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## An anxiolytic-like effect of hyperbaric oxygen in the mouse light/dark exploration test

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### Abstract

**Aims**—We studied whether hyperbaric oxygen (HBO<sub>2</sub>) treatment, which is known to increase production of nitric oxide (NO) in the brain, might also produce an NO-dependent anxiolytic-like behavioral response.

**Main methods**—Male NIH Swiss mice (20–25 g) were subjected to a 60-min HBO<sub>2</sub> treatment at different absolute atmospheres, and anxiety was assessed using the light/dark exploration test at different time intervals following the cessation of HBO<sub>2</sub> treatment. To ascertain the underlying mechanism of action, other groups of mice were pretreated with the NO synthase inhibitor N<sup>G</sup>-monomethyl-L-arginine acetate, the NO scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (carboxy-PTIO), the soluble guanylyl cyclase-inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or the benzodiazepine antagonist flumazenil to determine their influence on the HBO<sub>2</sub>-induced anxiolytic-like effect.

**Key findings**—A 60-min HBO<sub>2</sub> treatment at 3.0 absolute atmospheres increased the time spent by mice in the light compartment that lasted up to 90 min following the end of HBO<sub>2</sub> treatment. This anxiolytic effect of HBO<sub>2</sub> was significantly reduced by pretreatment with L-NMMA, carboxy-PTIO, ODQ and flumazenil.

**Significance**—Based on these findings, we conclude that a 60-min HBO<sub>2</sub> treatment is capable of inducing an anxiolytic effect that possibly involves NO, cyclic GMP and the benzodiazepine binding site.

### Keywords

hyperbaric oxygen; anxiolysis; light/dark exploration test; nitric oxide; cyclic GMP; benzodiazepine binding site; mouse

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#### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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## Introduction

Hyperbaric oxygen (HBO<sub>2</sub>) therapy is the clinical application of 100% oxygen at greater than normal pressures to achieve therapeutic outcomes. The increased tissue oxygen is presumed to be the reason for the clinical improvement observed when patients are exposed to HBO<sub>2</sub> therapy, such as in decompression sickness; carbon monoxide poisoning and smoke inhalation; gas gangrene; acute traumatic ischemia; wound-healing; necrotizing soft tissue infections; radiation tissue damage; compromised skin grafts; and thermal burns (Feldmeier 2003).

In addition to the above clinical indications that have been approved by the U.S. Food and Drug Administration (FDA), there are various other conditions for which HBO<sub>2</sub> reportedly produces beneficial effects, including improvement in neurological and cognitive function following cerebral ischemia (Neubauer and End 1980; Kapp et al. 1981; Shirachi et al. 2003, 2005; Hoggard et al. 2005).

Previous research has demonstrated that the medical gas nitrous oxide can induce an anxiolytic-like effect in animals that is dependent on nitric oxide (NO) (Li et al. 2003b). Since our research has provided evidence that HBO<sub>2</sub> treatment can also stimulate the production of NO in the brain (Ohgami et al. 2008), we studied whether HBO<sub>2</sub> is also capable of evoking an anxiolytic-like response in animals.

## Methods

### Animals

Male NIH Swiss mice, 20–25 g body weight, were purchased from Harlan Laboratories (Indianapolis, IN). Experiments were approved by an institutional animal care and use committee (IACUC) with post-approval review and carried out in accordance with The Guide for the Care and Use of Laboratory Animals, 8th Edition (National Academies Press, Washington, DC 2010).

Mice were housed five per cage in the Wegner Hall Vivarium with access to food and water ad libitum. The facility is maintained on a 12 h light:12 h dark cycle (lights on 0700–1900 h) under standard conditions (22 ± 1 °C room temperature, 33% humidity). Mice were kept in the holding room for at least four days following arrival in the facility. Each animal was used only once then discarded.

### Apparatus

The light/dark exploration box (450 mm length × 270 mm width × 270 mm height) was constructed of acrylic (Abbott Plastics, Rockford, IL). An acrylic divider with a 75 mm × 75 mm opening at floor level divided the box into a light compartment (three-fifths of the total length) and a dark compartment (two-fifths of the total length). The walls of the light and dark compartments were made of black and white acrylic, respectively. Behavioral observations and assessments were generally performed between 1000 and 1400 h. During all experiments, the light compartment was illuminated by two 40-watt white light fluorescent tubes mounted 180 mm directly overhead. In this paradigm, animals were individually placed in the center of the light compartment of the box then observed for 5 min. The time spent in the light chamber of the box was recorded for each mouse. A mouse was considered to have entered the new area when all four legs crossed the threshold into the compartment. An increase in the amount of time spent in the light compartment was indicative of an anxiolytic-like drug effect.

## Exposure to Hyperbaric Oxygen (HBO<sub>2</sub>)

Cages of five mice each were placed in a B-11 research hyperbaric chamber (Reimers Systems, Inc., Lorton, VA) as previously described (Zelinski et al. 2009; Ohgami et al. 2009; Chung et al. 2010). The chamber was ventilated with 100% O<sub>2</sub>, U.S.P. (A–L Compressed Gases, Inc., Spokane, WA) at a flow rate of 10 L/min. The pressure within the cylindrical clear acrylic chamber (27.9 cm diameter × 55.9 cm L) was increased over 2 min to the desired pressure (2.5, 3.0 or 3.5 absolute atmospheres, ATA) and maintained for 60 min. The mice were allowed to breathe spontaneously during HBO<sub>2</sub> treatment. After completion of the HBO<sub>2</sub> exposure, mice were then decompressed over 2 min. Control groups of mice were exposed to compressed air (A–L Compressed Gases) circulated through the chamber at 1.0 ATA and maintained for 60 min. In the time course experiment (Fig. 1A), different groups of mice were assessed for level of anxiety at different time intervals following the termination of HBO<sub>2</sub> treatment.

## Drugs

The following drugs were used in this research: flumazenil, N<sup>G</sup>-monomethyl-L-arginine acetate (L-NMMA) and 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, potassium salt (carboxy-PTIO) (Tocris Bioscience, Ellisville, MO); and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Axxora, San Diego, CA).

Flumazenil was suspended in 0.3% Tween 80. ODQ was dissolved in dimethylsulfoxide (DMSO), and distilled water was added to attain a final DMSO concentration of 1%. L-NMMA and carboxy-PTIO was dissolved in bacteriostatic saline. Based on previous experiments in our laboratory (McDonald et al. 1994; Quock et al. 2011), the dose of L-NMMA was 10 mg/kg, the dose of carboxy-PTIO was 0.6 mg/kg, the dose of ODQ was 0.01 mg/kg, and the dose of flumazenil was 10 mg/kg. The effect of pretreatment drugs alone on time spent in the light compartment of the light/dark box is shown in Fig. 2.

In the antagonism experiments (Fig. 1B), pretreatment drugs were administered i.p. to different groups of mice 30 min following termination of the 60-min HBO<sub>2</sub> treatment and 30 min prior to anxiolytic testing. This time interval was considered the midpoint of the anxiolytic-like response to HBO<sub>2</sub>.

## Statistical Analysis of the Data

The results are shown as mean ± S.D. Differences between means were analyzed by one-way ANOVA and a post hoc Bonferroni test.

## Results

Control mice spend an average of 84.6 ± 9.6 s (N=17) in the light compartment of the light/dark box. A 60-min HBO<sub>2</sub> treatment at 2.5, 3.0 or 3.5 ATA increased the amount of time spent in the light compartment. The most robust anxiolytic-like effect occurred following treatment with HBO<sub>2</sub> at 3.0 ATA. The HBO<sub>2</sub> treatment significantly increased the time spent in the light compartment to 127.7 ± 12.3 (N=14), 133.2 ± 11.2 (N=11) and 119.3 ± 8.9 s (N=13) at 30, 60 and 90 min after cessation of treatment, respectively (Fig. 3).

Separate groups of mice were subjected to a 60-min HBO<sub>2</sub> treatment at 3.0 ATA but received different drug pretreatment 30 min after HBO<sub>2</sub> treatment. Typically, at 60 min after HBO<sub>2</sub> treatment, mice spent an average of 133.2 ± 11.2 s (N=11) in the light compartment of the light/dark box. Groups of mice that were pretreated with L-NMMA, carboxy-PTIO, ODQ and flumazenil spent 77.3 ± 14.8 (N=12), 96.2 ± 9.7 s (N=21), 99.5 ± 14.5 s (N=16) and 99.8 ± 11.6 s (N=12) in the light compartment (Fig. 4). The reduction in time spent in

the light compartment was statistically significant for all four drug pretreatments. The different vehicles for each drug pretreatment (i.e., bacteriostatic saline for L-NMMA and carboxy-PTIO, 1% DMSO in saline for ODQ and 0.3% Tween 80 for flumazenil) alone did not produce any significant influence on the time spent in the light compartment.

It should also be noted that mice treated with L-NMMA alone spent  $81.8 \pm 10.1$  s (N=12) in the light compartment; mice treated with carboxy-PTIO alone spent  $84.7 \pm 10.0$  s (N=15) in the light compartment; mice treated with ODQ alone spent  $130.9 \pm 11.2$  s (N=14) in the light compartment; and mice treated with flumazenil alone spent  $89.4 \pm 9.2$  s (N=19) in the light compartment. Only the ODQ effect was significantly different from control ( $P < 0.05$ ).

## Discussion

Earlier research implicated NO in anxiolytic-like drug effects (Quock and Nguyen 1992). The anxiolytic-like effects of chlordiazepoxide in the mouse elevated plus-maze were antagonized by systemic pretreatment with the nitric oxide synthase-inhibitor L-<sup>N</sup><sup>G</sup>-nitro arginine (L-NOARG); this inhibitory effect of L-NOARG was stereospecifically and completely reversed by intracerebroventricular administration of L-arginine but not D-arginine (Quock and Nguyen 1992). The anxiolytic-like effects of nitrous oxide (N<sub>2</sub>O) were similarly antagonized by pretreatment with a variety of NOS-inhibitors (Caton et al. 1994; Li and Quock 2001; Li et al. 2003b) or an antisense oligodeoxynucleotide (AS-ODN) against neuronal nitric oxide synthase (nNOS) (Li et al. 2003a). Conversely, the intracerebroventricular administration of an NO-donor into the brain was found to induce anxiolytic-like behavioral effects in the mouse light/dark exploration test (Li and Quock 2002). Systemic or central treatment with a NOS-inhibitor produced anxiogenic-like activity in the rat elevated plus-maze (De Oliveira et al. 1997; Monzón et al. 2001). These findings suggest that NO may play an anxiolytic role in the modulation of anxiety.

Previous in vivo rat brain microdialysis studies showed that HBO<sub>2</sub> (3.0 ATA for 2 h) increased oxidation products of NO six- and four-fold in the hippocampus and striatum, respectively, indicative of an increased turnover of NO under HBO<sub>2</sub> (Elayan et al. 2000). Direct measurement of NO using NO-sensitive electrodes placed in the cerebral cortex revealed a pressure-dependent elevation in levels of NO induced by HBO<sub>2</sub> (2.5 and 2.8 ATA) (Thom et al. 2002; Thom and Buerk 2003). It has also been shown that HBO<sub>2</sub> (3.5 ATA for 60 min) increased NO oxidation products in specific rat brain regions and spinal cord (Ohgami et al. 2008). Some of the effects of HBO<sub>2</sub> may be mediated through the production of NO.

Against this background that HBO<sub>2</sub> is a demonstrated promoter of NO production, this study was conducted to ascertain the effects of HBO<sub>2</sub> on anxiety-like behavior. The results show that HBO<sub>2</sub> treatment induced anxiolytic-like behavior in mice for up to 90 min following removal of the animals from the hyperbaric chamber. Administration of carboxy-PTIO, a NO scavenger that inhibits extracellular transit of NO (Akaike et al. 1993), antagonized the anxiolytic-like effect of HBO<sub>2</sub>. Pretreatment with ODQ, an inhibitor of the soluble guanylyl cyclase enzyme that converts GTP to cyclic GMP (Garthwaite et al. 1995), also antagonized anxiolytic-like effect of HBO<sub>2</sub>. This result is consistent with earlier findings of an NO-cyclic GMP pathway that mediates drug-induced anxiolytic activity (Li et al. 2004, 2005).

Flumazenil, a benzodiazepine antagonist, also attenuated the anxiolytic effect of HBO<sub>2</sub>. This implicates benzodiazepine receptors in the drug effect. Exactly what is the relationship between the NO-cyclic GMP pathway and the benzodiazepine receptor, which is part of the GABA<sub>A</sub> receptor (Sigel and Buhr 1997), remains to be seen. However, the anxiolytic effect

of N<sub>2</sub>O was also found to involve the NO-cyclic GMP pathway and the benzodiazepine receptor (Li and Quock 2001; Li et al. 2004).

In a recently reported study, HBO<sub>2</sub> treatment (2.5 ATA daily for 5 consecutive days) was administered 24 hr prior to the application of a single prolonged stress (SPS) in an animal model of PTSD (Peng et al. 2010). Compared to non-HBO<sub>2</sub>-treated control rats, the HBO<sub>2</sub>-preconditioned rats exhibited less anxiety-like behavior and cognitive impairment. The HBO<sub>2</sub> effect was correlated to an up-regulation in the expression of thioredoxin reductase (TrxR) to inhibit neuronal apoptosis. However, the authors did not investigate mediation of the anxiolytic-like effect by known anxiolytic mediators and pathways in the brain.

There are several important points of discussion attendant to these findings. Some previous studies have suggested that HBO<sub>2</sub>-induced production of NO is short-lived. In one study utilizing NO-specific electrodes implanted in the rat cerebral cortex, the HBO<sub>2</sub>-induced increase in NO was reduced by approximately one-third 10 min after the end of HBO<sub>2</sub> treatment (Thom and Buerk 2003). Another investigation reported that the HBO<sub>2</sub>-induced increase in dialysate levels of NO oxidation products collected in the parietal cortex similarly declined by 40% 10 min after HBO<sub>2</sub> treatment (Sato et al. 2001). A third study found that HBO<sub>2</sub>-induced increase in dialysate levels of nitrite collected from the corpus striatum and hippocampus fell by roughly one-third at 1 h and two-thirds at 2 h following HBO<sub>2</sub> treatment (Elayan et al. 2000).

In the present study, L-NMMA was administered 30 min following the end of HBO<sub>2</sub> treatment and 30 min prior to testing in the light/dark box. If the increase in NO levels is short-lived, why should inhibition of NOS after the fact cause antagonism of the HBO<sub>2</sub> effect? There was no measurement of NO or nitrite/nitrate levels 1 hr after the HBO<sub>2</sub> treatment. Those levels are likely to be site-specific in the brain and remain to be determined. However, the declining but still significant pattern of change in dialysate nitrite levels in the Elayan et al. (2000) study appear to approximate the duration of the anxiolytic-like effect of HBO<sub>2</sub> at 3.0 ATA.

A second point is that L-NMMA, a nonselective NOS-inhibitor, was utilized in the present study to ensure inhibition of all three isozymes of NOS (neuronal, endothelial and inducible). Other studies have implicated non-neuronal forms of NOS in behavioral responses. For example, it has been shown that nonselective inhibition of NOS reduced the anxiogenic-like behavioral response induced by transient cerebral ischemia in the elevated plus-maze and the social interaction test (Nakashima et al. 2003). This anxiogenic-like response was blocked by the selective inducible NOS-inhibitor S-ethylisothiourea but not the by neuronal-selective NOS-inhibitor 7-NI, implicating iNOS rather than nNOS in the anxiogenesis. Conversely, it has been reported that the anxiolytic-like effects of nitrous oxide were sensitive to antagonism by the neuronal-selective NOS-inhibitor S-methyl-L-thiocitrulline (SMTTC) but not by regular doses of the endothelial-selective inhibitor N<sup>5</sup>-(1-iminoethyl)-L-ornithine (1-NIO) or the inducible-selective inhibitor 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (Li et al. 2003).

A third caveat is that there are published reports of a possible anxiogenic effect of NO. Results from other laboratories have demonstrated that NOS-inhibitors alone can evoke anxiolytic-like behavioral responses (Guimarães et al. 1994; Volke et al. 1997; Dunn et al. 1998). The observation that the nNOS-inhibitor 7-nitroindazole (7-NI) or ODQ alone elicit anxiolytic-like responses in the elevated plus-maze and the Vogel test (Spolidório et al. 2007). This is also consistent with the finding in this study that ODQ alone caused a significant increase in the amount of time spend in the light compartment of the light/dark box.

There is a perplexing dichotomy of evidence that NO is promotor and inhibitor of neurogenesis (Cárdenas et al. 2005), pronociceptive and antinociceptive (Miclescu and Gordh 2009; Schmidtko et al. 2009), proconvulsant and anticonvulsant (Ferraro and Sardo 2005), neuroprotective and neurotoxic (Calabrese et al. 2007) and thermogenic and thermolytic (Scammell et al. 1996; Quock et al. 2007). A plausible explanation for these discrepancies lies in the dual role of NO as a signaling molecule in multiple and possibly counteracting neuronal pathways.

## Conclusion

Our results demonstrate that a 60-min HBO<sub>2</sub> treatment produces anxiolytic-like activity that persists for at least 90 min following the end of treatment and that this anxiolytic-like effect appears to involve NO, cyclic GMP and benzodiazepine binding sites, not unlike the anxiolytic-like response of mice to nitrous oxide. These findings may be a harbinger of potential use of HBO<sub>2</sub> therapy in treatment of certain anxiety states.

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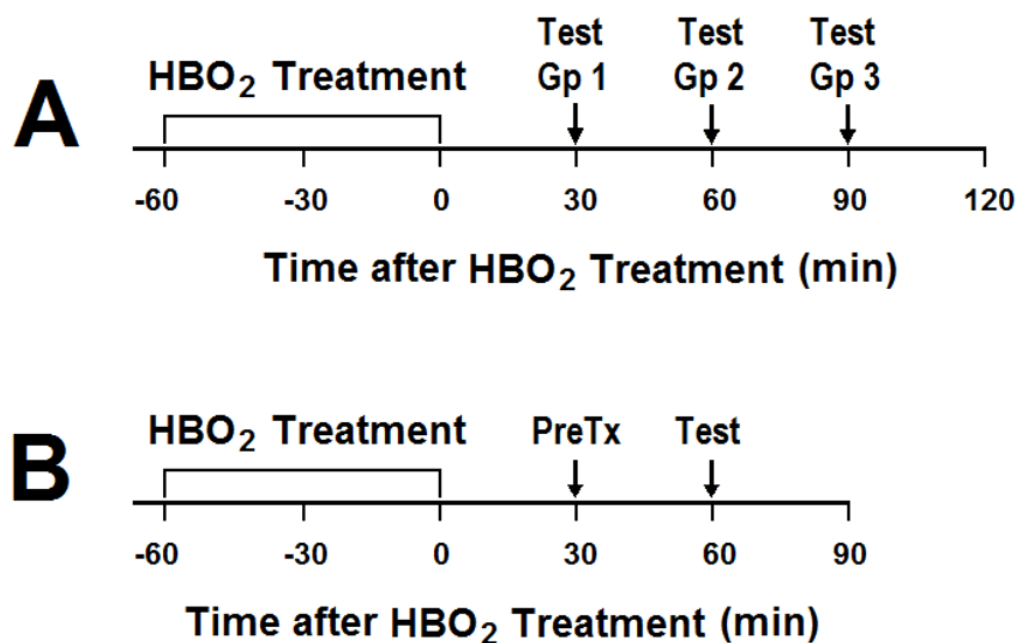
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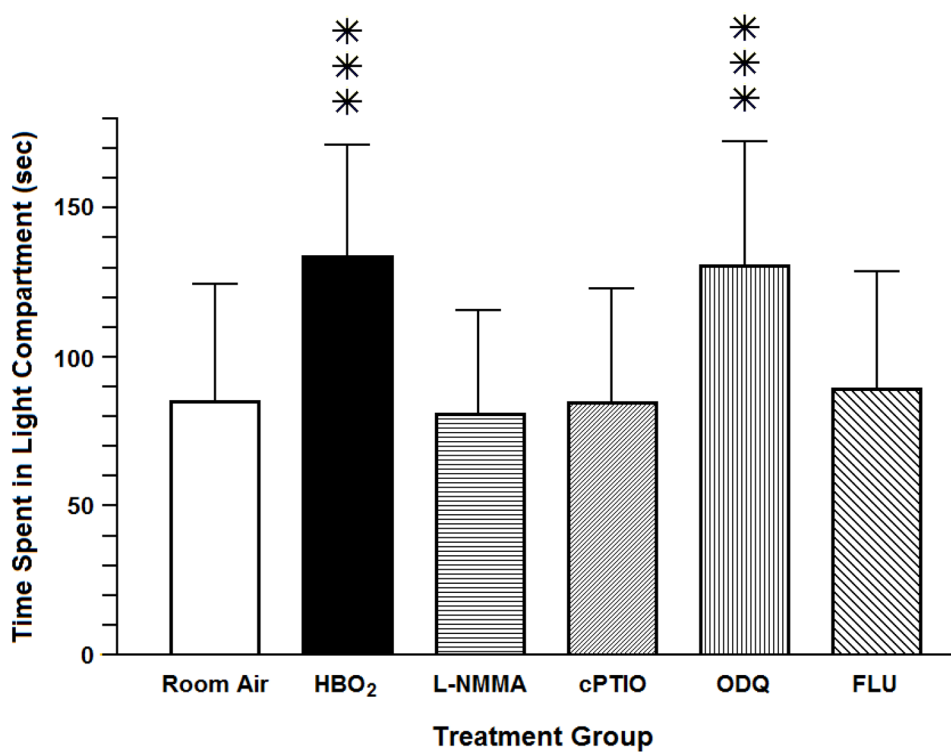
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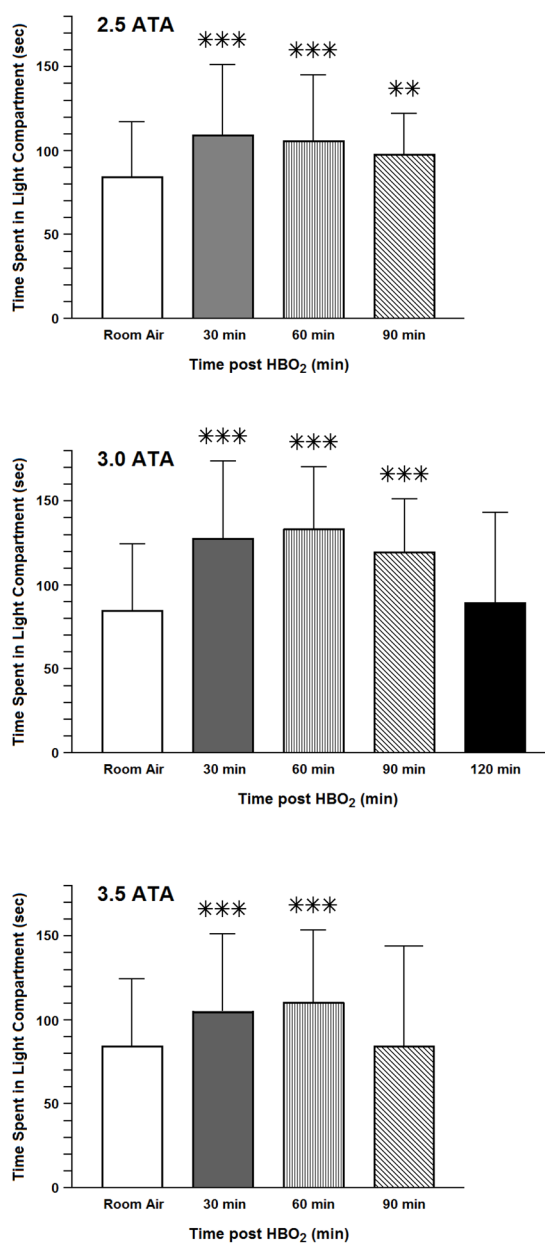




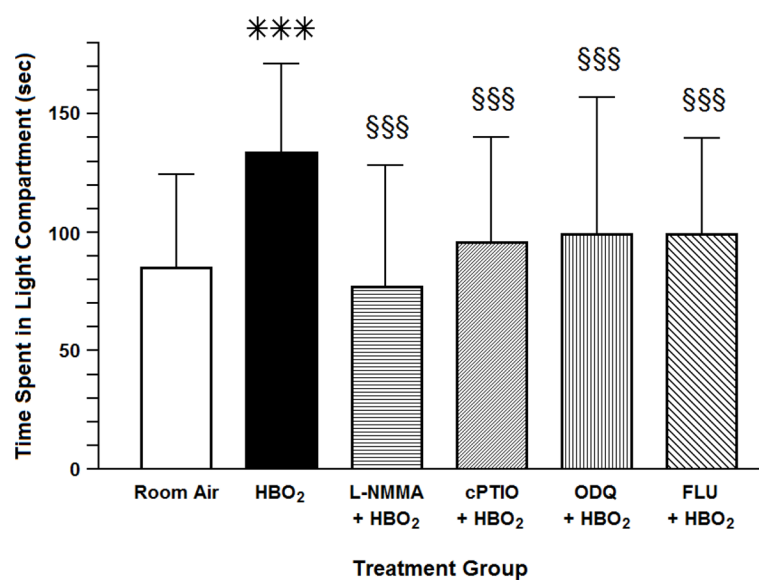
**Fig. 1.**  
Experimental protocol for (A) time course experiments and (B) antagonism experiments.



**Fig. 2.** The influence of treatment with L-NMMA, carboxy-PTIO (cPTIO), ODQ and flumazenil (FLU) on behavior. Each bar represents the mean time spent in the light compartment  $\pm$  S.D. of 10–19 mice per group. Significance of difference: \*\*\*,  $p < 0.001$ , compared to the room air (RA) control group (post-hoc Bonferroni test).

**Fig. 3.**

Time course of the anxiolytic-like response of mice to HBO<sub>2</sub> at 2.5, 3.0 or 3.5 ATA. Each bar represents the mean time spent in the light compartment  $\pm$  S.D. of 11–20 mice per group. Significance of difference: \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ , compared to the room air (RA) control group (post-hoc Bonferroni test).



**Fig. 4.** The influence of pretreatment with L-NMMA, carboxy-PTIO (cPTIO), ODQ and flumazenil (FLU) on HBO<sub>2</sub>-induced anxiolytic-like behavior. Each bar represents the mean time spent in the light compartment  $\pm$  S.D. of 12–21 mice per group. Significance of difference: \*\*\*,  $p < 0.001$ , compared to the room air (RA) control group; and §§§,  $p < 0.001$ , compared to VEH pretreatment group (post-hoc Bonferroni test).