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Intranasal Treatment with Neuroprotective Peptides Prevented Memory Performance Degradation in Sprague-Dawley Rats Subjected to Repeated Hypoxic or Hyperoxic Stress

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Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF)

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In this study, we report successful protection of cognitive function using neuroprotective peptides. Male, Sprague-Dawley rats were prophylactically treated with ADNP8, Semax, or Ang1-7 daily for four weeks by intranasal administration. In the fourth week, they were exposed to normobaric hypoxia (7.5% O2), hyperoxia (95% O2), or oscillating hypoxiahyperoxia (cycling between 95% and 5% O2) daily for five days. Episodic memory performance of control and treated rats were assessed using the novel object recognition test. All three peptides provided some level of protection. Semax was effective under all three exposure conditions, while ADNP-8 showed some protection in hypoxia and oscillating hypoxiahyperoxia. Ang1-7 appeared to be effective with hypoxia. These results suggest that nasal administration of neuroprotective peptides might be a useful prophylaxis against cognitive degradation induced by abnormally low and high levels of oxygen.

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TABLE OF CONTENTS

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1.0 SUMMARY

Aerospace professionals commonly experience hypoxia, which is frequently countered by hyperoxic exposure. Both conditions can have short- and/or long-term impact on neurological and cognitive functions (including the impairment of memory, concentration and attention shifting) and involuntary muscle movement, resulting in the slowing of motor tasks. The literature frequently reports deficits in working and spatial memory in animal models, and some effective countermeasures involving antioxidants or anti-inflammatory agents have been demonstrated. In this study, we report successful protection of cognitive function using neuroprotective peptides. Male, Sprague-Dawley rats were prophylactically treated with activity-dependent neuroprotective protein (ADNP)-8, Semax, or Ang_{1-7} daily for four weeks by intranasal administration. In the fourth week, they were exposed to normobaric hypoxia (7.5 percent (%) oxygen (O_2)), hyperoxia (95% O_2), or oscillating hypoxia-hyperoxia (cycling between 95% and 5% O_2) daily for five days. Episodic memory performance of control and treated rats were assessed using the novel object recognition test. All three peptides provided some level of protection. Semax was effective under all three exposure conditions, while ADNP-8 showed some protection in hypoxia and oscillating hypoxia-hyperoxia. Ang_{1-7} appeared to be effective with hypoxia. These results suggest that nasal administration of neuroprotective peptides might be a useful prophylaxis against cognitive degradation induced by abnormally low and high levels of oxygen.

2.0 INTRODUCTION

Hypoxia is a condition of inadequate oxygen supply to the cells, tissues, or organs in the body. It can result from poor diffusion of oxygen across the pulmonary epithelium due to a low partial pressure of oxygen in the inspired air. Oxygen availability decreases with barometric pressure (BP), which declines as altitude increases. For example, at sea level, BP is 760 millimeters of mercury (mmHg) (i.e., normobaric) and the partial pressure of oxygen (PO_2) is 159 mmHg, but at 3,048 meters (m) above sea level, BP is 523 mmHg (i.e., hypobaric), and PO_2 is 110 mmHg. Hypobaric hypoxia is a persistent threat for military personnel. Pilots and aircrews utilize a variety of airborne vehicles including unpressurized helicopters operating below 3,048 m, fighters that fly above 7,620 m, with positive-pressure, supplemental oxygen. Surveillance aircrafts that reach altitudes above 21,336 m, require fully pressurized suits similar to astronauts.[22, 41] The lack of sufficient oxygen induces a cascade of physiological, cellular, and molecular responses to compensate oxygen delivery to tissues, and optimize energy production and consumption. The physiological mechanisms of hypobaric hypoxia and its effects are well known from the studies of high-altitude residents, mountain climbers, and aviation professionals.[22,41, 66-69]

Low oxygen availability impairs brain function. Acute hypoxia, at moderate levels, slows cognitive and motor task performance, while sustained hypoxia can cause lasting impairment of short-term memory, concentration, attention shifting, and motor control.[18] Cognitive impairment has been demonstrated at simulated altitudes from 2,438 m to 10,000 m.[33, 44] A negative impact on piloting ability and cognitive functions have been observed,[57] and memory defects persisted after returning to sea level,[40] despite that subjects reported confidence in their performance.[2] Since psychomotor performance deteriorates at 2,438 m, commercial aircraft cabins are pressurized to simulate lower altitudes. Military aircraft are pressurized to a lesser extent and thus are more susceptible to a loss of cabin pressure. Oxygen supplementation is equipped to prevent hypoxic events. The percent of oxygen in the supplemental air is increased with the altitude up to 12,192 m, above which positive pressure breathing of 100% oxygen is necessary.[45] Nonetheless, hypoxic mishaps could still occur as the result of malfunctions of the oxygen delivery system.[8]

In order to recover from a hypobaric hypoxia event, a pilot is expected to descend to low altitude that has a higher BP and/or increase the amount of oxygen in the inspired air, often to

100%. Breathing pure oxygen may resolve the symptoms of hypoxia, but it can also result in hyperoxia, a condition of excess oxygen in the tissues. Extended normobaric exposure to high oxygen levels can cause alveoli deflation and collapse (a condition known as atelectasis), as well as oxygen toxicity that damages the respiratory tract. The effects of hyperoxia on the nervous system increase with time, beginning with small-muscle twitching, followed by vertigo, nausea, clumsiness, and then convulsions (reviewed by Chawla and Lavania[10]). Both hypoxia and hyperoxia can increase cellular oxidative stress by inducing the production of reactive oxygen species, causing cellular damage and altering tissue functions.[31, 42] Nevertheless, brief exposure to 100% oxygen reverses hypoxia and may have immediate, short-term, cognitive benefits including improved memory and reaction time.[39, 51]

Given the prevalence of hypoxic and hyperoxic events in the military setting, this project sought to prevent the persistent effect of low and high oxygen exposures on memory performance in order to improve the preparedness of pilots and aircrews. Herein we report on the potential for three different neuroprotective peptides to prevent such negative effects on cognition. The 8-amino acid fragment of activity-dependent neuroprotective protein (ADNP-8, also known as NAP or davunetide) is thought to preserve neuronal function and viability by stabilizing microtubules [17]. Angiotensin fragment 1-7 (Ang_{1-7}) is the primary depressor component of the renin-angiotensin system. It might protect the brain by reducing the inflammatory response[47] or by increasing capillary density through angiogenesis.[23] Semax is a synthetic analogue of adrenocorticotropic hormone fragment 4-10 that might exert its protective role by enhancing the expression of neurotrophic or modulating immune factors.[38, 53].

In this study, the peptides were administered prophylactically to Sprague-Dawley rats, by intranasal instillation, a method commonly used to target the central nervous system. In fact, Semax is typically applied intranasally,[1] and ADNP-8 has been found in the brain and other tissues after intranasal dosing.[16] The animals were then exposed daily to normobaric atmospheres with altered oxygen content: 60 minutes of hypoxia (7.5% O_2) or hyperoxia (95% O_2); or 10 cycles of oscillating hypoxia-hyperoxia (95% O_2 for 8 minutes, followed by 5% O_2 for 3 minutes), the most stressful conditions tested that did not affect the viability of the rat subjects. Following exposure, learning and memory were tested using the novel object recognition (NOR) test, an assessment of episodic memory, taking advantage of the innate curiosity of rodents for exploring a novel stimulus. In addition, various markers of hypoxic/ hyperoxic exposure and

organ injury were examined including red blood cell and hemoglobin concentration; S100 calcium binding protein (S100B); copeptin; erythropoietin (EPO); vascular endothelial growth factor (VEGF); Angiotensin II; 1,3-Bisphosphoglyceric Acid (1,3-BPG); 2,3-Bisphosphoglyceric Acid (2,3-BPG); and Bisphosphoglycerate Mutase (BPGM).

We report here that treatment with each of the neuroprotective peptides provided some degree of memory performance protection. All three peptides reduced the impact of hypoxia; Semax protected the rats from hyperoxia; and ADNP-8 and Semax both alleviated the effects of oscillatory hypoxia-hyperoxia exposure. Given the potential for these three peptides to prevent cognitive deficits caused by the abnormal oxygen exposures commonly encountered in aerospace occupations, further study to define their safe and effective use is warranted.

3.0 METHOD, ASSUMPTION AND PROCEDURES

Animals and Husbandry

The study protocol was approved by the Wright-Patterson Air Force Base Institute of Research, Institutional Animal Care and Use Committee (IACUC), and by the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Research, National Research Council, National Academies Press, 2011. They were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC).

The animals were Specific Pathogen Free (SPF), Male, Sprague Dawley (SD) Rats (*Rattus Norvegicus*) (Charles River Laboratories, Wilmington, MA). They were 6 weeks old on arrival and placed in quarantine for 2 weeks while being tested for ten specific rodent pathogens. They were examined visually at least twice daily during the study for signs of distress. Only males were used to avoid the compounding effect of estrous cycles and sex-specific behavioral differences and responses to hypoxia. The rats were socially housed (2/cage) in clear plastic cages with conventional bedding (CellZorb, Cincinnati Lab Supply, Cincinnati, OH) and provided tunnels, nesting material, and nylon bones for enrichment. Food (LabDiet Formulab Diet 5008, Cincinnati Lab Supply) and water were freely available. The animal rooms were climate controlled (20 – 26 degrees Celsius (°C), 30 – 70% humidity) with a 12-hour light/dark cycle (on at 0600).

Experimental Design

The animals were divided randomly into 14 different groups using a computerized random number generator, 8 animals in each group, as shown in Table I. The study was divided into 3 experiments, according to the exposure conditions: hypoxia, hyperoxia, and oscillatory hypoxia-hyperoxia. The hypoxia and hyperoxia experiments had five groups each: normal open-air control (with no peptide treatment), and exposed groups treated with control peptide, ADNP-8, Semax, and Ang_{1-7}. The oscillatory hypoxia-hyperoxia experiment had the same treatment groups excluding Ang_{1-7}. The study design is shown in Figure 1. Animals were treated daily for 4 weeks by intranasal administration of the peptides at the beginning of each day. During the

fourth week of the experiment, the animals were exposed daily to hypoxia or hyperoxia for 60 minutes or 10 cycles of oscillatory hypoxia-hyperoxia. The NOR test was conducted on the final day of the experiment, preceded by six days of habitation and 1 day of training. Necropsy was conducted 3 days after the final treatment, exposure, and NOR test. Multiple-event days were handled in this order: peptide treatment, hypoxic/ hyperoxic exposure, then NOR testing.

Table I: Experimental Groups Included in This study

The rats subjects were treated with the peptides indicated in the first column and exposed to a specific oxygen concentration in each experiment. Eight, male, Sprague-Dawley rats were assigned to each treatment/exposure group. Each experiment had one open-air control group that received no peptide treatment.

Peptide Treatment	Experiment 1	Experiment 2	Experiment 3
None	Open Air	Open Air	Open Air
Control Peptide	Hypoxia (7.5% Oxygen)	Hyperoxia (95% Oxygen)	Oscillatory Hypoxia-Hyperoxia (5% ↔ 95% Oxygen)
Ang_{1-7}	Hypoxia (7.5% Oxygen)	Hyperoxia (95% Oxygen)	N/A
ADNP-8	Hypoxia (7.5% Oxygen)	Hyperoxia (95% Oxygen)	Oscillatory Hypoxia-Hyperoxia (5% ↔ 95% Oxygen)
Semax	Hypoxia (7.5% Oxygen)	Hyperoxia (95% Oxygen)	Oscillatory Hypoxia-Hyperoxia (5% ↔ 95% Oxygen)

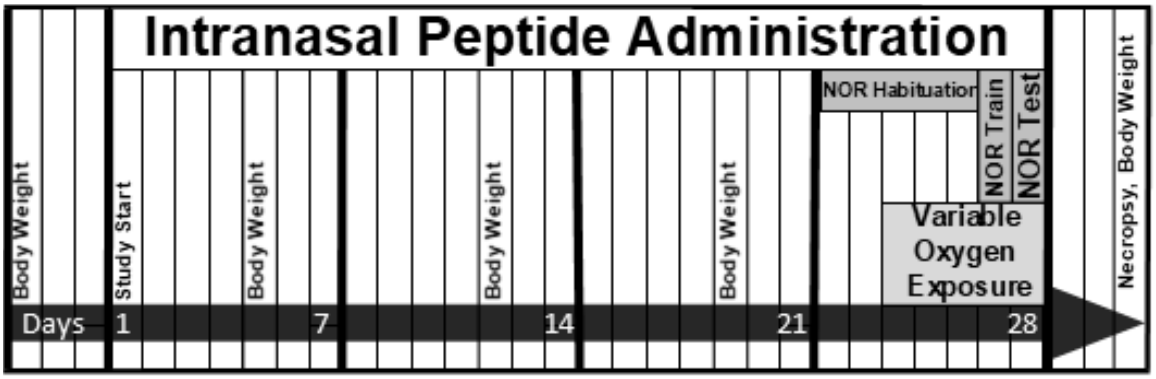

Figure 1: Experimental design

Each vertical rectangle represents one day. Animals were treated daily with one of the four peptides tested in this study for 4 weeks, and subjected to hypoxia, hyperoxia or oscillating hypoxia-hyperoxia during the final week of peptide treatment. Memory performance was assessed using the novel object recognition (NOR) test. Necropsy occurred 3 days after the NOR test.

Peptide Treatment

The source, amino acid sequence, lot number, purity, and dose concentration of each of the peptides, which were custom synthesized by Selleckchem (Houston, TX) or Thermo Fisher Scientific (Thermo) (Carlsbad, CA), are shown in Table II. They were dissolved in phosphate buffered saline, pH 7.0 (PBS) (Thermo), at 1.25 milligrams/milliliter (mg/mL) (ADNP-8) or 6.25 mg/mL (Ang_{1-7}, Semax, and Control) and administered daily for 4 weeks by intranasal instillation. The dose volume (maximum 0.02 mL) was calculated weekly based on individual body weight (BW) and divided evenly between the two nares.

Table II: The four peptides employed in the study

The amino acid sequence (single-letter-abbreviations), intranasal dose concentration, source of custom synthesis, lot number, and purity (provided by suppliers) of the peptides used in this study are shown. A 9-mer peptide, which contains small amino acids (glycine and alanine) and is expected to form minimal secondary structure, was designed empirically and used as control peptide.

Peptide Treatment	Amino Acid Sequence	Dose (µg/kg BW/day)	Source	Lot	Purity
ADNP-8	NAPVSIPQ	50	Selleckchem	P103375	> 98%
Ang_{1-7}	DRVYIHP	250	Selleckchem	P1703005	> 98%
Semax	MEHFPGP	250	Selleckchem	P1703006	> 98%
Control peptide	GGAAGSSGG	250	Life Technologies	NF100030.1	> 99%

Hypoxic and/or Hyperoxic Exposures

Normobaric exposure to altered-oxygen atmospheres were conducted in custom-built, clear, polycarbonate chambers approximately 40 centimeters (cm) wide, 35 cm long, and 21 cm high (Coy Laboratory Products, Grass Lake, MI), with real-time control over the internal gas concentrations. The chambers were calibrated daily using compressed, medical grade Nitrogen and Oxygen gases (Airgas, Dayton, OH). The oxygen level was set for each exposure and nitrogen completed the chamber atmosphere. Two, cohoused animals were transferred to the same chamber daily for 5 days and exposed to one of three atmospheres. Hypoxia exposure was 7.5% oxygen, corresponding to an oxygen level at altitude of 8200 meters (ppO_2 = 57.7 mmHg) for 60 minutes. Hyperoxia subjects breathed 95% oxygen for 60 minutes. The oscillatory hypoxia-hyperoxia group started with 15 minutes at 95% oxygen, and then experienced 10 cycles of 95% for 8 minutes followed by 5% for 3 minutes. It normally takes less than 2 minutes to complete each change in oxygen concentration in the chamber, so that the total exposure lasted

about 2.5 hours. The animals were monitored closely for signs of distress. An additional group, open-air control, was exposed to normal, room air and housed in clear plastic cages near the Coy chambers.

Novel Object Recognition (NOR) Test

Animals underwent NOR testing similar to Barnes, *et al.*[4], at the end of 5 days of altered-oxygen exposure. The NOR field was an open-top, 60 cm square, black plastic box with 40 cm walls. Activity was recorded by video camera and analysis using Ethovision XT 12 tracking software with a three-point body-tracking module (Noldus, Leesburg, VA). Fluorescent tube lights supplied 3 to 6 lux to the NOR field. Habituation consisted of 30 minutes in the testing room and ten minutes in an empty NOR field, daily. Exploration time for training was ten minutes and for testing, was five minutes. Three similarly sized objects were used: rubber duck, round wooden tower, square plastic tower and exploration included a 2 cm buffer around the object. Familiar and novel objects were assigned randomly while ensuring equal distribution to the groups in a counter-balanced design. They were placed equal distances from opposite corners of the field and held in place by Velcro. Rats with a positive discrimination ratio (DR)[19] passed the test while the magnitude of the ratio along with the percent of time exploring each object served to indicate the degree of effect and it mitigation by peptide treatment.

Bioanalysis for Physiological Effects

The animals were euthanized by exsanguination or decapitation under anesthesia (Ketamine/Xylazine), three days after the final NOR test. Euthanasia methods were reviewed and approved by the IACUC and that methods followed the AVMA (American Veterinary Medical Association) guidelines on euthanasia, The heart, lung, brain, liver, and spleen were weighted, fixed in 4% paraformaldehyde and stored at -80 °C. Whole blood was collected in K_3EDTA Vacuette tubes (Greiner, Monroe, NC) to measure hematocrit, hemoglobin, 1,3-BPG, 2,3-BPG, and BPGM. Hemoglobin was measured using the Hemoglobin Assay Kit (MilliporeSigma, St. Louis, MO). Blood for the hematocrit was processed using the StatSpin MP (Iris, Chatsworth, CA) and measured using the CritSpin Digital Hematocrit Reader (Iris). Red blood cells (RBCs) were collected by centrifugation and lysed using RBC lysis buffer (Abcam, Cambridge, MA) in order to measure the concentrations of 1,3-BPG and 2,3-BPG and BPGM, using analyte-specific, rat ELISA kits (MyBiosource, San Diego, CA).

Serum was isolated using centrifugation of post-mortem blood collected in BD Vacutainer SST tubes (BD, Franklin Lakes, NJ) and stored at -80 °C until it was used to quantify the level of Angiotensin II, Copeptin, S100B, and VEGF (MyBiosource), and EPO (Biomatik, Wilmington, DE), using ELISA kits for the respective analytes.

Statistical Analysis

The data was analyzed using Prism 7 for Windows, version 7.05 (GraphPad Software, La Jolla, CA). The means were compared using one-way ANOVA with alpha = 0.05, followed by the Bonferroni's multiple comparison test when a significant effect was identified. The mean for each peptide treatment was compared to the open-air control and to the exposure-specific control peptide group. The open-air control results were pooled across experiments except for the behavior data, where greater day-to-day variation is more likely. Body weights were analyzed also by two-way ANOVA using treatment and time as factors; however, since no animals were treated with Ang_{1-7} and exposed to oscillating hypoxia-hyperoxia, the analysis first included Control Peptide, ADNP-8, and Semax with each exposure and then a second analysis included all four peptides with hypoxia and hyperoxia exposures.

4.0 RESULTS AND DISCUSSIONS

Physiological Effects of Hypoxic and/or Hyperoxic Exposure

The animals gained weight normally during the study and the difference between each treatment group was not statistically significant (Figure 2). During the week of altered-oxygen exposures, the oscillatory hypoxia-hyperoxia group gained the least amount of body weight; however, only the difference between control peptide-treated animals in the oscillatory and the hypoxia exposure groups reached statistical significance ($p = 0.003$) (Figure 2). The only loss of weight detected in the study was one animal during hypoxic exposure in the Ang_{1-7} group. Because of that loss of weight, the difference between Ang_{1-7} and control peptide in the hypoxia group was also statistically significant ($p = 0.035$). If that animal was excluded from the analysis, the difference was no longer statistically significant ($p = 0.366$). One animal treated with control peptide died during oscillatory hypoxia-hyperoxia exposure, but the cause was not readily apparent based on gross, pathological assessment (data not shown). The animal monitoring criteria for humane endpoints were closely watched and this animal could not be recovered once signs were identified/observed.

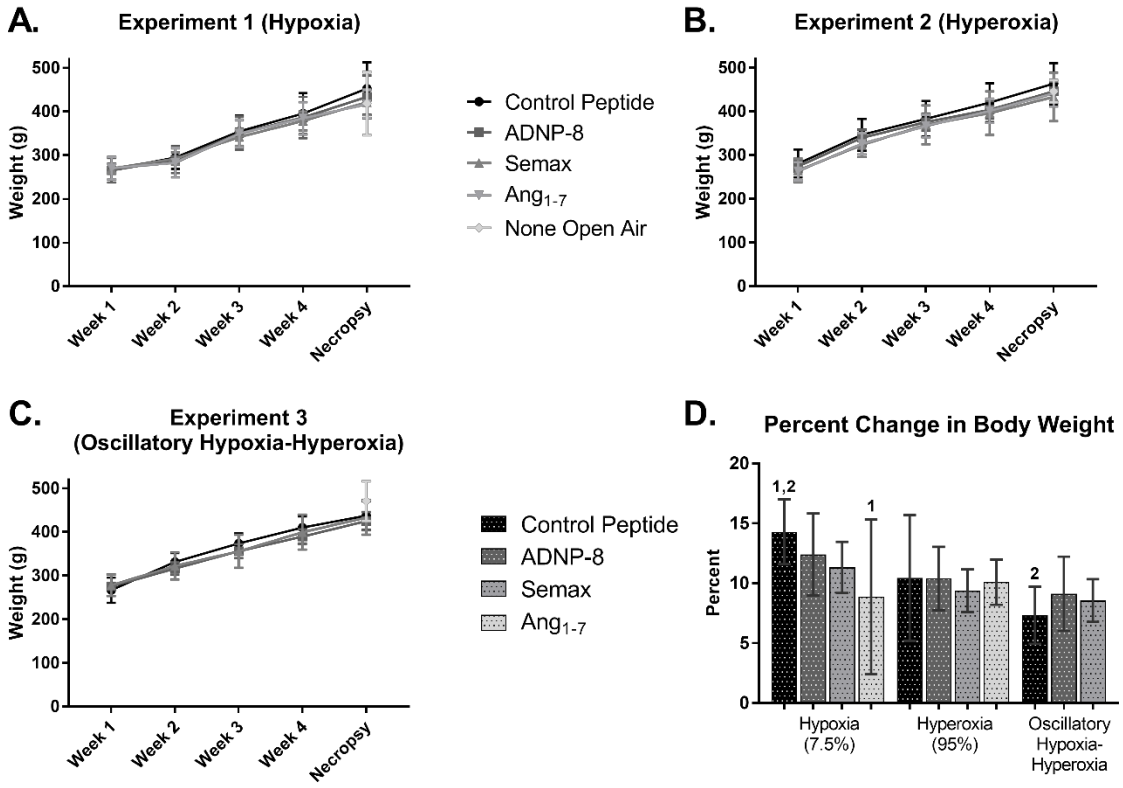

Figure 2: Body weight

The weekly mean body weight for each treatment group increased normally during each experiment (A, B, and C), but the mean percent gain in body weight during the exposure week (D) differed between exposures. The no-peptide, open-air control group (None Open Air) was only weighed at necropsy and the Ang_{1-7} treatment group was excluded in the oscillatory hypoxia-hyperoxia experiment. Otherwise, n = 8 for each treatment and exposure, except n = 7 for the control peptide treatment groups exposed to hyperoxia and oscillatory hypoxia-hyperoxia. The difference between the experimental groups labeled with the same number are statistically significant ($p < 0.05$). The legend between A and B applies to A, B, and C, while the legend between C and D applies to D only.

The differences in organ weights between treatments and exposures were small and only one reached statistical significance: brain weight in the open-air control versus rats treated with control peptide and exposed to hypoxia (Table III). This result suggests that hypoxia exposure is capable of decreasing brain mass, which could be successfully rescued by the treatment with the neuroprotective peptides used in this study.

Table III: Organ Weights

For most treatment/exposure group, n = 8. For the control-peptide group exposed to hypoxia or oscillatory hypoxia-hyperoxia, n = 7. The open-air control group results were pooled, n = 24. The mean organ weights are expressed in grams with the standard deviation in parentheses. [a]The difference between the no-peptide, open-air control group and the control-peptide hypoxia group was statistically significant (one-way ANOVA, Bonferroni's test, p = 0.0008).

Exposure	**Open Air**	**Hypoxia (7.5% Oxygen)**				**Hyperoxia (95% Oxygen)**				**Oscillatory Hypoxia-Hyperoxia (5% ↔ 95% Oxygen)**		
Peptide Treatment	**None**	**Control**	**ADNP-8**	**Semax**	**Ang_{1-7}**	**Control**	**ADNP-8**	**Semax**	**Ang_{1-7}**	**Control**	**ADNP-8**	**Semax**
Brain	2.04[a] (0.13)	1.67[a] (0.65)	1.79 (0.28)	1.96 (0.37)	1.93 (0.15)	2.06 (0.16)	2.12 (0.12)	2.00 (0.07)	2.03 (0.08)	2.20 (0.18)	2.16 (0.09)	2.15 (0.11)
Heart	1.64 (0.23)	1.73 (0.32)	1.59 (0.13)	1.67 (0.17)	1.73 (0.19)	1.52 (0.25)	1.57 (0.19)	1.54 (0.21)	1.50 (0.13)	1.57 (023)	1.61 (0.12)	1.67 (0.22)
Right Lung Lobes	2.65 (0.94)	2.45 (0.45)	2.01 (0.59)	2.05 (0.69)	2.63 (0.69)	2.24 (0.63)	2.21 (0.73)	2.16 (0.83)	2.29 (0.60)	3.01 (0.43)	2.63 (0.52)	2.78 (0.65)
Left Lung Lobe	1.30 (0.37)	1.20 (0.40)	1.09 (0.49)	1.10 (0.43)	1.35 (0.41)	1.14 (0.40)	1.27 (0.41)	1.16 (0.43)	1.25 (0.31)	1.39 (0.24)	1.20 (0.34)	1.51 (0.56)
Liver	18.6 (3.3)	20.3 (4.2)	18.2 (2.9)	18.6 (3.2)	18.6 (2.6)	18.4 (3.6)	18.4 (2.8)	18.2 (3.8)	18.7 (1.3)	17.7 (2.0)	16.7 (1.3)	18.7 (2.4)
Spleen	0.947 (0.22)	0.797 (0.26)	0.855 (0.30)	0.927 (0.19)	0.895 (0.27)	0.884 (0.11)	0.910 (0.15)	0.985 (0.22)	0.809 (0.09)	0.834 (0.10)	0.924 (0.14)	0.943 (0.15)

Table IV summarizes the results of select markers related to altered-oxygen exposures. The concentration of hemoglobin directly influences the oxygen-carrying capacity of the blood. It was increased in animals exposed to hypoxia, but decreased in oscillatory hypoxia-hyperoxia, for all peptide treatments groups (Table IV, Figure 3). In the hypoxia group, the increase over the open-air control reached statistical significance for animals treated with control peptide ($p = 0.04$), ADNP-8 ($p < 0.0001$), and Semax ($p = 0.040$), but not with Ang_{1-7} ($p = 0.099$). The changes induced by oscillatory exposure, however, did not reach statistical significance for rats treated with Semax ($p = 0.073$) or ADNP-8 ($p = 0.119$) or control peptide ($p > 0.999$). The affinity of oxygen for hemoglobin can be lowered by the binding of 2,3-BPG, which releases the bound oxygen into the surrounding tissues. The enzyme BPMG catalyzes the conversion of 1,3-BPG, a normal metabolic product of glycolysis, to 2,3-BPG. The serum concentration of 1,3-BPG was not altered by any exposures or treatments, but 2,3-BPG was lower in the animals exposed to hypoxia than the open-air control, and the difference between the control rats and those treated with ADNP-8 or Ang_{1-7} reached statistical significance ($p < 0.05$) (Table IV). In contrast, 2,3-BPG in rats treated with control peptide and exposed to oscillatory hypoxia-hyperoxia was greater than the open-air control and the difference was statistically significant ($p = 0.014$). The control-peptide, oscillatory exposure also had more 2,3-BPG than the ADNP-8 and Semax groups with the same exposure, and only the difference from the ADNP-8 group was statistically significant ($p = 0.035$). This suggests that hypoxia caused lower levels of 2,3-BPG, while oscillatory hypoxia-hyperoxia increased its level that was somewhat reversed by the treatment with ADNP-8 or Semax (Table IV). In apparent agreement with the reduction of 2,3-BPG in hypoxia, the concentration of BPMG in rats exposed to hypoxia was lower than that of the open-air control. BPMG in the ADNP-8 group with hyperoxia exposure were also lower than the open-air control, with a concomitant decrease in the 2,3-BPG levels; however, these changes did not reach statistical significance.

VEGF, which stimulates angiogenesis and increases vascular permeability, was found at lower levels in all treatment groups exposed to hypoxia or oscillatory hypoxia-hyperoxia when compared to open-air controls, but only the difference with the oscillatory exposure groups reached statistical significance ($p < 0.003$) (Table IV, Figure 3). The serum concentration of Angiotensin II, which stimulates vasoconstriction and water retention by provoking the release

of aldosterone, appeared to increase compared to the open-air control group in animals treated with control peptide or ADNP-8 that were exposed to hypoxic conditions, and in animals treated with Semax or Ang_{1-7} that were exposed to hyperoxia. In contrast, it was lower in all animal groups exposed to oscillatory hypoxia-hyperoxia (Table IV). Due to high variability in the results for this analyte, the changes were not statistically significant except for the ADNP-8-treated group in the hypoxia experiment.

Table IV: The Biological Effects of Peptide Treatment and Altered Oxygen Atmosphere Exposures

*The number of animals is the same as Table III. The mean value is shown with the standard deviation in parentheses. The difference between results with the same superscripted letters are statistically significant (1-way ANOVA, Bonferroni's test, $p \leq 0.05$). *Significant effect by ANOVA but not significant after the multiple comparison correction. **Due to levels below the limit of detection, the number of samples is reduced for copeptin and are indicated in brackets.*

Exposure	Open Air	Hypoxia (7.5% Oxygen)				Hyperoxia (95% Oxygen)				Oscillatory Hypoxia-Hyperoxia (5% ↔ 95% Oxygen)		
Peptide Treatment	None	Control	ADNP-8	Semax	Ang_{1-7}	Control	ADNP-8	Semax	Ang_{1-7}	Control	ADNP-8	Semax
Hemoglobin (g/dL)	20.1[a,b,c] (7.0)	29.1[a] (5.1)	35.3[b] (8.8)	27.5[c] (7.5)	26.8 (8.2)	20.2 (4.2)	19.7 (3.0)	19.2 (2.2)	19.3 (4.4)	16.3 (3.8)	13.6 (1.6)	13.2 (2.3)
1,3-BPG (nM/mL)	5.13 (4.4)	7.16 (1.5)	5.61 (1.5)	5.96 (1.5)	5.31 (1.2)	4.10 (1.8)	6.33 (3.9)	4.35 (1.4)	6.02 (3.2)	4.97 (2.3)	3.68 (1.3)	3.13 (2.1)
2,3-BPG (nM/mL)	0.377[h,i,k] (0.16)	0.245 (0.10)	0.215[i] (0.06)	0.216 (0.08)	0.215[k] (0.07)	0.324 (0.10)	0.282 (0.10)	0.290 (0.12)	0.307 (0.07)	0.569[h,l] (0.08)	0.360[l] (0.16)	0.459 (0.13)
BPMG (ng/mL)	2.04 (0.88)	1.33 (1.2)	1.61 (0.69)	0.933 (0.97)	1.39 (0.51)	1.53 (0.71)	1.23 (0.88)	1.48 (0.59)	1.30 (0.88)	2.27 (0.54)	1.82 (0.57)	1.09 (1.1)
VEGF (pg/mL)	172[d,e,f] (79)	110 (38)	145 (70)	119 (34)	121 (41)	221 (35)	173 (28)	219 (106)	226 (60)	70.6[d] (39)	71.7[e] (29)	81.0[f] (48)
Angiotensin II (pg/mL)	244[g] (142)	584 (549)	634[g] (587)	330 (169)	207 (125)	418 (231)	284 (109)	564 (458)	497 (247)	83.1 (10.1)	75.7 (10.1)	93.1 (39.3)
Hematocrit* (% RBC vol.)	41.9 (3.0)	44.0 (4.3)	45.0 (2.4)	43.5 (2.4)	43.5 (3.8)	43.7 (3.2)	41.9 (2.3)	42.0 (2.6)	41.8 (2.0)	38.9 (5.5)	40.4 (4.4)	39.8 (2.0)
Erythropoietin (μg/mL)	0.788 (0.29)	1.02 (0.98)	0.760 (0.13)	0.845 (0.11)	0.972 (0.68)	0.924 (0.27)	0.828 (0.20)	1.02 (0.40)	0.955 (0.48)	0.492 (0.16)	0.616 (0.22)	0.660 (0.22)
S100B (pg/mL)	64.4 (31.0)	83.9 (25.8)	65.3 (28.9)	79.5 (23.8)	78.0 (22.3)	80.5 (20.0)	59.9 (24.5)	53.2 (19.8)	60.0 (20.9)	57.6 (18.0)	61.6 (20.4)	50.2 (33.4)
Copeptin** (pg/mL)	136 (80)[7]	89.3 (84)[5]	131 (118)[4]	59.1 (5.9)[3]	105 (59)[6]	104 (78)[3]	70.6 (53)[2]	119 (147)[4]	81.3 (35)[4]	155 (115)[2]	108 (96)[3]	34.3 (49)[3]

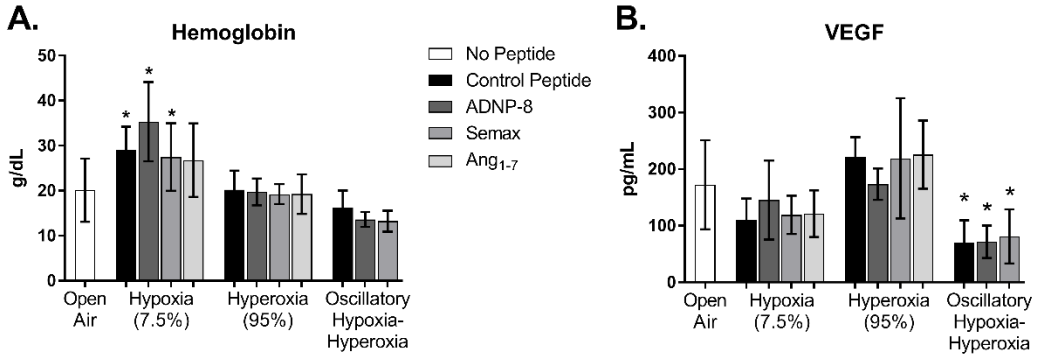

Figure 3: Serum hemoglobin and VEGF

The number of animals is the same as in Table 4. The asterisks (*) indicate the experimental groups that are significantly different from the open-air control group ($p < 0.05$).

The hematocrit was lower in all oscillatory exposure groups than the open-air control (Table IV). Although the changes were relatively small, the ANOVA result revealed a statistically significant effect, but no significant differences were found after the Bonferroni's multiple comparison test. Consistently, all animal groups in this experiment also showed decreased levels of EPO (that were not statistically significant). There were no overt or statistically significant changes in S100B, an indicator of damage to the blood brain barrier, or Copeptin, which regulates blood volume by affecting the resorption of water.

Memory Performance

Memory performance was assessed using the NOR test, which determines the animal's ability to distinguish between a previously encountered, familiar object and a novel, not previously seen object. An animal with normal memory functions will spend more time exploring a novel object than a familiar one, and so the difference in exploratory time served as an indicator of episodic memory formation and retrieval. In the open-air control groups, the percentage of exploratory time spent with the novel object was greater than that with the familiar object for the oscillatory and hypoxia exposures ($p < 0.0001$ and $p = 0.0539$, respectively, $n = 8$) (Figure 4). When all the open-air results were pooled, the animals spent 60% of exploratory time with the novel object and 40% with the familiar ($p = 0.0304$, $n=24$) (data not shown). The rats treated with control peptide in all three experiments explored the two objects nearly equally

(Figure 4), indicating that hypoxia, hyperoxia, and oscillatory hypoxia-hyperoxia exposures inhibited episodic memory function. Under hypoxic conditions, at least two of the peptides prevented memory deficit: the difference in the percent exploratory time with the familiar and novel objects was statistically significant for ADNP-8 ($p = 0.0072$) and showed a positive trend for Semax ($p = 0.0636$), but not significant for Ang_{1-7} ($p = 0.1989$). Under hyperoxic conditions, the only group that appeared to spend more time exploring the novel object was the Semax group, but the difference between the objects did not reach statistical significance ($p = 0.1270$). During exposure to oscillatory hypoxia-hyperoxia, both ADNP-8 and Semax protected animals from memory deficits, having statistically significant differences between the novel and familiar objects ($p < 0.0001$). A similar conclusion can be drawn from the number of rats in each group that passed the NOR test (Figure 4, panels B, E and H) and from the DR (Figure 4, panels C, F and I). Seventy-nine percent (79%) of the open-air control rats passed, but for the rats treated with control peptide, only 38% of hypoxia rats, 43% of hyperoxia rats, and 57% of oscillatory hypoxia-hyperoxia rats passed the NOR test. The peptides that prevented memory deficits based on the percent of exploratory time also improved the rate of NOR passage, making them more similar to the open-air control. In fact, 100% of the rats treated with Semax and exposed to oscillatory hypoxia-hyperoxia passed the NOR test. Additionally, the analysis of DR suggests that exposure to oscillatory hypoxia-hyperoxia caused the greatest memory impairment, since the difference between the rats treated with control peptide and the open-air control was statistically significant ($p = 0.0362$), and treatment with ADNP-8 or Semax protected rats from this insult. The ADNP-8 and Semax treatment groups were more similar to the open-air control, and their difference from the control peptide treatment was statistically significant ($p = 0.0209$ and $p = 0.0053$, respectively).

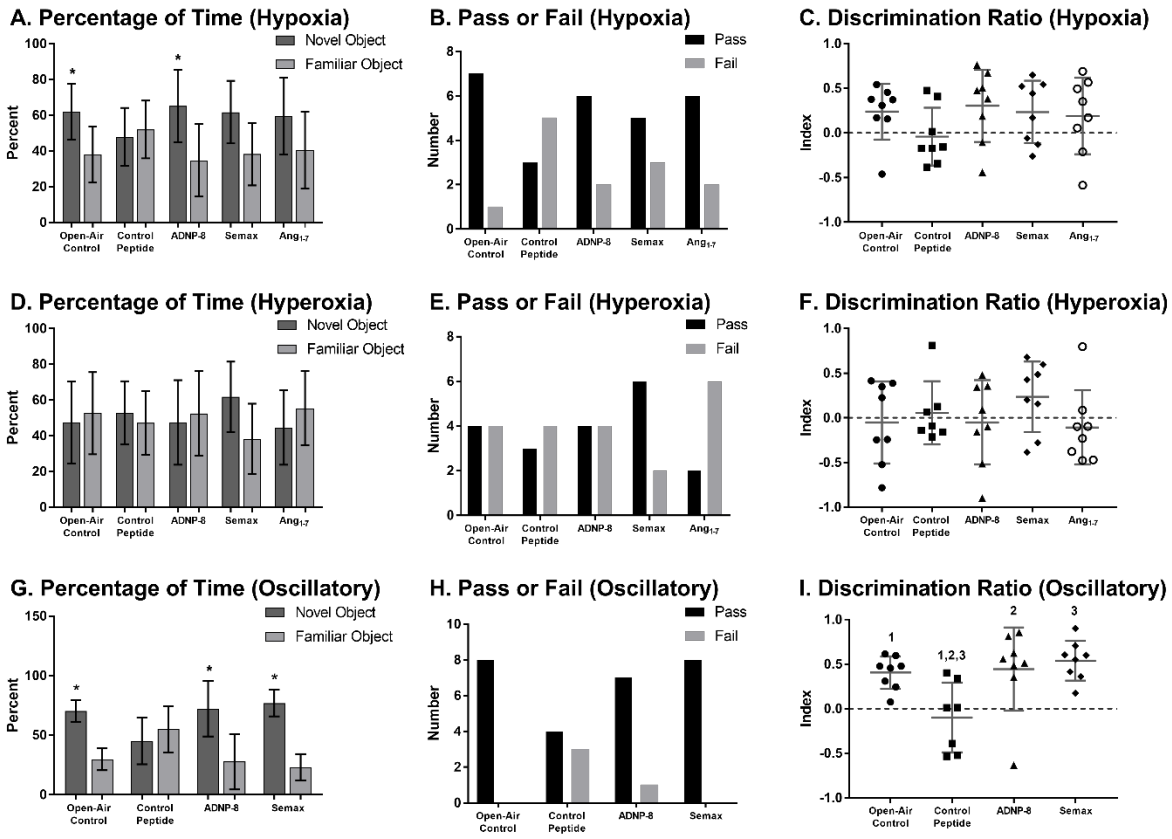

Figure 4: Novel Object Recognition (NOR) Behavior Test

The percentage of exploratory time spent with each object (A, D, and G), the rate of test passage (B, E, and H), and the discrimination index (C, F, and I), show that memory was impaired by hypoxia (A, B, and C) and oscillatory hypoxia-hyperoxia (G, H, and I) exposures, and reduced somewhat by hyperoxia (D, E, and F), in the control peptide treatment group. The asterisks (*) in A and G indicate the experimental groups in which the exploration of novel object is significantly higher than that of familiar object ($p < 0.05$). The difference between experimental groups labeled with the same number in I are statistically significant ($p < 0.05$). The number of animals is the same as Figure 2

5.0 CONCLUSION

In this study, episodic memory was determined in rats treated with neuroprotective peptides, and then exposed to hypoxia, hyperoxia or oscillatory hypoxia-hyperoxia in the last week of peptide treatment. Although treatments with these neuroprotective peptides prevented memory performance deficits, the results of bioassays suggested that certain exposure-induced effects persisted even after 3 days of recovery, including the changes in physiological markers such as hemoglobin, 2,3-BPG and VEGF, as well as reduced brain weight following hypoxic exposure.

Hemoglobin greatly increases the oxygen-carrying capacity of the blood. The concentration of hemoglobin can be upregulated under hypoxic conditions[55] or downregulated during hyperoxia[25], in order to help regulate oxygen delivery to the tissues. The initial increase in concentration may be caused by a decrease in the blood volume,[55] which concentrates the RBC's, but a sustained change is brought about by an increase in RBC production induced by EPO.[55]

In this study, the concentration of hemoglobin increased in the rats exposed to hypoxia, as expected; however, it did not decrease in rats exposed to hyperoxia, perhaps due to the relatively brief exposure used here (60 minutes daily) compared to others. In fact, less than 16 hours daily treatment was reported to be ineffective.[29] The oscillatory hypoxia-hyperoxia exposure resulted in lower hemoglobin concentration (which did not reach statistical significance). Although it is not clear if this represents a response directly resulting from the high oxygen exposure or a specific detrimental effect caused by the oscillating oxygen levels, the result of the hyperoxia experiment however suggests that the latter seems to be a more plausible explanation. Interestingly, the level of EPO was also deceased in the oscillatory hypoxia-hyperoxia groups (although this does not reach statistical significance), while the hematocrit showed significant decreases in the same animal groups before the correction for multiple testing. Reports indicate that RBC synthesis[46] and EPO levels[58] are dependent on both the partial pressure of oxygen and the length of exposure. This study used relatively brief exposures (60 minutes daily for five days) that did not result in statistically significant changes in EPO levels and hematocrit. Thus, the change in hemoglobin observed under hypoxia was likely the result of reduced blood volume.

The supply of oxygen to the tissues can also be adjusted by the BPMG-catalyzed conversion of 1,3-BPG to 2,3-BPG in the RBC's. When hemoglobin is bound by 2,3-BPG, its affinity for oxygen decreases so that more oxygen is released to the local tissues. Exposure to hypoxia causes 2,3-BPG to increase in RBC's[5] while hyperoxia causes it to decrease.[60] In this study, 2,3-BPG was decreased with hypoxic exposure and increased in animals treated with control peptide and exposed to oscillatory hypoxia-hyperoxia. BPGM appeared to decrease with hypoxic and hyperoxic exposures, but the results were not statistically significant. It is possible that these results reflect a rebound after the 3-day recovery period.

When blood pressure decreases due to a lower blood volume, Angiotensin II causes vasoconstriction to reduce venous blood volume and increase arteriole resistance, which in turn increases blood pressure and consequently its supply to tissues. At the same time, Angiotensin II triggers the vasopressin production and secretion, as well as the release of aldosterone to encourage fluid retention and reestablish normal blood volume.[3] In this study, Angiotensin II increased with select treatments in the hypoxia and hyperoxia exposures, and decreased under oscillatory hypoxia-hyperoxia. The changes suggest activation of compensatory mechanisms, but the pattern is not clear and many of the changes did not reach statistical significance, which could be the result of the three-day recovery prior to serum collection. Copeptin, which is a surrogate for vasopressin that regulates blood volume and a sensitive indicator for acute hypoxia exposure, was unchanged in this study. It has been reported that copeptine returns to baseline levels within 16 hours after hypoxic exposure.[43] In this study, the serum was collected three days after the final exposure. Probably due to this reason, copeptine was frequently below the level of detection, thus reducing the number of samples included in the analysis.

VEGF functions as a transcription factor to stimulate endothelial cell growth and migration, increase vascular permeability, and induce angiogenesis under hypoxic stress.[59] It was downregulated during hyperoxia.[6] In this study, VEGF decreased in the oscillatory hypoxia-hyperoxia groups, suggesting that the high oxygen portion of the cycle might have greater impacts than the low oxygen portion; however, VEGF levels were unaffected in the hyperoxia and decreased under hypoxia. The 3-day recovery may have affected these results also.

Deficits in spatial, visual, and working memory, resulting from hypoxic exposures (including acute continuous,[64] chronic continuous,[9, 24, 30, 63, 65] acute intermittent,[54] and chronic intermittent, and oscillating,[14, 28]), have been revealed using the Morris water maze. Memory

impairment has also been demonstrated using a radial-arm maze[11] and a visual memory test similar to NOR.[26] Likewise, this study showed that episodic memory performance, as determined using the NOR test, was impaired in rats exposed to hypoxia. Similarly, exposure to oscillating hypoxia-hyperoxia and to some extent, hyperoxia also caused memory deficits. These behavioral effects caused by abnormal oxygen levels are expected, because the brain suffers oxidative insult during both hypoxia and hyperoxia, and oxidative stress is known to impair memory function.[13]

Others have reported alleviating hypoxia-induced memory deficits by treatment with a variety of agents. Metformin, an antidiabetic biguanide that improves glucose utilization and reduces gluconeogenesis, prevented spatial memory impairment[64]. The antioxidants Crocin,[63] *Cyperus rotundus* root extract,[24] and quercetin[30] prevented spatial memory deficits. Mac-1 siRNA, which is believed to prevent microglial type switching, reduced the impact of hypoxia on working memory.[11] Spatial and working memory were protected by breathing a mixture of 67% hydrogen and 33% oxygen immediately prior to hypoxia.[28] Voluntary exercise also prevented visual and spatial memory impairment caused by hypoxia.[26]

In this study, we tested the ability of three neuroprotective peptides to reduce the effects of abnormal oxygen levels on memory function. Treatment with ADNP-8 prevented the deficit in memory performance resulted from exposure to hypoxia and oscillatory hypoxia-hyperoxia. Others have reported that ADNP-8 protects the brain from various insults. It prevented tissue damage, reduced mortality, prevented cognitive impairment, and improved neurobehavioral recovery in rodent models of traumatic brain injury,[7] ischemic injury,[27] and Alzheimer's disease.[62] ADNP-8 was also reported to improve short-term memory in rats after 3 to 8 months of intranasal administration.[15]. Based on its ability to protect neurons and preserve cognitive function, ADNP-8 is a good candidate for use as a preventative or countermeasure against the adverse effects of hypoxic, as well as hyperoxic exposures.

Treatment with Semax protected memory function in rats exposed to hypoxia, hyperoxia, and oscillatory hypoxia-hyperoxia. Its protective effect under hypoxia and oscillatory exposure reached statistical significance. Under hyperoxic conditions, the effect was highly evident, although it did not reach the statistical significance threshold. Other researchers have also reported on the neuroprotective effects of Semax. It reduced tissue damages, and learning and memory deficits caused by focal ischemic lesions in rats.[49, 56] Semax also prevented behavioral defects caused by gestational exposure to valproic acid.[34] Combined with an opiate-like peptide,

Semax prevented some of the growth and behavioral effects of gestational hypoxia.[36] It can improve learning and memory in rats exposed to heavy metals, but impaired these functions when used independent of the toxins.[20, 21] It has been reported Semax improves memory, attention, and brain circulation in humans without negative side effects (reviewed by Asmarin, et al.[1]). Its neuroprotective effects demonstrated in this study strongly suggest its potential to be used as a countermeasure for hypoxic and hyperoxic stressors that are commonly encountered in the aerospace environment. To realize its benefits, dose optimization however will be needed due to its relatively narrow therapeutic window.[35] Additional studies aimed to address this issue will likely facilitate its development into a standard prophylaxis for hypoxic and/or hyperoxic exposure.

Treatment with Ang_{1-7} appeared to improve memory performance after exposure to hypoxic conditions: a greater number of rats passed the NOR test and the rats spent noticeably more time exploring the novel than the familiar object, though the difference was not statistically significant. Others have demonstrated the potential for Ang_{1-7} to prevent tissue damage[37] and reduce cognitive impact[48, 61] in rodent models of ischemia. However, Ang1-7 failed to protect memory performance under hyperoxia, and in fact this group of animals showed the lowest performance in this exposure experiment. For this reason, Ang_{1-7} was excluded from the oscillatory hypoxia-hyperoxia experiment.

This study used normobaric exposures to air with different concentrations of oxygen in order to study the effects of hypoxia and hyperoxia. There is some debate over the use of normobaric hypoxia to simulate high-altitude, hypobaric hypoxia. The temporal, physiological response to hypoxia at different BPs appears to differ slightly. For example, a 40-minute exposure to 12% O_2 or its equivalent PO_2 = 90 mmHg, resulted in greater hypoxemia, hypocapnia, and blood alkalosis under hypobaric conditions, but greater arterial oxygen saturation under normobaria.[50] Yet, after 24 hours at 13.4% O_2 or PO_2 = 102 mmHg, there was little difference between hypobaria and normobaria.[12] It seems that the pattern of the changes in blood parameters is different under the two exposure conditions, but over time, both exposures will cause a similar pattern of physiological changes and hypoxic symptoms.[52] Moreover, even though the physiological response is slightly different, both exposures inhibit working memory in a similar manner.[32] The repeated, acute exposures to normobaric hypoxia, hyperoxia, and oscillatory hypoxia-hyperoxia used herein therefore should be acceptable, as surrogates for

studying the cognitive effects involved in military flight. In fact, this study found that oscillatory hypoxia-hyperoxia had the greatest impact on body weight, hemoglobin and VEGF levels, and on memory performance. This is in agreement with Malle, *et al.,*[32] who similarly found that breathing 100% oxygen while recovering from hypoxic exposure hastened the return to normal vascular oxygen levels, but caused slowing of the EEG and poor performance on cognitive assessments.

This study showed that the neuroprotective peptides, ADNP-8, Semax, and Ang_{1-7} each has the potential to be used as neuroprotective agents for abnormally low or high oxygen concentrations frequently encountered in the aerospace environment. It is tempting to speculate that a combination of these peptides will result in a more robust protection or even an enhancement of cognitive functions. In aerospace careers, such exposures can occur frequently and unexpectedly. Prophylactic treatments and countermeasures to protect aerospace professionals are necessary and continue to be investigated. The encouraging results presented here emphasize the value of further study to establish the safe and effective use of these neuroprotective peptides.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

%	percent
°C	degrees Celsius
cm	centimeters
m	meters
mmHg	millimeters of mercury
mg/mL	milligrams per milliliter
O_2	oxygen
PO_2	pressure of oxygen
ADNP	activity-dependent neuroprotective protein
BP	barometric pressure
BPG	Bisphosphoglyceric Acid
BPGM	Bisphosphoglycerate Mutase
EPO	erythropoietin
IACUC	Institutional Animal Care and Use Committee
NOR	novel object recognition
ORISE	Oak Ridge Institute for Science and Education
VEGF	Vascular Endothelial Growth Factor