findings also strongly support a direct mechanism for hyperbaric antagonism. This work demonstrated that exposure to 12 ATA heliox antagonized ethanol-, flunitrazepam- and pentobarbital-induced activation of GABA<sub>A</sub> receptor function in an isolated membrane preparation without altering the sensitivity of the receptor to GABA. These findings replicate and extend recent studies which found that exposure to 12 ATA heliox selectively antagonized ethanol potentiation of GABA-activated  $^{36}\text{Cl}^-$  uptake, but did not significantly

alter the effects of GABA, picrotoxin or bicuculline on  $^{36}\text{Cl}^-$  uptake (Davies and Alkana 1998). The present in vitro findings with ethanol also indicate that the antagonistic effects of pressure cannot be attributed to the effects of helium. Taken together, present and past in vitro findings show that the antagonistic effects of pressure do not result from: 1) pressure-induced changes in baseline GABA<sub>A</sub> receptor function or 2) pressure-induced changes in the responsiveness of the receptor to GABA. Further, these in vitro findings closely parallel the present and previous behavioral findings and provide an important new line of evidence that the antagonistic effects of 12 ATA heliox on ethanol's, diazepam's and pentobarbital's behavioral effects cannot be explained by pressure-induced changes in drug absorption, distribution or elimination, or to changes in body temperature. Moreover, these in vitro and behavioral findings provide critical evidence supporting a cause-effect link between pressure antagonism of allosteric modulators at the behavioral level and pressure antagonism of the effects of these drugs on GABA<sub>A</sub> receptor function. Collectively, these behavioral and in vitro studies consistently indicate that pressure directly and selectively antagonizes the effects of allosteric modulators on GABA<sub>A</sub> receptor function.

The specific site(s) of pressure antagonism on the GABA<sub>A</sub> receptor complex is (are) not known. Pressure could act by altering the binding event that initiates allosteric coupling, by altering coupling or by altering the responsiveness of the GABA effector (Fig. 1). However, it is unlikely that pressure's antagonistic effects on diazepam, flunitrazepam and pentobarbital were due to a generalized alteration of binding at the initiation site, since pressure antagonized some (diazepam, pentobarbital), but not all (THIP,  $3\alpha,5\beta$ -P, picrotoxin) GABA<sub>A</sub> receptor active drugs. This conclusion is supported by recent preliminary studies in which 12 ATA heliox did not significantly effect [ $^3\text{H}$ ]flunitrazepam binding. In addition, the present experiments found that exposure to 12 ATA heliox did not significantly affect the ability of GABA to activate  $^{36}\text{Cl}^-$  uptake or the ability of  $3\alpha,5\beta$ -P to potentiate this effect of GABA allosterically. These findings argue strongly against pressure-induced changes in GABA binding, or the responsiveness of the receptor at or downstream from the GABA site as targets for pressure antagonism. Therefore, although further investigation is necessary, the available evidence suggests that pressure may act on the GABA<sub>A</sub> receptor complex by selectively blocking allosteric coupling between binding sites on the complex (Fig. 1).

In summary, the results from the present and previous studies with 12 ATA heliox fit predictions expected of a direct ethanol antagonist and support the hypothesis that allosteric coupling pathways in the GABA<sub>A</sub> receptor complex represent initial sites of action for ethanol. Although further concentration response studies are necessary to characterize the differences in sensitivity to pressure antagonism among allosteric modulators of the GABA<sub>A</sub> receptor complex before definitive conclusions can be drawn, the pattern of selective antagonism between pres-

sure and allosteric modulators of GABA<sub>A</sub> receptor function suggest common physicochemical and underlying structural characteristics between ethanol's site(s) of action and the coupling pathways for the benzodiazepines and barbiturates, but not for the neuroactive steroids. Moreover, these findings suggest the exciting possibility that there may be common physicochemical and underlying structural characteristics that define the ethanol sensitive regions of receptor proteins and/or their associated membranes that can be identified by pressure within a given receptor (e.g., GABA<sub>A</sub>) and possibly across ligand-gated ion channels currently purported to show ethanol sensitivity (e.g., GABA<sub>A</sub>, NMDA, 5HT<sub>3</sub>).

**Acknowledgements** The authors thank Dr. Nandita Pal, Ms. Christina de Luna, Ms. Tahira Mirza, Mr. Minh Ha and Mr. Kheang Long for their technical assistance. This work was supported by United States Public Health Service Research Grants RO1 AA03972, AA05234 and F31AA0436, National Institute on Alcohol Abuse and Alcoholism, NIH. This work was conducted as partial fulfilment of the requirements for the Ph.D. degree in Molecular Pharmacology and Toxicology, School of Pharmacy, University of Southern California (D. L. D.).

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