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## Ethanol Withdrawal in Mice Precipitated and Exacerbated by Hyperbaric Exposure

**Abstract.** *Mice were fed an ethanol-containing liquid diet for 9 days. On removal of the diet, exposure to 12 atmospheres absolute of a mixture of helium and oxygen precipitated earlier withdrawal, increased withdrawal scores for the first 6 hours, and increased the peak withdrawal intensity compared to dependent animals exposed to control conditions. The enhanced withdrawal did not appear to reflect alterations in ethanol elimination, oxygen or helium partial pressures, body temperature, or general excitability. These results extend to chronically treated animals the evidence that hyperbaric exposure antagonizes the membrane actions of ethanol.*

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Current molecular theories of general anesthesia are based on the physicochemical properties of anesthetics rather than on specific receptor interactions (1-4). These theories postulate that general anesthetics, including ethanol, act by penetrating hydrophobic lipid or protein regions in brain cell plasma membranes. The resultant perturbations are thought to lead to conformational changes in membrane-bound proteins, altering their function and thereby causing cellular depression and anesthesia. The exact site of anesthetic action is unknown, but evidence suggests that perturbations in discrete microenvironments in the membrane are more likely to be the critical events than are changes in the bulk membrane (3, 4). Perturbation theories are

supported, in part, by the ability of increased hydrostatic and atmospheric pressure to reverse or reduce the acute depressant effects of ethanol and other general anesthetics (2, 3, 5). It is hypothesized that the increased pressure blocks or counteracts anesthetic-induced perturbations, thereby returning the membrane components to their original conformational and functional states.

Chronic exposure to ethanol can lead to the development of tolerance and physical dependence (6). These phenomena, including the hyperexcitability and convulsions seen after ethanol is removed from dependent animals, are thought to reflect compensatory changes that offset the acute effects of ethanol (7). If increased atmospheric pressure antagonizes the acute effects of ethanol by blocking or counteracting its action on membranes, then exposure to hyperbaric environments should precipitate or enhance withdrawal in ethanol-dependent animals. The augmented withdrawal would be analogous to the effect of opiate antagonists on morphine-dependent animals (8).

In previous studies we observed that exposure to 12 atmospheres absolute (ATA) of a mixture of helium and oxygen antagonizes the acute depressant effect of ethanol in mice (5, 9-12). The antagonism does not reflect changes in the absorption, distribution, or elimination of ethanol; increases in the partial pressure of oxygen or helium; or alterations in body temperature. In the present study we found that exposure to 12 ATA of helium + oxygen precipitates and exacerbates withdrawal in ethanol-depen-

dent mice. To our knowledge, this is the first evidence that hyperbaric exposure behaves as an ethanol antagonist in chronically treated animals.

Drug-naïve adult male C57BL/6J mice were made physically dependent on ethanol by exposure for 9 days to a liquid ethanol-containing diet (F711A; Bio-Serv, Inc.) as the sole source of water and nutrients (13). The animals were housed individually in a room kept at  $21^\circ \pm 1^\circ\text{C}$  with a 12-hour light-dark cycle (lights on at 0700 hours). Diet consumption and intoxication scores (14) were recorded daily before fresh diet was supplied at 0800 hours.

The withdrawal period was initiated at 0200 hours on day 9 by removing the diet. The mice were moved to new individual housing consisting of clear plexiglass tubes (8 by 20.5 cm) and were randomly assigned to one of the following conditions: 1 ATA of air at  $25^\circ\text{C}$ ; 1 ATA of helium + oxygen at  $30^\circ\text{C}$ ; or 12 ATA of helium + oxygen at  $30^\circ\text{C}$  (15). We used a higher chamber temperature in the helium + oxygen conditions to offset the cooling effect of helium and thereby eliminate the potential for confounded results due to differences in body temperature (10, 14, 16). Three 18-liter chambers, holding up to seven mice each, were used to test the three treatment conditions simultaneously (17). The atmospheric gas content, pressure, and flow rate were adjusted with compressed air or helium + oxygen. The procedures ensured adequate oxygenation during and after compression and set the final partial pressure of oxygen at 0.2 ATA (5, 12). Withdrawal signs (14) were rated hourly for 14 hours by an observer who was unaware of the chamber conditions. The hourly score for each mouse was based on its behavior during a 2-minute observation period at the beginning of each hour. There were no statistically significant differences in withdrawal scores between the group exposed to 1 ATA of helium + oxygen and the group exposed to 1 ATA of air (Fig. 1 and Table 1). Therefore, the data for these two groups were pooled for statistical comparisons with the 12 ATA group.

Hyperbaric exposure precipitated and intensified the signs of ethanol withdrawal (Fig. 1 and Table 1). The mice in the 12 ATA group exhibited signs of withdrawal at the first rating period 1 hour after the ethanol diet was removed. In contrast, the 1 ATA air group did not show any signs of withdrawal until the second hourly rating (Fig. 1). The mean withdrawal scores for the 12 ATA group were 23 to 134 percent greater than those for

Table 1. Blood ethanol concentrations (BEC's) and intoxication ratings before withdrawal and withdrawal characteristics for the mice represented in Fig. 1. The results are means  $\pm$  standard errors except for the periods of maximum withdrawal, which represent the 3-hour periods over which maximum withdrawal intensity occurred. The maximum withdrawal intensity was computed for each group as the mean of all scores over the three highest consecutive hourly ratings at the peak of the withdrawal curve (18). Intoxication ratings and eye blood samples (24) for ethanol determinations (10) were taken immediately after the ethanol diet was removed and before the animals were exposed to the experimental treatments. Mice with BEC's  $<350$  mg/dl or exhibiting tremors upon handling when the diet was removed were eliminated from the experiment.

Treatment	n	Before withdrawal		Maximum withdrawal		Area under withdrawal curve	
		BEC (mg/dl)	Intoxication rating	Intensity	Period (hours)	Hours 1 to 6	Hours 7 to 14
1 ATA air	14	457 $\pm$ 10	1.9 $\pm$ 0.1	2.8 $\pm$ 0.3	5 to 7	8.8 $\pm$ 0.7	7.5 $\pm$ 1.7
1 ATA He + O <sub>2</sub>	12	446 $\pm$ 11	1.9 $\pm$ 0.2	3.0 $\pm$ 0.4	5 to 7	8.5 $\pm$ 1.6	5.2 $\pm$ 1.7
12 ATA He + O <sub>2</sub>	12	431 $\pm$ 9	1.8 $\pm$ 0.1	3.5 $\pm$ 0.2*	4 to 6	12.5 $\pm$ 0.7†	6.3 $\pm$ 1.5

\*  $P < 0.05$  compared to the combined value for the 1 ATA groups (unpaired *t*-test). †  $P < 0.005$ .

the 1 ATA air group in the five subsequent hourly ratings. The area under the withdrawal curve for this 6-hour period for the 12 ATA group was significantly greater than that for the 1 ATA controls (Table 1). In addition, the period of maximum withdrawal (18) began earlier and the maximum withdrawal intensity (18) during this period was significantly higher in the 12 ATA group than in the 1 ATA controls (Table 1). Four mice exposed to 12 ATA of helium + oxygen and four mice exposed to 1 ATA of air died during the rating period.

There were no statistically significant differences between groups in daily diet consumption, intoxication rating during acquisition of dependence, or intoxication rating and blood ethanol concentration at the initiation of withdrawal (Table 1). In separate experiments, mice that were fed Purina Lab Chow and water ad libitum and mice that were pair-fed isocaloric liquid diet without ethanol for 9 days did not manifest any observable signs of withdrawal when exposed to 1 ATA of air or to 1 or 12 ATA of helium + oxygen for 14 hours.

The mechanism by which hyperbaric exposure precipitated and enhanced withdrawal in the dependent animals is not known. The blood ethanol concentrations in the experimental and control groups at the initiation of the withdrawal period were not significantly different. Furthermore, exposure to 12 ATA of helium + oxygen did not have a statistically significant effect on the rate of ethanol elimination in mice injected acutely (9, 10) or chronically (19) with ethanol, nor did it change the distribution or absorption of ethanol in a manner that could explain these results (9, 10). Therefore, pharmacokinetic factors do not appear to mediate the enhanced withdrawal seen in the hyperbaric condition.

It also appears unlikely that the enhanced withdrawal in the hyperbaric group reflects helium- or compression-induced increases in general excitability

(20). First, treatment with 12 ATA of helium + oxygen did not induce any observable signs of withdrawal or hyperexcitability in mice that had received control liquid diet or Lab Chow. Second, withdrawal in ethanol-dependent mice exposed to 1 ATA of helium + oxygen was not significantly different from withdrawal in dependent mice exposed to 1 ATA of air. Finally, the enhanced withdrawal in the 12 ATA group did not continue throughout the withdrawal period, as would be expected with a generalized increase in excitability. Rather, the increase in withdrawal signs disappeared at about the same time (7 hours into the withdrawal period) that blood ethanol would be expected to approach zero (21).

The acceleration and enhancement of withdrawal during hyperbaric exposure could reflect a direct antagonistic action of pressure against ethanol, in agreement with extrapolations from membrane perturbation theories of anesthesia. Thus, hyperbaric exposure may partially counteract the acute actions of ethanol in critical microenvironments in brain cell membranes (3) or force the ethanol out of critical sites of action (22). The resultant reduction in the effective concentration of ethanol would unmask the adap-

tive changes that occurred during chronic exposure to offset the acute effects of ethanol (7), thereby precipitating and enhancing withdrawal.

Studies involving acute exposure to ethanol support the contention that hyperbaric exposure acts as a direct antagonist to ethanol at the membrane level. As mentioned above, indirect mechanisms have been eliminated as critical factors mediating the antagonism (5, 9-11). In addition, the characteristics of hyperbaric ethanol antagonism mimic those of classical pharmacological agonist-antagonist relationships in that the degree of antagonism is directly related to pressure and inversely related to the dose of ethanol (5). Furthermore, the effects of hyperbaric exposure on ethanol intoxication interact with body temperature in a manner suggesting a common action in the membrane (10). Finally, hyperbaric exposure reduces the membrane-perturbing effects of ethanol on membrane preparations in vitro (23).

The biochemical and physiological mechanisms leading to the acute and chronic behavioral effects of ethanol are not well understood. The paucity of information partially reflects the absence of a specific ethanol antagonist that could be used to identify the biochemical

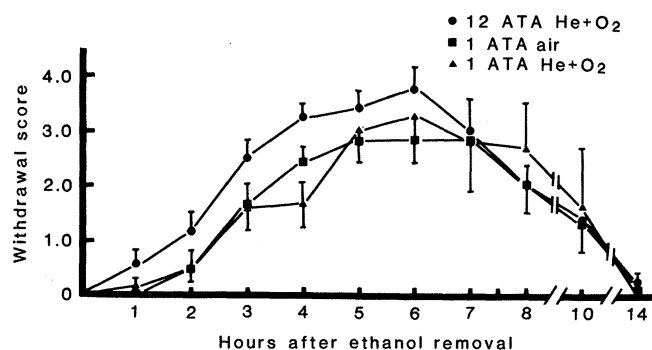


Fig. 1. Withdrawal scores for ethanol-dependent mice exposed to 12 ATA of helium + oxygen, 1 ATA of air, or 1 ATA of helium + oxygen for 14 hours after their ethanol-containing diet was removed. Hyperbaric exposure significantly increased withdrawal signs for the first 6 hours after removal of the diet. See Table 1 for statistical comparisons. Values are means  $\pm$  standard errors for 12 or 14 animals per group.

underpinnings of ethanol's behavioral effects. Our findings extend to chronically treated animals the evidence that hyperbaric exposure antagonizes the acute, membrane actions of ethanol which lead to intoxication. This indicates that hyperbaric exposure can be used in place of a more traditional, specific, receptor antagonist to investigate the cascade of events leading from the membrane to acute intoxication, tolerance, and physical dependence.

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mg/dl per minute in C57BL/6 mice exposed to 12 ATA of helium + oxygen (10, 19). For the present situation, in which the mice presumably started the withdrawal period with ethanol in their gastrointestinal tracts, it is reasonable to assume that ethanol concentrations approached zero 6 to 7 hours after the diet was removed.

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## Regeneration of Functional Synapses Between Individual Recognizable Neurons in the Lamprey Spinal Cord

**Abstract.** In 4- to 5-year-old sea lamprey larvae that had recovered from complete transection of the spinal cord, pairs of giant interneurons on opposite sides of the scar were impaled with microelectrodes. In 4 of 30 pairs, stimulation of the caudal cell elicited a monosynaptic electrochemical excitatory postsynaptic potential in the rostral cell. Fifty percent of such pairs were synaptically linked in control lampreys without transections. These results show regeneration of functional synaptic connections between individual neurons in a vertebrate central nervous system.

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Spinal cord injury in mammals results in abortive regeneration of axotomized neurons and permanent functional disability (1). Use of peripheral nerve grafts and bridges (2) and fetal tissue transplants (3) has produced morphological evidence of neurite growth for short distances (1 to 2 mm) in the mature central nervous system (CNS). However, a demonstration of the reestablishment of functional synaptic connections between known neuronal populations across the lesion site has been lacking.

A similarly limited distance of regeneration accompanies recovery of function in the spinally transected sea lamprey larva (4). Most fibers grow up to 5 mm across the scar (5), and their distal segments are electrically excitable (6, 7). We now report that giant interneurons (GI's; second-order, rostrally projecting sensory neurons) can reestablish functional synaptic contacts with other GI's across a healed spinal transection in sea lamprey larvae 4 to 5 years old.

Spinal cords from larvae that had recovered for more than 7 weeks after spinal transection and from control larvae without transections were studied in a transilluminated perfusion chamber (4). Pairs of GI's were impaled simultaneously with microelectrodes filled with 6 percent horseradish peroxidase (HRP) in 0.2M potassium acetate (pH 7.4) (Fig. 1A). The cells were stimulated intracellularly at frequencies of 0.2 to 33.3 Hz.

After the responses were recorded, HRP was injected iontophoretically and the identities of the impaled cells were verified in whole mounts of the cord (5). In control animals, stimulation of the more caudal cell elicited a composite electrochemical excitatory postsynaptic potential (EPSP) in approximately 50 percent of GI pairs (Fig. 1B and Table 1). In adult lampreys 70 percent of such cell pairs are synaptically linked (8). The EPSP's followed stimulation at frequencies up to 33.3 Hz with a fixed latency and persisted in Ringer's solution with 20 mM Ca<sup>2+</sup> (Fig. 1, C to F), which was added to eliminate polysynaptic activity (9). Thus the connections were monosynaptic.

In animals that had recovered from spinal transection, one GI located less than 8 mm rostral to the scar was impaled with one of two microelectrodes. Whole cord stimulation with a bipolar electrode positioned approximately 30 mm caudal to the scar evoked EPSP's of both variable and fixed latency in 17 of 20 cells studied (Fig. 2A). In Ringer's solution with 20 mM Ca<sup>2+</sup> the variable-latency EPSP's disappeared (even after a tenfold increase in the stimulus voltage), while fixed-latency EPSP's persisted. This suggested that synapses had been formed on GI's directly by axons regenerating rostralward. To test this hypothesis, we impaled a second GI located contralateral to the first and caudal to the transection site. The responses to intracellular stimulation of this cell were recorded in the GI rostral to the scar. In 4 of 30 pairs examined, a fixed-latency, composite EPSP was observed that persisted at higher stimulation frequencies (3.3 Hz) and in the high Ca<sup>2+</sup> Ringer's solution (Fig. 2B). The amplitude of the