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Mechanisms of the Combined Effects of Inhaled Chemical Contaminant Exposure and Hypoxia on Real-Time Performance in Rats

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STATEMENTS

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The views expressed in this technical report are those of the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government.

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Animal Care and Use statement

The study protocol was reviewed and approved by the Wright-Patterson Air Force Base Institutional Animal Care and Use Committee in compliance with all federal regulations governing the protection of animals and research.

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Executive Summary

Physiological episodes (PEs) have been attributed to, among other things, hypoxia and exposures to chemical contaminants. The consensus to date is that most, if not all, of the contaminants to which aircrew are exposed are not present at sufficient levels to induce PE-like symptoms. There is also scant evidence that classic hypoxia alone is the cause. The potential additivity or synergy of the effects of simultaneous exposure to chemical contaminants and hypoxia has not been closely examined, particularly with respect to subtle central nervous system (CNS) or motor coordination effects that are relevant to aviator performance.

The objective of this research was to understand the mechanisms of combined inhalation of chemical contaminants and hypoxia in an *in vivo* rat model to better assess risk of the combined exposures as a potential cause of PEs. Following a previously performed dedicated analysis and review of chemical compounds detected in and around Navy aircraft, trimethylsilanol (TMS) and carbon monoxide (CO) were identified as top candidates for combination studies to test for any additivity or synergy of exposure-related effects between themselves and hypoxia that could potentially contribute to the occurrence of PEs. TMS exposure has been shown to have depressant effects on the CNS, while CO is known to preferentially bind to hemoglobin and prevent oxygen delivery to tissues within the body. Both were seen as potential candidates to have additive or synergistic effects with hypoxia exposures that are also known to impact CNS function.

The project was divided into two separate experiments. The goal of the first experiment was to study the differences between the effects of individual and combined exposures to TMS, CO, and hypoxia on a motor coordination task in a rat model. The second experiment then investigated the differences in physiological changes associated with the same sets of individual and combined exposures in the same rat model. During the first experiment, a minimum effective concentration (MEC) for CO and hypoxia exposure was identified that produced an effect on a motor coordination neurobehavioral task. Using these results, and the results of an equivalent experiment performed with TMS in a prior study, the chemicals were then tested in combination to determine their effect on motor coordination performance and whether this effect was synergistic, additive, or antagonistic compared to a modeled prediction of the combination response based on the individual results. A subsequent experiment exposed separate groups of rats to the MECs of CO, TMS, and hypoxia identified during the neurobehavioral experiments to assess physiological changes in the respiratory, cardiovascular, and neurological systems that could be attributed to the reduction in performance. The exposures to TMS, CO, and hypoxia were performed individually and in combination to attempt to determine mechanistic explanations for additive or synergistic neurobehavioral decrements of combination exposures relative to individual exposures.

During the first experiment, separate groups of adult Sprague Dawley rats were exposed whole-body to one of a series of concentrations of each individual chemical for two hours. At 90 and 110 minutes into the exposure, the rats were tested using an accelerod motor coordination task that determined how long they could stay on a constantly accelerating rotating rod. The MECs for latency to fall in the accelerod test were determined to be 513 ppm CO and 11.7% oxygen, based on the lowest concentration of CO and highest concentration of oxygen in each of the series of

exposures that produced a significant reduction in latency to fall at either time point. A previous set of experiments using the same methodology determined a MEC of 750 ppm for TMS. The results of the combined exposures at the MEC level demonstrated some evidence of additivity but weak to no evidence of synergy when comparing the performance in the same task to the individual exposures. The combination exposures were more detrimental to performance than the individual exposures, as evidenced by the clear significant difference from baseline in accelerod latency to fall during the combination exposures as compared to the borderline significance for the individual exposures. Combination analysis using a Bliss Independence model found no clear evidence of synergy of the combination exposures, supporting the conclusion that the additional risk of effects of combined exposures to TMS, CO, and hypoxia on complex motor tasks that would not be greater than the response predicted by straightforward additivity of the individual exposure risks.

During the physiology experiments, there was an effect of exposure to CO and hypoxia in combination on hippocampal responses to input stimuli at a series of intensities that was not present in either of the individual CO or hypoxia exposures. This result suggests that there is an impact on hippocampal function in the presence of combined CO and hypoxia that is not observed with either CO or hypoxia exposures individually. Although the accelerod testing performed in this study does not specifically assess hippocampal performance, given the foundational data provided from this project, further analyses could use this information in more nuanced cognitive tests that would provide specific outcomes in regards to spatial reasoning, learning and memory, as well as attentional processing that would provide supporting behavioral data for the hippocampal changes observed during this study. While this study provided an initial first screening for the effects of these chemicals using a general motor coordination task, the use of one other more targeted cognitive tests could provide a more sensitive result and give more information on specific cognitive deficits that would be translatable to the military aviator.

There were other physiological responses that were only observed with the CO and hypoxia combination. Arterial partial pressure of carbon dioxide ($p\text{CO}_2$) was significantly reduced during the CO and hypoxia combination exposure, which was not observed in the individual exposures or any other combination exposures. The effect of the CO and hypoxia combination exposure on $p\text{CO}_2$ corresponded to the reduction in hippocampal activity in the same animals, suggesting a correlation of the two effects that could be related to the established effect of a reduction in $p\text{CO}_2$ on cerebral blood flow, and is an intriguing target for future study that could provide a causal link between the combination exposure and the effect in the CNS.

The %COHb (percent carboxyhemoglobin, a measure of the amount of CO bound to hemoglobin in the blood that is inversely related to the oxygen carrying capacity of the blood) was approximately 15% higher, about 50% above pre-exposure baseline, in the rats exposed to the CO and hypoxia combination and TMS, CO, and hypoxia triple combination than it was in the group of rats exposed to CO alone. One possible explanation for these increases was the heightened minute volume of 25-35% above baseline levels that was present in these groups, in particular during the first half of the exposure, that was not present in the group only exposed to CO. These

increases could also be a contributing factor for the reduction in performance in the motor coordination task.

The combination exposure of all three chemicals produced a drop in blood pressure to approximately 50% below the pre-exposure baseline value, which was a significantly greater reduction than all other exposure groups. A sustained reduction in blood pressure, and no compensating increase in heart rate, would over time limit perfusion to the tissues and, along with the lower atmospheric oxygen and reduced oxygen carrying capacity, could severely limit oxygen delivery. This pathway would potentially explain why there was not much of a difference in accelerated performance between the TMS, CO, and hypoxia exposure and the other combinations at 90 minutes into the exposure, yet the persistence of the reduced oxygen delivery in the triple-exposure may have made it difficult for oxygen-starved tissues to perform in the second run at 110 minutes.

In summary, although there was no apparent synergy detected during this study, there were clear additive effects in both the motor coordination and physiology experiments in the rat model. Even though there is a gap in concentration levels between the MECs in the two-hour exposures performed during this study and real-world exposures present in military aviation, what are individually thought of as “safe” exposure levels might sum to elicit observable effects when present in combination, especially for CO and hypoxia. The results of the present study have identified this combination exposure as a potential target for further research. Additional experimentation would be necessary to specifically identify the minimum individual concentrations of CO and hypoxia needed to induce an effect in a combination exposure, and determine if there are potentially more sensitive effects on the hippocampus and cognitive function.

Background

Physiological Episodes

Physiological Episodes (PEs) remains a top priority for Navy, Marine Corps, and Air Force tactical aviation. PEs have been potentially attributed to, among other things, hypoxia and exposures to chemical contaminants (Cragg et al., 2017; Mumy, 2018). There is scant evidence that classic hypoxia is the cause of PEs, and chemical contaminants, although present, are mostly within occupational exposure guidelines / limits (Mumy, 2018). However, the potential additivity or synergy of the effects of simultaneous exposure to chemical contaminants and hypoxia has not been closely examined, particularly with respect to subtle central nervous system (CNS) effects that are relevant to the aviation environment. Although exposures to extreme hypoxia or high concentrations of contaminants may be necessary for readily observable toxicity, mild CNS impairments may interfere with the ability to perform a technically challenging task such as flying a high-performance aircraft. It is possible that hypoxia could exacerbate the CNS toxicity of chemical exposure by increasing ventilation, absorption of chemical via the lungs, and altering the distribution of the chemical to the brain. Likewise, chemical exposure could increase susceptibility to hypoxia by altering pulmonary function, directly increasing CNS impairment, or impacting normal cardiovascular responses to hypoxia.

PEs characterized by cognitive dysfunction are attributed to effects on the CNS (Cragg et al., 2017). Hypoxia in the brain can lead to cognitive impairment, which has been reported by pilots via symptoms that include impaired decision making, disorientation, and difficulty reading instruments. In addition, these symptoms occur contemporaneously with reports of numbness, tingling, euphoria, lightheadedness, and fatigue (Cragg et al., 2017). In particular, the hippocampal region of the brain is essential for navigation of spatial domains via cognitive maps (Eichenbaum, 2017) and it is susceptible to chemical exposure (Silvers et al., 2003) and hypoxia (Yamaoka et al., 1993). Subtle changes in hippocampal neuronal activity can be detected by electrophysiology methods *in vivo* (Fowler et al., 2003) that provide quantitative measurements including synaptic transmission efficiency, synaptic plasticity, and spontaneous activity.

The objective of this research was to understand the effects and mechanisms of combined inhalation of chemical contaminants and hypoxia in an *in vivo* rat model to better assess risk of the combined exposures as a potential contributor to PEs. Following a dedicated analysis and review of chemical compounds detected in and around Navy aircraft (Mumy, 2018), trimethylsilanol (TMS) and carbon monoxide (CO) were identified as top candidates for investigation as contributors to the occurrence of PEs.

Trimethylsilanol

Trimethylsilanol (TMS), a tertiary alcohol, includes a silicon-atom core bound to three methyl groups and one hydroxyl group (Organization for Economic Cooperation and Development, 2014). TMS, which has been previously detected in onboard breathing gases (Mumy, 2018), is a by-product of the degradation of aircrew flight equipment, life support systems equipment, and other materials containing silicone onboard aircraft (James, 2008).

PE symptoms indicate disruption of normal CNS functioning, and TMS is reported to cause CNS depression (James, 2008; Organization for Economic Cooperation and Development, 2014). The current value for the Spacecraft Maximum Allowable Concentration (SMAC), 15 ppm for a one-hour exposure, was established by the National Aeronautics and Space Administration (NASA) (James, 2008). However, there is elevated uncertainty associated with this exposure limit because it was based on studies that used oral and injectable administration of TMS (Dow Corning Corp., 1991). TMS has been measured in breathing air, therefore aircrews are vulnerable to the effects of TMS exposure via inhalation (Mumy, 2018). There is also potential that, even if an individual TMS exposure does not occur at a high enough concentration to induce an effect on the CNS, overlaying a hypoxia or other chemical exposure could be sufficient to cause PE-like symptoms if those exposures act additively or synergistically.

Data published by the Organization for Economic Cooperation and Development (OECD) demonstrated a concentration capable of causing death in 50% of study animals (Lethal Concentration 50%; LC₅₀) of 3151 ppm for inhaled TMS (Organization for Economic Cooperation and Development, 2014). Other experiments listed by the OECD investigated 14-day and 28-day inhalation exposures and observed that reduced activity, impaired balance, and pale lungs at TMS concentrations above 300 ppm were evident following the 14-day exposure. No adverse effects were observed for TMS concentrations at 0, 60, 300, or 600 ppm following a 28-

day exposure, although it is unclear in the reference if the same behavioral endpoints as the 14-day study were tested (Organization for Economic Co-Operation and Development, 2014). It is unclear what effect, if any, inhaled TMS has on blood oxygenation, either with or without concurrent hypoxic exposure.

Carbon Monoxide

Carbon monoxide (CO) has been detected in Navy jet aircraft at a maximum concentration of 11 ppm, a level that exceeds technical military standards for the breathing systems (Mumy, 2018). MIL-STD-3050 defines 10 ppm as the maximum allowable concentration in breathing gas (Department of Defense, 2015). However, the American Conference of Governmental Industrial Hygienists (ACGIH) suggest a Threshold Limit Value – Time-Weighted Average of 25 ppm CO exposure during a 40-hour week consisting of 8-hour workdays (ACGIH, 2023). NIOSH and OSHA recommend higher occupational exposure values of 35 ppm and 50 ppm, respectively (United States Department of Labor, 2023).

The lack of distinctive cues that occur during CO exposure make it particularly dangerous in short-term exposures. CO is not irritating, has no taste, and lacks odor and color as a gas (Raub & Benignus, 2002). Symptoms of CO exposure such as headache, dizziness, and confusion suggest a direct CNS effect but overlap broadly with any number of medical conditions. Acute Exposure Guideline Levels (AEGLs) of 83 ppm, 33 ppm, and 27 ppm have been recommended for single exposures of one hour, four hours, and eight hours to prevent irreversible or long-lasting health effects in the general population. However, AEGLs for concentrations of CO that can produce discomfort and other transient symptoms have not been developed. While not as concerning to the general population, these transient symptoms could be sufficient to cause impairment that would interfere with operation of high-performance aircraft. Based on existing information, CO levels of 11 ppm detected in T-45 aircraft were not expected to elicit CNS symptoms in a short-term exposure (Mumy, 2018). However, given the limited margin of safety of measured exposure levels relative to existing short-term limits for more severe effects, a combined CO, hypoxia, and/or TMS exposure could be sufficient to impair performance during a complicated task such as flying an aircraft.

CO binds to hemoglobin in the blood to form carboxyhemoglobin (COHb), thus reducing the oxygen carrying capacity of the blood and undermining tissue and/or organ level oxygenation (Benignus & Annau, 1994). In the face of reduced oxygen, cerebral autoregulation facilitates vasodilation of the local vascular supply to increase blood flow and oxygen delivery (Kogure et al., 1970). This auto-regulatory response corrects oxygen deficits to a point. When COHb levels reach ~25% in the blood (~220 ppm CO in the atmosphere), metabolism of oxygen decreases and CNS dysfunction is already occurring (Benignus 1994; Raub & Benignus, 2002). Behavioral alterations following CO exposure are evident as soon as blood COHb levels reach 20% (Raub & Benignus, 2002). In addition, exposure to higher concentrations of carbon monoxide may lead to reduced brain blood flow (Raub & Benignus, 2002), a scenario that may produce cognitive impairment (Joris, et al., 2018). ACGIH has defined a Biological Exposure Index, or the blood concentration of COHb below which all workers should not experience adverse health effects, of

3.5% (ACGIH, 2023). The reported LC₅₀ of CO in rats during whole-body exposure for 5 minutes is 10,151 ppm and 3,954 ppm for 60 minutes (E.I. du Pont de Nemours and Co., 1981). Reports suggest that blood COHb levels were between 50% and 80% in rats that died during LC₅₀ experiments (Committee on Acute Exposure Guideline Levels, 2010).

Hypoxia

Hypoxia is a lack of oxygen at the tissue or organ level (Samuel & Franklin, 2008). Hypoxemia – abnormal arterial oxygen tension below 80 mmHg – is a typical precursor to tissue hypoxia and it is measured in blood to identify onset of hypoxia (Samuel & Franklin, 2008). Previous research demonstrated that hypoxia has the potential to impair cognition; however, there is much debate on the impact of acute hypoxia due to methodological and population discrepancies (McMorris, et al., 2017). Acute normobaric hypoxia impairs neural activity in motor executive functioning and inhibitory processing, with no significant deficits in somatosensory processing (Nakata, et al., 2017). It is unknown if hypobaric hypoxia, a greater risk to aviators, elicits functionally specific decrements in neural activity. Chronic hypobaric hypoxia in rats has demonstrated sustained cognitive deficits as measured by increased latencies in the Morris Water Maze, a behavioral test of learning and memory, and disrupted neural functioning as measured by fMRI (Yuan, et al., 2019). While a chronic hypoxia study may not hold transitional validity as an accurate model of hypoxia experienced by aviators, acute hypobaric hypoxia studies in rats have also demonstrated significant cognitive deficits as measured by the Morris Water Maze (Zhang, et al., 2018). However, a problem with relating these rodent studies to real-time effects experienced by aviators is the delay between hypoxia exposures and testing in the study designs. The lack of real-time data on neurobehavioral and physiological outcomes during hypoxic conditions, and how they are impacted by co-exposures, are gaps in the rodent model literature that this study was designed to address.

Approach

The objective of this research project was to understand the mechanisms of combined inhalation of chemical contaminants TMS, CO, and hypoxia in a rat model to support better assessment of the risk of the combined exposures as a potential cause of or contributor to PEs. Experiments were designed to investigate neurological and motor impairment, indicated by locomotor deficits, to determine the level of real-time impairment during combined exposures to TMS, CO, and hypoxia to identify if combined exposures cause an additive or synergistic effect relative to individual exposures. Cardiovascular effects, respiratory changes, and atypical patterns of electrical activity in the hippocampus were investigated as potential mechanistic physiological causes of increased neurobehavioral deficits in the combination exposures.

The project was divided into separate experiments to first study the differences between individual and combined exposures on a motor coordination task and then investigate the differences in physiological changes between the individual and combined exposures in a rat model. The goal of the first experiment was to identify the minimum effective concentration (MEC) for each of CO and hypoxia exposures that produced an effect on a motor coordination neurobehavioral task. Using these results, and the results of an equivalent experiment performed with TMS exposure in

a prior study, the chemicals were then tested in combination to determine their effect on motor coordination performance and whether this effect was synergistic, additive, or antagonistic compared to a modeled prediction of the combination response based on the individual results. A subsequent experiment exposed separate groups of rats to the MECs of CO, TMS, and hypoxia identified during the neurobehavioral experiments to assess physiological changes in the respiratory, cardiovascular, and neurological systems that could be attributed to the reduction in performance. The exposures to TMS, CO, and hypoxia were performed individually and in combination to attempt to determine physiological explanations for additive or synergistic neurobehavioral decrements of combination exposures relative to individual exposures.

Combinations Analysis

Combinations analysis can be used to determine if the responses to simultaneous exposures to multiple chemicals are similar to, greater than, or less than would be expected from a prediction that is based on additivity of the responses during individual exposures. If the effect measured during the combination exposures was greater than the predicted effect, the combination exposure would be considered synergistic. If the opposite occurred, with the effect measured during the combination exposure being less than the prediction, the combination exposure would be antagonistic. An effect in the combination exposure that was not significantly different from the prediction would indicate that the combination exposure was additive. There are several different methods listed in the literature for determining the predicted combination responses from individual responses, however, two commonly used models are the Loewe Additivity model and Bliss Independence model (Foucquier and Guedj, 2015; Roell et al., 2017). The Loewe Additivity model predicts the combined responses using individual concentration-response curves that can be developed for each individual exposure. Rather than arithmetically adding the responses, it takes into account the shape of the concentration-response curve in determining the predicted combined response. The limitation of the Loewe Additivity model, however, is it assumes that the individual exposures have similar potency, meaning that the minimum and maximum asymptotes on the concentration-response curves (assuming a sigmoidal-shaped curve) approach similar values for each exposure (Foucquier and Guedj, 2015). Because of the reliance of the model on the concentration-response curve, a sufficient number of exposures need to be performed to properly build the curve out to near its minimum and maximum responses, requiring more testing than what was performed in the present study. The expected dissimilarity of the concentration response curves of hypoxia, TMS, and CO, in addition to the added concentrations necessary to fully characterize the concentration-response curve, resulted in the Loewe Additivity model not being chosen as the model used to predict the level of combined responses for the present study.

The Bliss Independence model differs from the Loewe Additivity model in that it assumes that the individual exposures are acting on independent targets and uses a probability-based calculation to predict a combined response of independent actors (Foucquier and Guedj, 2015; Roell, et al., 2017). If synergy is present, the effect will be greater than this predicted effect. If the individual exposures are acting independently in the combined exposure, then there would be an additive effect of the combination. The Bliss Independence model fits better with the present experimental design, because although the exposures are all ultimately targeting the CNS, the primary targets of

each individual exposure are different. TMS exposure is thought to directly depress neuronal activity in the CNS, while hypoxia impacts the CNS through a reduction in available oxygen. CO exposure causes a lack of availability of hemoglobin to bind oxygen for delivery to the tissues, ultimately impacting the CNS. Therefore, the Bliss Independence model was chosen as the model used to determine predictions of combined effects from the individual exposures in the combinations analysis performed during this study.

Methods

Husbandry

Adult male Sprague-Dawley (CD) rats (*Rattus norvegicus*) were obtained from Charles River Laboratories (Wilmington, MA) at approximately six weeks of age. Rats were acclimated to the USAF 711 HPW RHDV vivarium on Wright-Patterson Air Force Base for approximately seven days following arrival at the animal facility. Food and water were provided *ad libitum* while rats were housed in the vivarium prior to exposure. Animal rooms were maintained at a temperature and relative humidity in accordance with the recommendations of the National Research Council's Guide for the Care and Use of Laboratory Animals (2011), with approximately 15 complete air changes per hour, and a 12-hour/12-hour electronically controlled light/dark cycle. All animals were observed twice daily by vivarium staff for any signs of pain, distress, or other abnormalities.

Rats were weighed at the end of acclimation and again on the day of exposure. Following acclimation, rats were randomized into exposure groups. Rats were individually housed at the start of training for animals assigned to neurobehavioral tests (i.e., accelerod) or following surgical procedures necessary for the physiology experiments. On the day of exposure, the rats were transported to the testing room from the vivarium and then taken to necropsy immediately following completion of exposure.

All procedures were performed in accordance with animal protocol FWA-2019-0189-A approved by the Wright-Patterson Air Force Base Institutional Animal Care and Use Committee.

Accelerod Performance

Experimental Design

A total of 128 rats were exposed to CO, hypoxia, or TMS either individually or in combination for two hours inside of a whole-body inhalation exposure chamber. Two hours was chosen as the exposure period because it was comparable to the typical duration of a military tactical aircraft flight. Rats underwent a neurobehavioral test called an accelerod test to evaluate motor coordination at multiple points during the experiment. The accelerod test involved the rats walking on a rotating rod that increased in speed over the course of five minutes, and the length of time that the rat was able to stay on the rod was used as a metric of functioning motor coordination. The accelerod test was chosen over the more traditional rotarod test, during which the rat is tasked with walking at a constant speed on the rod, because the accelerod test has been shown to be more sensitive to neurotoxic effects (Bogo, et al., 1981).

Each test proceeded as described in Figure 1, with all activities occurring inside of the exposure chamber. Once the rats were loaded into the chamber, they received a 15-minute acclimation to

the chamber before undergoing a five-minute baseline accelerod test. Upon completion of the test, the rats were exposed for 90 minutes before being subjected to the first accelerod test while being exposed. A second accelerod test was performed approximately 20 minutes later at 110 minutes into the exposure. The exposure ended after 120 minutes at which point the rats were removed from the chamber. Rats were euthanized and necropsied within 30 minutes following the end of exposures.



Figure 1. Summary of timeline for accelerod performance exposures.

After a 15-minute acclimation period, a baseline accelerod test was performed five minutes prior to the start of exposure. Two additional accelerod tests were performed at 90 minutes and 110 minutes inside of the chamber while the exposure was ongoing.

Five total concentrations of CO from 250-750 ppm and five total oxygen concentrations from 10-21%, with normal room air being 21% oxygen, were tested to obtain concentration-response curves and determine the MEC for each type of exposure. Each chamber contained two caging units and two rotarod units. Two chambers were used simultaneously, therefore a total of four rats could be exposed during each run. One concentration of one type of exposure was tested during each run, with two total tests occurring each day as described in Table 1. The MEC of TMS, 750 ppm, was determined by way of prior experiments at NAMRU-D that were conducted using the same experimental design as the present study.

Table 1. Exposure schedule for individual accelero rod performance exposures.

Numbers of animals used in each exposure are represented in the table. Two sets of two-hour exposures occurred each day. Target concentrations are listed for each exposure.

CO Conc. (ppm)	Day 1				Day 2				Day 3				Day 4				Day 5				Day 6				n
	Hour				Hour				Hour				Hour				Hour				Hour				
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	
0	4		4																						8
250					4		4																		8
375									4		4														8
500													4		4										8
625																	4		4						8
750																					4		4		8
Hypoxia	Day 7				Day 8				Day 9				Day 10				Day 11				Day 12				n
	Hour				Hour				Hour				Hour				Hour				Hour				
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	
21%	4		4																						8
14%					4		4																		8
13%									4		4														8
12%													4		4										8
11%																	4		4						8
10%																					4		4		8
Note: Days were not consecutive.																						Animal Subtotal: 96			

Note: Days were not consecutive.

After the MECs were obtained for each type of exposure, CO, TMS, and hypoxia were tested using the MECs in different pairs of combinations, and in a combination of all three chemicals, to determine if additive, synergistic, or antagonistic responses were present compared to the individual exposures. The schedule of the different combinations tested is listed in Table 2. The same exposure chambers that were used to test the individual exposures were also used to test the combinations, so once again only four rats could be exposed during each run. One combination was tested during each run, with two total tests occurring each day.

Table 2. Schedule of combined exposures for accelerod performance experiment.

Animals were exposed to MECs of TMS, CO, and/or hypoxia during each combination exposure. Numbers of animals used in each exposure are represented in the table. Two sets of two-hour exposures occurred each day.

Combined MEC Exposures	Day 1 Hypoxia MEC				Day 2 Hypoxia MEC				Day 3 Hypoxia MEC				Day 4 Normoxia				n
	Hour				Hour				Hour				Hour				
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	
TMS MEC	4		4														8
CO MEC					4		4										8
TMS MEC+CO MEC									4		4		4		4		16
Note: Days were not consecutive.															Animal Subtotal: 32		

Chemicals and Test Atmosphere Generation

Carbon Monoxide (CO)

CO gas cylinders were purchased from Matheson Tri-Gas (Dayton, Ohio). One 1A, 175 cubic feet cylinder of 99.99% CO was obtained and used with a CGA350 stainless steel dual-stage regulator to calibrate the Nicolet Fourier-Transform Infrared Spectroscopes (Analytical Instruments) used for concentration measurements. In addition, one 190 cubic feet, 1% certified CO with the balance air was used with a CGA590 SS dual-stage regulator to generate the test atmospheres. CO gas was controlled with a mass flow controller (MFC), (Teledyne Hastings Instruments, Model HFC-202, Hampton, VA) and mixed with the clean, humidified supply air of the chamber to rapidly achieve the five target concentrations used to build the dose-response curve. CO concentrations were controlled by changing the CO delivery flow rate of the MFC.

Hypoxia

Clean, humidified air was mixed with nitrogen from an in-house nitrogen generator (Parker Balston, Model DB-10, Lancaster, NY) to rapidly achieve the five target oxygen concentrations used to build the concentration-response curve. Oxygen concentrations were adjusted by changing the nitrogen delivery flow rate with a MFC (Teledyne Hastings Instruments, Model HFC-202, Hampton, VA). Generation occurred inside a closed system with the exposure chamber in series with the generator.

Trimethylsilanol (TMS)

TMS vapor was generated by aerosolizing liquid TMS (CAS Number 1066-40-6, >97.5% purity, Sigma-Aldrich, St. Louis, MO) with a nebulizer (Spray Systems Co., Model SU11 with a fluid cap 2050 and air cap 67147, Wheaton, IL) to spray the TMS into the headspace of a custom-fabricated stainless-steel (SS) generation tube (SSGT) (See Figure 2). TMS was pumped into the nebulizer with a fluid metering (FMI) pump (Fluid Metering, Inc., Model QVG50-V06231AC83, Syosset, NY) and stroke rate controller (Fluid Metering, Inc., Model V300-C100597 and V300-C100595, Syosset, NY) where it was mixed with clean, compressed air and flowed out a common spray port into the SSGT as a diluted vapor. The TMS vapor flowed out of the SSGT as it vaporized at room

temperature and entered into the humidified, clean air supply of the exposure chamber, where it was diluted to rapidly reach the target TMS concentration. The concentrations of TMS were controlled by altering the FMI pump flow rates. The TMS generation system was contained in generation box fabricated from aluminum frame with slots for polycarbonate panels. An exhaust port was added to create a negative static pressure to prevent any potential leakage from escaping into the room.

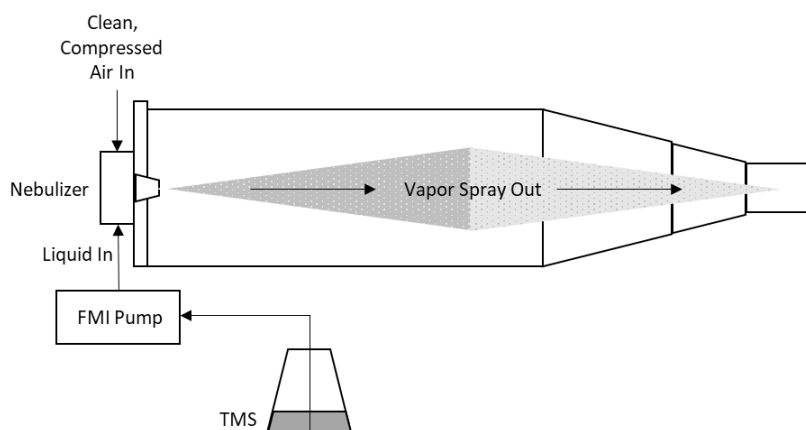


Figure 2. Diagram of TMS generation system.

Stainless-steel generation tube for TMS through which TMS vapor was generated from a nebulizer fed by compressed air and an FMI pump that controlled the flowrate of liquid TMS.

Combined Exposures

Following the determination of the MEC for each of the individual TMS, CO, and hypoxia exposures, combined exposures were generated by mixing MECs of TMS, CO, and/or hypoxia using the procedures described above for each individual test substance.

Exposure Chamber

Rats were exposed to the test atmospheres in a modified Toxic Hazards Research Unit (THRU) chamber with an internal volume of approximately 690 L (see Figure 5). The stainless-steel chamber had four sides, three of which originally consisted of glass windows, and a back wall that was all stainless steel. The front chamber door had the glass removed from the frame and replaced with a 3/8" polycarbonate panel. The polycarbonate panel was sealed with a gasket and secured with clamps. Additional gaskets were added to the inside edge of the chamber opening to create a leak-tight seal between the door and the chamber when clamped closed. Two four-inch holes were removed from the door and in-house, 3-D printed glove port brackets were installed. Butyl rubber glovebox gloves were placed in the glove port brackets and extended into the THRU chamber to create a tight seal. The gloves enabled safe animal handling by laboratory personnel to manipulate the rats (moving rats from cage to rotarod, then back) inside the chamber filled with the test atmospheres.

An aluminum grate floor (McNichols, Columbus, OH) was added to support the rotarod apparatus (Ez-Rod, Omnitech Electronics, Inc., Columbus, OH) and animal cages. The grate floor was selected to allow unimpeded flow of the test atmosphere from top to bottom of the THRU chamber. Each exposure chamber contained two conventional rodent cages and two rotarods. The rodent cages were wire-mesh cages, 7" tall by 8" wide and 16" long (Klubertanz Equipment Co., Inc., Model CO5E, Edgerton, WI), with a lid that opened on the top. A barrier made of stainless steel mesh (0.25" by 0.25" mesh) was added beginning near the front of the rotarod apparatus and covered from the bottom floor to near the top of the rotarod apparatus to prevent the rodents from traveling to the back of the chamber in the event they escaped during transport from the animal cage to the rotarod apparatus. Two modified THRU chambers were used to simultaneously to expose four rats (two rats per chamber) to the test substances during each run.

The individual rotarod apparatuses were customized so that the enclosure had perforations on the top and airflow being pulled through an outlet port near the bottom of the chamber to draw chemical vapors through the enclosure. A clean air flow was also administered to the compartment containing the electronic control mechanisms at the back of the unit to prevent excess buildup of volatile chemicals that could present a combustion hazard.

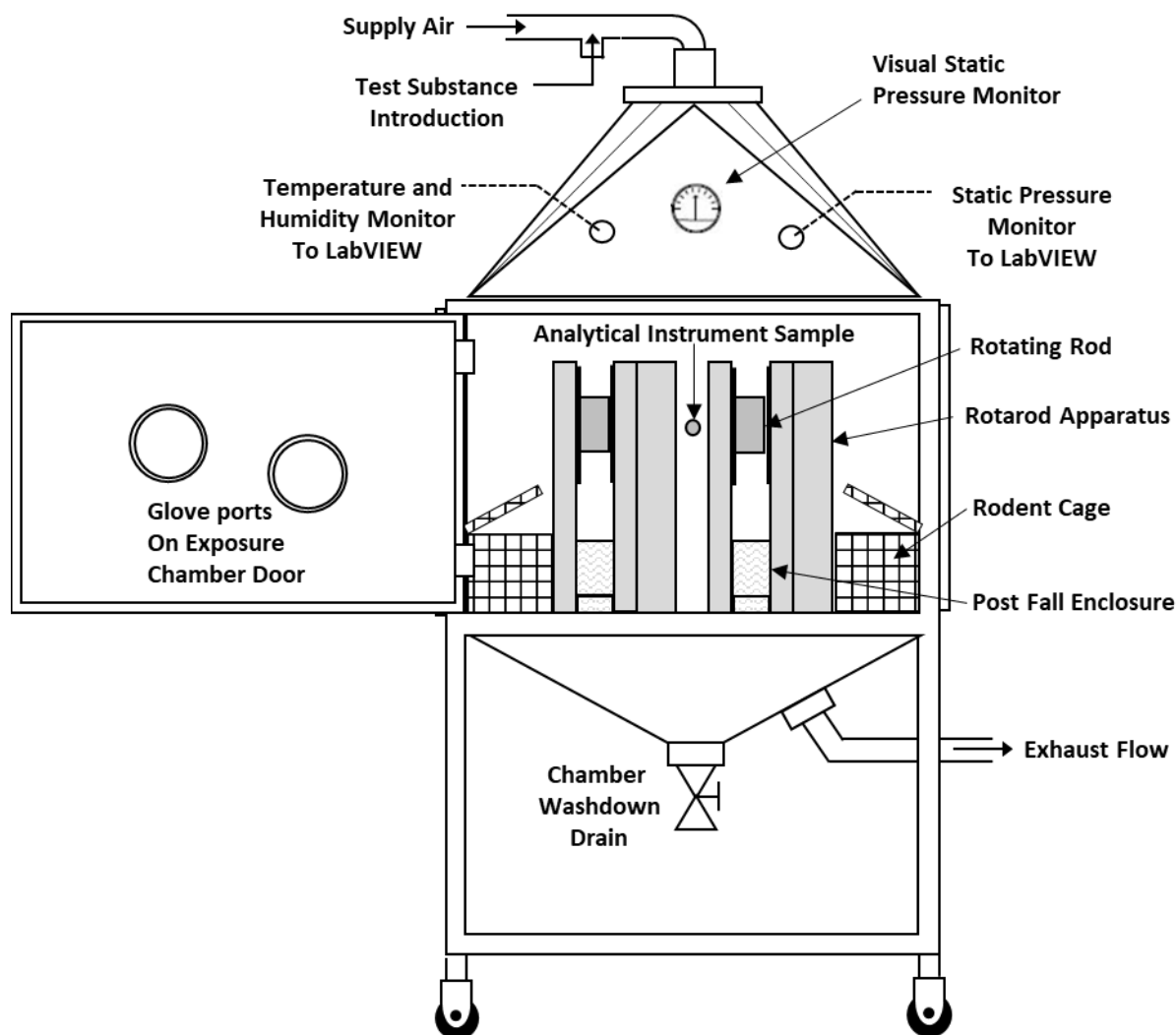


Figure 3. Diagram of exposure chamber for accelerated performance exposures.

Test Atmosphere Monitoring

All test atmosphere samples were taken near the center of the chamber from a 1/4" stainless steel tube that passed through the back of the chamber, between the two rotarod apparatuses (See Figure 3).

TMS

TMS concentrations inside of the chamber during the exposure were monitored using two Nicolet Fourier Transform Infrared Spectrometer (FTIR) instruments, (Thermo Fisher Scientific Inc., Model is10, Waltham, MA) tuned to a peak at 2996 cm⁻¹ based on their intra-assay precision and accuracy statistics. The FTIRs were characterized by analyzing a qualitative bag of test article in air. Gas bags (SKC Inc. SamplePro PVDF Sample Bag, SKC Inc., Eighty Four, PA) were used with a known mass/volume of material and a known volume of compressed air. The spectrum that

was produced gave prominent peaks at specific wavenumbers with absorbance units (AU) correlating to known TMS concentrations. The FTIRs were calibrated for a range of 22 to 2,180 ppm TMS. The calibration curves for each FTIR used (one for each chamber) were $\text{TMS Conc} = \text{FTIR AU} \times 1111, \pm 0.3 \text{ ppm}$ and $\text{TMS Conc} = \text{FTIR AU} \times 1096, \pm 0.3 \text{ ppm}$. The FTIR data were logged using a macro running in Omnic software (Thermo Scientific, Waltham, MA) that used the calibration formulas calculated for each of the peaks. The macro ran an approximately 20 second loop with one sample being collected during that time and the data logged to a text file. A new clean air background for the macro was collected at the start of each day, after the system had had approximately 45 minutes to equilibrate before any test chemical was introduced to any part of the system. The sample flow for each FTIR was generated by the house vacuum system and regulated by a ruby orifice (Bird Precision, Waltham, MA) downstream of the FTIR gas cell. A Magnehelic (Dwyer Instruments, Michigan City, IN) pressure gauge was attached just after the gas cell to monitor the pressure in the cell which was set to $-0.2'' \text{ H}_2\text{O}$ at all times. The cell pressure was regulated with a needle valve on the inlet side of the FTIR gas cell. The FTIR sample pressure was maintained at $-0.2'' \text{ H}_2\text{O}$ during calibrations and chamber sampling.

Carbon Monoxide

CO concentrations inside the chamber during the exposure were monitored using two FTIR instruments (Thermo Fisher Scientific Inc., Model is10, Waltham, MA) tuned to a peak at 2185 cm^{-1} based on their intra-assay precision and accuracy statistics. The FTIRs were characterized by analyzing a qualitative bag of test article in air. Gas bags (SKC Inc. SamplePro PVDF Sample Bag, SKC Inc., Eighty Four, PA) were used with a known mass/volume of material and a known volume of compressed air. The spectrum that was produced gave prominent peaks at specific wavenumbers with absorbance units (AU) correlating to known CO concentrations. The FTIRs were calibrated for a range of 0 to 500 ppm CO (only zero and 500 ppm points). The calibration curves for the two FTIRs each instrument were $\text{CO Conc} = \text{FTIR AU} \times 11,363, \pm 7 \text{ ppm}$ and $\text{CO Conc} = \text{FTIR AU} \times 12,382, \pm 3 \text{ ppm}$. FTIR data for CO were collected and logged during the exposure in the same manner as TMS as described in the preceding section.

Hypoxia

Oxygen concentrations inside of the chamber during the exposure were monitored using two oxygen analyzers (Horiba Instruments Inc., Model VA-3113, Irvine, CA) with a range of 0 to 25% oxygen. The instruments were zero calibrated with nitrogen and spanned on Room Air (20.9% oxygen).

Temperature and humidity

Chamber temperature, humidity, airflow rate and static pressure were monitored continuously and recorded during the exposure using sensors within the chamber or air-stream immediately entering the chamber (Model HF532WB6XD1XX, Model HC2-S, Rotronics Instruments, Inc., Hauppauge, NY) linked to LabView software. Chamber temperature and relative humidity was maintained, to the maximum extent possible, between 20 to 24°C (68 to 75°F) and 30 to 70% , respectively.

Data Collection with LabVIEW

Data collection and MFC flow set-point control was performed by LabVIEW software (National Instruments, Austin TX). The LabVIEW software ran on a custom-built, UPS-connected PC and interfaced with the data logging equipment via a USB-connected data acquisition (DAQ) cradle (National Instruments model cDAQ-9178, Austin TX) containing the necessary modules. One analog input module (National Instruments model NI 9207, Austin TX) per chamber allowed logging of data from the temperature/humidity and pressure sensors. The four MFCs interfaced with LabVIEW through a proprietary hub (Alicat Scientific, model BB9-232, Tucson, AZ) that connected to the computer via USB a connection. LabVIEW logged the data from the MFCs as well as set the target flow rates. The FTIR data were also captured and recorded in LabVIEW by reading from text files that were stored on the local lab network and written to in real time by each of the instruments.

Accelerod Performance Testing

Accelerod Test Description and Training Procedures

The accelerod test is a reliable and validated test used to evaluate motor coordination, endurance, and balance while the animal walks on a rotating cylinder (called a rotarod) that accelerates at a pre-determined constant rate for a pre-determined amount of time (Shiotsuki et al., 2010, Curzon et al., 2009). Motivation to stay on the seven centimeter (cm) diameter textured rod was provided by rod height (40 cm) and the animal's natural fear of falling. The rotarod apparatuses are self-contained enclosures that have lower drop areas, accessible via a lower door, to hold the animals following a fall and to prevent them from escaping during operation. Falls are automatically detected by photo-beam breaks in the post-fall enclosure at the bottom of the apparatus as well as visually observed and noted accordingly. Latency to fall is the primary dependent measure.

All animals were trained two or three times per day, 5-10 minutes per session, for three days to ensure adequate baseline performance on the accelerod test. The primary objectives of the training were to ensure that the animal can maintain balance on the rod and that the animal can maintain balance and walk/run on the rod while it accelerates to 20 revolutions per minute (RPM) over the course of 73 seconds, adapted from the methods of Ramirez, *et al.*, 2013. Training occurred inside the enclosed exposure chambers, with technicians transporting the animals between the cage units and rotarod apparatus using the glove ports in the same manner as would occur during the exposure.

On the first day of training, rats underwent an initial training session during which they were placed on the motionless rod and allowed to acclimate themselves to sitting on top of it for a few minutes. A second training session was performed a few minutes later, during which the rats were placed on the motionless rod, and once they were comfortably sitting on the rod, the rod began rotating at a slow speed of five RPM. After 1-2 minutes of learning to comfortably walk on the rotating rod, the rotation rate was increased to eight RPM. Subsequently, after another 1-2 minutes it was further increased to 11 RPM. During each training session, the rats were encouraged to face forward on the rod by tapping their tails if they turned around or keeping their attention forward by waving fingers in front of the rotarod apparatus. Running on the rod backward was more

difficult for the rats, especially at higher speeds, so part of the training was teaching them to stay in the forward position. If the rats fell, they were placed back on the rod by the technician immediately after falling. The rats were given a break of 20-30 minutes, then they underwent a final training session during which they were tasked with walking on the rod at 11 RPM for five minutes. Rats were placed back on the rod if they fell, but if a rat fell ten times before finishing the five-minute testing period, that rat's training was ended for the day.

On the second day of training, the rats were tasked with walking on the rod at 11 RPM for ten minutes. During the training session the rats were encouraged to face forward on the rod by tapping their tails if they turned around or keeping their attention forward by waving fingers in front of the rotarod apparatus. Rats were placed back on the rod if they fell, but if a rat fell ten times before finishing the ten-minute testing period, that rat's training session was ended. The rats were given a break of 20-30 minutes, then were given a second training session that acclimated them to acceleration from an initial speed of 4 RPM up to 20 RPM over a span of 73 seconds. Rats were only given one of these training sessions, even if they fell prior to reaching 20 RPM. The third day of training was a repeat of the second day of training. Total number of falls during constant speed training and latencies to fall during acceleration training were documented and recorded. At the end of the third day, 87.5% rats had demonstrated that they could reach 20 RPM during the acceleration training, with the remaining rats who did not reach 20 RPM having reached an average of 16 RPM. Rats that did not reach 20 RPM during the final acceleration testing were still included in the experiment because their baseline performances were still deemed sufficient for the experiment.

Accelerod Performance Testing During Exposure

During experimental data acquisition, accelerod testing was conducted as described in Figure 1. Rats were loaded into the wire-mesh cages inside of the chamber and the chamber door was then closed. The rats were allowed to acclimate to the chamber for 15 minutes before a five-minute baseline accelerod test was performed. Using the glove ports, the technicians removed each rat from the wire-mesh cage and placed them on the rod, which was slowly rotating at 4 RPM. After ensuring that the rat was comfortably walking on the rod, the acceleration began with the rod constantly accelerating at 0.12 RPM per second from 4 RPM to 40 RPM over five minutes. The latency to fall was recorded, and if the rat fell prior to the end of the five-minute period, he remained in the post-fall enclosure until the five-minute period had ended. At the end of five minutes, the technicians moved the rats back into the wire-mesh cages using the glove ports, and the exposure was started. After 90 minutes of exposure, another accelerod test was conducted in the same manner as the baseline accelerod test. A final test was conducted at 110 minutes into the exposure. Two tests were used to ensure that representative data collection occurred from all animals in the event an animal had an early fall during one of the tests. After the final test, technicians used the glove ports to return the rats to the wire-mesh cages until the chemical concentration had dropped to safe enough levels to open the chamber doors and remove the rats from the chambers. The rotarod apparatuses and wire mesh cages were cleaned between each run. All accelerod protocols were controlled via a laptop computer using Accurotor software (Omnitech Electronics, Inc., Columbus, OH). Data were stored on the computer for later export and analysis.

Physiology Analysis

Experimental Design

Using the MECs of TMS, CO, and hypoxia necessary to elicit neurobehavioral changes during the accelerated tests, animals were exposed in individual whole-body plethysmograph exposure chambers for two hours to one of the three types of exposures or their combinations. Prior to the exposures, rats were implanted with indwelling arterial catheters that were used to collect blood samples for analysis of chemical constituents as well as monitor blood pressure real-time during the exposure. Additionally, the rats were surgically implanted with electrophysiological probes in the hippocampus to monitor how hippocampal activity was altered by the exposures. Respiration was also monitored real-time during the exposure because the exposure chambers also served as plethysmographs that could monitor breathing through changes in pressure within the chamber.

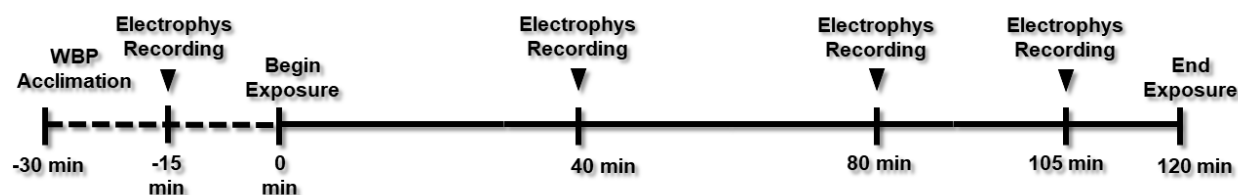


Figure 4. Summary of timeline for electrophysiology measurements.

Summary of the timeline for electrophysiology measurements taken while animals were being exposed in whole-body plethysmograph (WBP) chambers. Baseline electrophysiology recordings occurred 15 minutes prior to the start of exposure, and during exposure electrophysiology was measured at 40-min, 80-min, and 105-min time-points.

Each run proceeded as described in Figure 4 and Figure 5. Animals were acclimated to the chamber for 15 minutes before a ten-minute baseline period was conducted for pre-exposure measurements of electrophysiology, blood pressure, and respiration (Figure 4). At the end of this baseline period, a blood sample was drawn during the five minutes immediately preceding the start of exposure (Figure 5). Electrophysiology recordings were taken at 40, 80, and 105 minutes into the exposure, while respiration and blood pressure were monitored continuously throughout the exposure. The electrophysiology time points were chosen to allow for up to ten minutes of recording prior to the blood draws, which were taken at 90 and 115 minutes to match the approximately timing of the neurobehavioral testing in the previous experiments. The exposure ended at 120 minutes, at which point the rats were removed from the chambers. Rats were euthanized and necropsied within 30 minutes following the end of exposures.

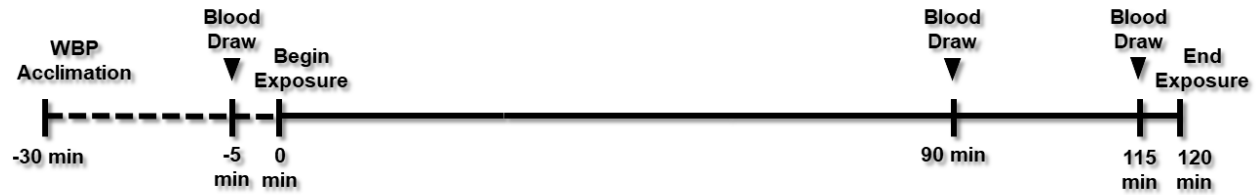


Figure 5. Summary of timeline for blood sampling during exposure.

Blood sampling was performed at three time points for each exposure while animals were inside of whole-body plethysmography chambers: 5-minutes prior to exposures beginning, 90 minutes during exposure, and 115 minutes during exposure.

Each individual exposure (CO, TMS, and Hypoxia) was tested along with a control group that received clean air at normoxic (21%) oxygen levels (Table 3). Additionally, all pairs of combinations (TMS+CO, TMS+Hypoxia, and CO+Hypoxia) in addition to a combination of all three exposures (TMS+CO+Hypoxia) were tested. Two sets of two-hour exposures were tested per day, with a maximum of three rats tested during each run. During each run, the exposure start times of each rat were staggered by ten minutes to prevent overlap of electrophysiology recording and blood draw time points between rats. This staggering allowed for a maximum of three rats to be tested without overlap of earlier time points for one rat with the later time points of another rat. Due to some lack of catheter patency and some electrophysiological recordings with poor quality data, additional rats had to be tested to obtain at least four rats per group with satisfactory cardiovascular, respiratory, and electrophysiology datasets. Additionally, ten animals were used for surgical method development prior to the start of experimentation.

Table 3. Exposure schedule for physiology analysis exposures.

Animals were exposed to MECs of TMS, CO, and/or hypoxia during each individual and combination exposure. Numbers of animals used in each exposure are represented in the table. Two sets of two-hour exposures occurred each day.

Control & MEC Exposures		Day 1					Day 2					Day 3					Day 4					n																
		Hour					Hour					Hour					Hour																					
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5																	
Normoxia	Control	1												1						1		3																
	CO				1							1										2																
	TMS+CO						1							1								2																
	TMS	1										1										2																
Hypoxia	Hypoxia				1							1										2																
	CO						1													1		2																
	TMS+CO									1							1					2																
	TMS									1							1					2																
Control & MEC Exposures		Day 5					Day 6					Day 7					Day 8					n																
		Hour					Hour					Hour					Hour																					
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5																	
Normoxia	Control						1										1			1		3																
	CO	1													1							2																
	TMS+CO				1										1							2																
	TMS	1								1										1		3																
Hypoxia	Hypoxia	1								1										1		3																
	CO				1												1					2																
	TMS+CO						1										1					2																
	TMS						1								1							2																
Control & MEC Exposures		Day 9																				Total n																
		Hour																																				
		1	2	3	4	5																																
Normoxia	Control																																					6
	CO	1				5																																
	TMS+CO					4																																
	TMS					5																																
Hypoxia	Hypoxia																																					5
	CO																																					4
	TMS+CO	1																																				5
	TMS	1																																				5
Practice & Training																																						10
Note: Days were not consecutive.										Overall Animal Total: 49 (39 Experimental, 10 Training)																												

Chemicals and Test Atmosphere Generation

Trimethylsilanol

The chemical vapor generation system was a vapor head-space type generator and consisted of three 5-gallon pressure vessels (Alloy Products Corp, Waukesha, WI) containing liquid TMS (CAS Number 1066-40-6, >97.5% purity, Sigma-Aldrich, St. Louis, MO) within ventilated hoods in the exposure lab. Three pressure vessels were necessary to have a large enough volume to keep concentrations stable at the required flow rates for the exposures. The vessels were connected to a delivery line that passed through each of the two exposure fume hoods in which it was utilized. The pressure vessels were pressurized to approximately 10 PSIG with filtered compressed air supplied through 1/4" LLDPE tubing (McMaster-Carr, Aurora, OH) from a common air line to keep the pressure even. They were also wrapped in heat tape (BriskHeat Corporation, model BS0051120, Columbus, OH) which was plugged into a variable voltage transformer (ISE Inc., model Variac TDGC2-2KM, Cleveland, OH). The heat tape and variable voltage transformer allowed the temperature of the vessels to be controlled within a target range of 75 – 80 °F (approximately 23.9 – 26.7 °C) as measured by a thermocouple probe attached to the exterior of the vessel and ensured similar vapor generation conditions in the vessels day-to-day. The combined headspace and compressed air mixture from each of the three pressure vessels then passed through 1/4" PTFE tubing (Cole-Parmer, Vernon Hills, IL) and was recombined at a cross before entering the delivery line through a 1/2" stainless steel needle valve (Hoke Inc., Spartanburg, SC). There was an identical needle valve opposite a tee that supplied the filtered compressed air dilution to the delivery line. The test chemical-laden exposure gas then passed through a three-way valve that allowed either that atmosphere or filtered compressed air to be selected to flow to the delivery line and into the exposure system.

A diagram of the exposure system is shown in Figure 6. The delivery line coming from the generator consisted of 3/8" 316L stainless steel tubing. There was a tee fitting on the delivery line in each of the exposure hoods that allowed the exposure gas to flow through a 1/4" PTFE tube to a high precision 150mm variable area flow meter (Cole-Parmer, Vernon Hills, IL) that was used to regulate test chemical gas flow to the exposure system in each hood. The exposure gas from the outlet of each variable area flow meter flowed through 1/4" PTFE tubing to a tee where it was diluted to the target concentration by clean compressed air supplied from another variable area flow meter before being delivered to the exposure chambers. A period of a few minutes was necessary for concentrations in the chambers to reach the target TMS concentration.

Carbon Monoxide

The CO gas supplied from the cylinder was mixed with clean air to a concentration of approximately 10% using variable area flow meters, then flowed through Teflon tubing to the exposure chambers. The CO gas was diluted with clean air to the target concentration, then delivered to the exposure chamber in each of the two fume hoods utilizing CO for each exposure. A period of a few minutes was necessary for concentrations in the chambers to reach target CO concentration. Both generation and exposure occurred inside of a fume hood to minimize potential for exposure to lab personnel. CO concentrations were monitored using a Horiba gas analyzer at the inlet of the exposure chambers (see Figure 6).

Hypoxia

Clean air from house compressed air was mixed with nitrogen gas from an in-house nitrogen generator (Parker Balston, Model DB-10, Lancaster, NY) to create the appropriate concentration of oxygen for exposure (MEC or 21%). The mixture was delivered to the exposure chambers. A period of a few minutes was necessary for concentrations in the chambers to reach target oxygen MEC. Both generation and exposure occurred inside of a fume hood to minimize potential for exposure to lab personnel. Oxygen concentrations were monitored using a Horiba gas analyzer at the inlet of the exposure chambers (see Figure 6).

Chemical Combinations

Combined exposures were generated by mixing TMS, CO, and/or hypoxia generated using the procedures described above. The MECs of TMS, CO and oxygen determined in the accelerated performance experiment were used as the exposure concentrations for each of the combined exposures. Generation of combined exposures also occurred inside a closed system with the exposure chambers in series via Teflon tubing. A period of a few minutes was necessary for concentrations in the chambers to reach target concentrations for chemicals and hypoxia. Combined exposure concentrations were monitored using an FTIR and a Horiba gas analyzer at the inlet of the exposure chambers (see Figure 6).

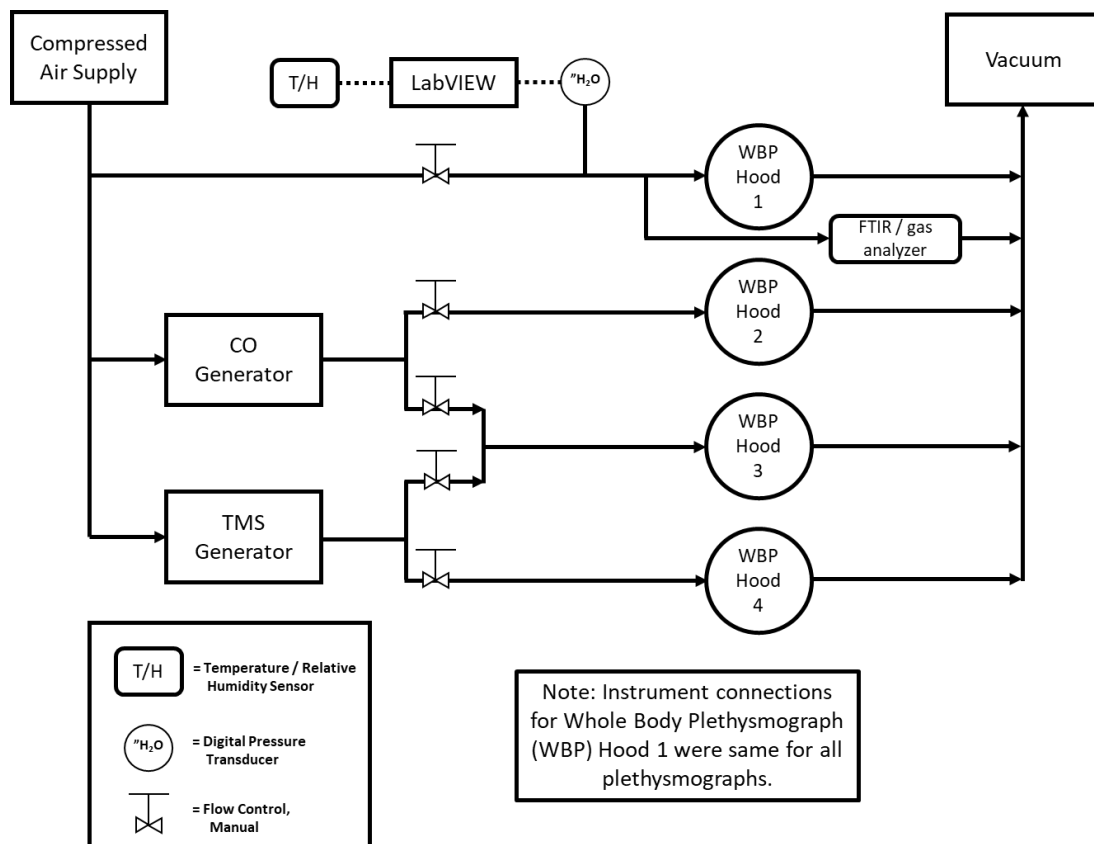


Figure 6. Diagram of exposure system for physiology analysis exposures.

Exposure Chamber

Rats were exposed to the test atmospheres in whole-body plethysmography chambers (WBP) (Buxco Respiratory Products, Data Sciences International, St. Paul, MN) which enabled real-time respiratory measurements during the exposures. The plethysmographs were cylindrical-shaped containers that gave the animal a space of approximately 20 cm in height and 18.5 cm in diameter. The “on” and “off” exposure gas flows to the exposure chambers in each hood were directed using a pair of 120 VAC 3-way solenoid valves (NoShok Inc., model S95A334R, Berea, OH) controlled by a switch. The rats were exposed for two hours, after which the exposure concluded by shutting off the chemical(s) and hypoxia exposure systems. The chamber was constantly monitored until less than 10% of the target chemical concentration remained and/or oxygen concentration returned to greater than or equal to 90% of the pre-exposure levels.

Test Atmosphere Monitoring

All test atmosphere samples were taken at the inlet to each chamber from a 1/4” Teflon tube (Figure 6).

Trimethylsilanol

TMS concentrations during the exposure were monitored using FTIRs fitted with short-path two-meter gas cells (Thermo Fisher Scientific Inc., Model is10, Waltham, MA). They were tuned to a peak at 2996 cm^{-1} based on their intra-assay precision and accuracy statistics. The FTIRs were characterized by analyzing a qualitative bag of test article in air. Gas bags (SKC Inc. SamplePro PVDF Sample Bag, SKC Inc., Eighty Four, PA) were used with a known mass/volume of material and a known volume of compressed air. The spectrum that was produced gave prominent peaks at specific wavenumbers with absorbance units (AU) correlating to known TMS concentrations. TMS was calibrated through a range of 407 to $13,845\text{ mg/m}^3$ (131 to 4,445 ppmv at $20\text{ }^{\circ}\text{C}$). The wavenumber for TMS = 2996.7 cm^{-1} was chosen based on intra-assay precision and accuracy statistics.

The FTIR data was logged using a macro running in Omnic software (Thermo Scientific, Waltham, MA) that used the calibration formulas calculated for each of the peaks. The macro ran an approximately 20 second loop with one sample being collected during that time period and the data logged to a text file. A new clean air background value for the macro was collected at the start of each day, after the system had had approximately 45 minutes to equilibrate, and before any test chemical was introduced to any part of the system. The sample flow for each FTIR was generated by the house vacuum system and regulated by a ruby orifice (Bird Precision, Waltham, MA) downstream of the FTIR gas cell. A Magnehelic (Dwyer instruments, Michigan City, IN) was attached just after the gas cell to monitor the pressure in the cell which was set to $-0.2''\text{ H}_2\text{O}$ at all times. The cell pressure was regulated with a needle valve on the inlet side of the FTIR gas cell. The FTIR sample pressure was maintained at $-0.2''\text{ H}_2\text{O}$ during calibrations and chamber sampling.

Carbon Monoxide

CO concentrations during the exposure were monitored using gas analyzers (Horiba Instruments Inc., Model VA-3113, Irvine, CA) with a range of 0 to 1000 ppm by volume. The instruments

were zero calibrated with nitrogen. The gas analyzers were characterized by analyzing a qualitative bag of test article in air. Gas bags (SKC Inc. SamplePro PVDF Sample Bag, SKC Inc., Eighty Four, PA) were used with a known mass/volume of material and a known volume of compressed air. The Horiba instruments were calibrated internally with 1000 ppm CO. This was created with 10,000 ppm CO stock cylinder and a 10x dilution with 99.99% nitrogen gas with a steady sample flow to the instrument. Target flows were set by BIOS (DryCal BIOS Defender 530+, Mesa Labs, Butler, NJ) and compressed air, with the CO flow using a 1.017 correction factor (0.9670 relative gas density, with air = 1). Precision of this setup gave 3% error (1% BIOS, 2% rotameter), or ± 30 ppm CO.

Hypoxia

Oxygen concentrations during the exposures were monitored at the inlets to the chambers using oxygen analyzers (Horiba Instruments Inc., Model VA-3113, Irvine, CA) with a range of 0 to 25% oxygen. The instruments were zero calibrated with nitrogen and spanned on room air (20.9% oxygen).

Temperature and humidity

Chamber temperature, humidity and static pressure were monitored continuously and recorded during the exposure using sensors linked to LabView software. The temperature and relative humidity were measured by an electronic probe (Model HF532WB6XD1XX, Model HC2-S, Rotronics Instruments, Inc., Hauppauge, NY) located in the top, front of each exposure fume hood. The temperature in the fume hoods was maintained between 68 and 79 °F with a target of 72 °F, and the relative humidity was maintained between 30 and 70% with a target of 50%. Airflow in each WBP was maintained at a target of approximately 14 air changes per hour, within a range of 12 to 15 air changes per hour. This corresponded to a target flow rate of 1.3 L/min in the chambers. The airflows were monitored by using calibrated ball heights on high precision variable area flow meters. The flows were set before exposures and checked with a BIOS (DryCal BIOS Defender 530+, Mesa Labs, Butler, NJ) primary flow standard.

Data Collection with LabVIEW

Data collection was performed by LabVIEW software (National Instruments, Austin TX). The LabVIEW software ran on a custom-built, UPS-connected PC and interfaced with the data logging equipment via a USB-connected DAQ cradle (National Instruments model cDAQ-9178, Austin TX) containing the necessary modules. One analog input (AI) module (National Instruments model NI 9207, Austin TX) logged data from the temperature/humidity and pressure sensors. The FTIR data were also captured and recorded in LabVIEW by reading from text files that were stored on the local lab network and written to in real time by each of the instruments. The TMS test chemical concentrations were reported by the FTIRs in mg/m³ then converted to ppmv by LabVIEW before being recorded. The CO and oxygen data from the Horiba instruments was transmitted to LabVIEW via RS232 cables that plugged into a hub that interfaced with the LabVIEW computer via USB.

Surgeries for Intra-arterial Catheter and Electrode Implantation

Surgical Methods

Arterial catheters were implanted to allow for real-time arterial blood sampling and blood pressure measurements during exposure. Catheter implantation surgery was performed using published methods (Feng, et al., 2015), briefly described here. Rats were anesthetized using isoflurane (5% at induction) delivered inside an induction chamber. Fur was removed on the ventral side of the animal overlying the throat and on both sides of the midline, and on the dorsal side of the animal from the scapular region, with electric clippers. An analgesic agent, buprenorphine, and the antibiotic gentamicin were administered subcutaneously immediately prior to surgery. Exposed skin was disinfected with betadine and sterile ophthalmic ointment was applied to the surface of the cornea to prevent drying of the eyes during anesthesia.

Anesthesia was maintained with a nose cone for continuous delivery of isoflurane (1.5-2%), and the animal was kept warm via a heating pad. An incision no greater than 2.5 cm was made on the left side of the midline, with care to avoid severing significant subcutaneous arteries and minimize bleeding. Blunt dissection using small scissors was used to separate the skin from the underlying tissue and fascia to locate the left common carotid artery. A bulldog clamp was used to temporarily arrest blood flow just below the caudal end of the artery incision area. Microscissors were then used to create a small incision that did not transect the artery, and the carotid artery was cannulated with a polyurethane catheter (Instech Laboratories, PA, USA), anchored to the surrounding fascia with sutures, and then filled with lock solution (500 U/mL heparinized saline/glycerol).

The external portion of the carotid artery catheter was tunneled subcutaneously to the scapular region of the rat, exteriorized through a puncture in the skin, secured and protected by threading it through a sterile button that was placed subcutaneously with an exteriorized chimney protruding from the skin that contained the catheter. All incisions were closed using sutures. A 1 mL syringe was used to flush the catheter with 0.15 mL heparinized glycerol. The exteriorized portion of the arterial catheter was sealed with a sterile stainless-steel plug.

A subset of rats also underwent surgical implantation of a neural probe for *in vivo* electrophysiology recording following completion of catheter insertion. Each rat was placed in a motorized stereotaxic apparatus with atlas integration (Neurostar, Tübingen, Germany). A midline incision was made along the dorsal surface of the skull. The exposed skull was cleaned of periosteal tissue and an anchor screw was positioned in the lower right quadrant. The drill (used to penetrate the skull) and electrode were calibrated and positioned as outlined in apparatus manual. A 16-channel electrode array probe (Buzsaki16-CM16LP, Neuronexus, Ann Arbor MI) was stereotactically implanted in the CA1 region of the hippocampus (-5.04 mm AP, -3.40 mm ML, -3.5 mm DV). The probe was secured using Fuji glass ionomer and dental cement. Sutures were added to incision in front and behind the probe.

Post-operative care and catheter maintenance

Animals were monitored daily for body weight recovery and behavioral indications of pain or distress. Animals were allowed seven days to recover before testing or exposure. Catheters were

flushed with heparin lock solution (500 U/ml heparinized saline/glycerol) at a minimum two times per week to ensure catheter patency.

Plethysmography

Changes in respiratory function were measured using a whole-body plethysmography system (Buxco Respiratory Products, Data Sciences International, St. Paul, MN) that recorded respiratory parameters continuously in real-time during the exposure. The whole-body plethysmograph is designed to measure changes in pressure and flow in/out of the chamber that correlate to changes in volume and frequency of the respiration of the animal. The respiratory rate, tidal volume, and minute volume were calculated from the waveform, recorded, and averaged in a two-second moving interval by Finepointe software (Buxco Respiratory Products, Data Sciences International, St. Paul, MN). Following a 15-minute acclimation period in the whole-body chambers, respiration was measured at baseline for 8-10 minutes to determine the levels of pre-exposure respiratory parameters. Respiratory data were averaged for the baseline period, as well as every 15 minutes during the exposure. Artifacts in respiratory parameters that resulted from movement of the rats were removed from the dataset prior to averaging.

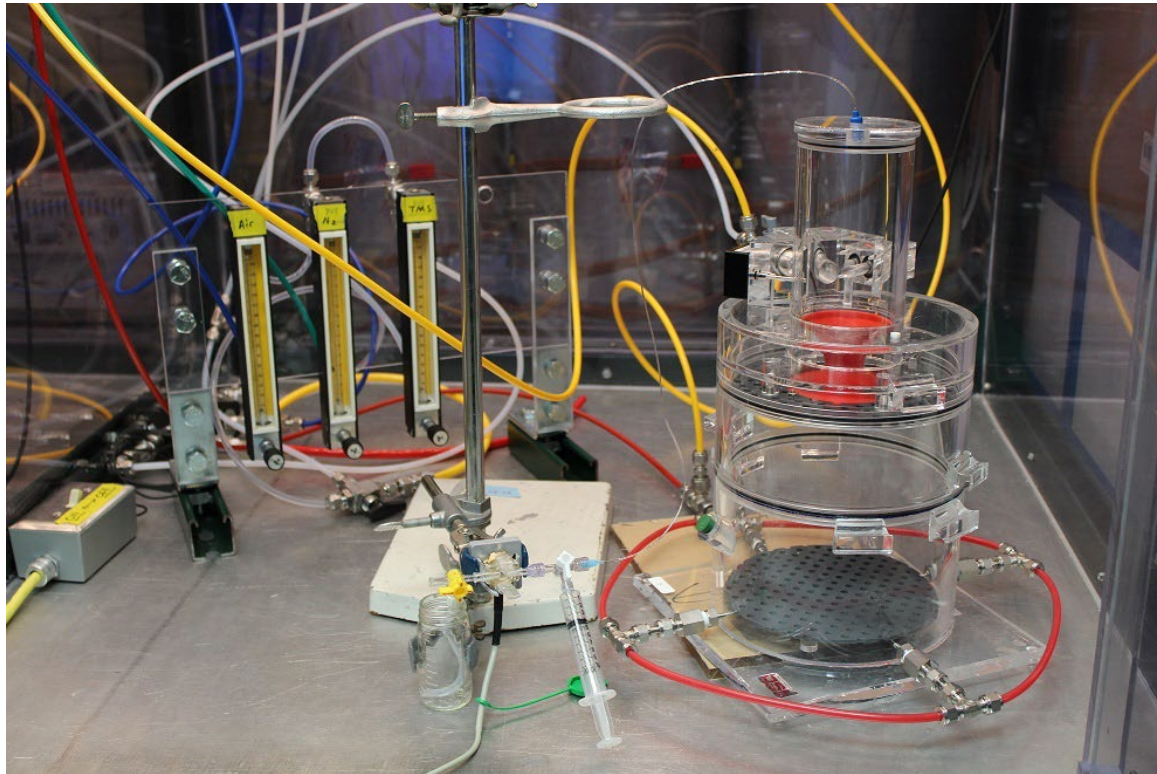


Figure 7. Image of one exposure setup used during physiology analysis exposures.

Image of exposure setup including whole-body plethysmograph with exteriorized blood pressure and blood sampling line, pressure transducer, and heparinized saline-filled syringe that was used as a reservoir.

Blood Pressure and Heart Rate Measurements

During the exposure, the exterior end of the catheters was attached to polyurethane tubing 0.025 x 0.055" in diameter and approximately 10" in length that was filled with heparinized saline. The tubing was protected inside of the whole-body plethysmograph using a metal tether (SAI Infusion Technologies, Lake Villa, IL) through which the tubing was threaded. The tubing was exteriorized from the top of the whole-body plethysmograph through a 22-gauge single-channel port with a swivel attached to the interior side of the pass-through. The swivel, along with the tether, prevented the tubing from tangling inside of the whole-body plethysmograph and allowed the rat to be freely moving and conscious during all measurements. The tubing outside of the plethysmograph was connected to a blunt needle that was attached to a three-way stopcock. The three-way stopcock opened to either a heparinized saline reservoir in a 10 mL syringe or a pressure transducer (Physiological Pressure Transducer, ADInstruments, Inc., Colorado Springs, CO) for continuous monitoring of blood pressure in the carotid artery prior to and during the exposure. The pressure transducer connected to an Octal Bridge amplifier (PowerLab 8/35, ADInstruments, Inc., Colorado Springs, CO), and data were recorded in LabChart Pro software (ADInstruments, Inc., Colorado Springs, CO).

After the rat was allowed to acclimate to the exposure chamber for 15 minutes, baseline blood pressure measurements were taken for 8-10 minutes to determine pre-exposure blood pressure. Blood pressure was recorded as a one-second moving average of mean arterial blood pressure, which was calculated from the blood pressure waveforms by LabChart Pro software. Heart rate was determined from the blood pressure waveforms in the same manner. Both the heart rate and blood pressure data were averaged for the baseline period, as well as every 15 minutes during the exposure. Data was removed for timepoints that were recorded during blood draws because the three-way valve was turned off to the pressure transducer during those timepoints.

Blood Sampling During Exposure

Indwelling catheters were flushed with 0.08 mL sterile saline, approximately twice the dead volume of the catheter, immediately prior to exposure. Catheters were attached tubing inside of the whole-body plethysmograph chambers that was exteriorized through a port in the top of the chamber as described in the previous section and shown in Figure 7, allowing the plethysmograph chambers to remain a closed system and maintain the sterility of the catheter line for blood sampling and blood pressure measurements.

Blood samples were taken within five minutes prior to the start of exposures, at 90 minutes into the exposure, and at 115 minutes into the exposure. The tubing attached to the three-way stopcock was removed after clamping it approximately one inch from the stopcock. A 1.0 mL sterile syringe with a blunt needle was inserted into the tubing and the clamp was then released. The syringe was used to remove all saline from the tubing until the tubing only contained blood. That syringe was discarded, and another sterile 1.0 mL syringe was attached to collect approximately 0.5 mL blood. The blood sample was transferred from the syringe into a 0.8 mL lithium-heparin coated blood collection tube (Grenier Bio-one, Monroe, North Carolina). After each blood withdrawal, tubing was flushed with sterile heparinized saline to prevent clotting during the exposures. Blood samples

were analyzed in a GEM 4000 Premier (Werfen - Instrumentation Laboratory, Bedford, MA) for pO₂, pCO₂, pH, %COHb, and %O₂Hb. Two replicates were run for each sample, and the results of the replicates were averaged to give one value for each parameter at each timepoint per rat.

Electrophysiology Measurements

For *in vivo* electrophysiology experiments used to evaluate changes in neurological function resulting from the individual and combined exposures, rats underwent surgery to implant *in vivo* microelectrodes into the hippocampus region of the brain. Rats were given seven days of post-surgical recovery prior to baseline *in vivo* electrophysiology recording. All electrophysiology data were recorded using the Multi Channel Systems Wireless2100 (ALA Scientific Instruments Inc., Farmingdale NY). The implanted 16-channel electrode array was connected via a wireless headstage (W2100) in which two of the 16 channels were dedicated stimulation channels, used to deliver currents to generate evoked responses (channel #2 and channel #16). On the day of exposure, immediately prior to loading rats into the whole-body plethysmographs, rats were induced with light isoflurane anesthesia to briefly immobilize them to allow for attachment of the headstage to the implanted probe. Two to three headstages (2-3 rats) were recorded at a time, at a sampling frequency of 5 KHz per channel. Rats were then placed in the exposure chambers and awakened almost immediately. After rats had been acclimated to the whole-body plethysmographs for 15 minutes, baseline spontaneous recordings with intermittent evoked potentials were collected for approximately 8-10 minutes in the conscious rat. The wireless W2100 system (Multi Channel System distributed by ALA Scientific, Farmingdale, New York) was used to obtain *in vivo* electrophysiology recordings while the rat was inside of the exposure chamber. Concurrent with chemical and hypoxia exposures, *in vivo* electrophysiology measurements including spontaneous recordings with intermittent evoked potentials were made at approximately 40 minutes, 80 minutes, and 105 minutes into the exposures. All recordings took between 8-10 minutes and the 80- and 105-minute recordings were completed immediately prior to blood withdrawals.

Input/output (I/O) function and paired-pulse facilitation (PPF) were derived from the electrophysiology recordings. PPF is form of short term synaptic plasticity and thought to result from accumulation of residual calcium from the first stimulus that had not been pumped out completely by the time the second stimulus is delivered, hence more neurotransmitter containing vesicles are released inducing a facilitated postsynaptic response (Wu and Saggau, 1994). Enhancement of PPF has been commonly correlated with reduced probability of neurotransmitter release and conversely, a reduction in PPF can be correlated with greater probability of neurotransmitter release (Debanne et al., 1996, Capogna, 1998).

Evoked responses were elicited by delivering a current of 10-100 μ A and I/O curves were generated by calculating the amplitude of the evoked response (output) as a function of stimulation (input) intensity. PPF was elicited by delivering two consecutive stimuli of 50 μ A, at 40 milliseconds apart. Degree of PPF was quantitated as the ratio (PPF Ratio) of the amplitude of the evoked response amplitude from the second stimulation to that from the first stimulation.

Analyses of electrophysiology data were performed using Multi Channel Systems Experimenter and Analyzer software. Igor Pro v.7 from WaveMetrics, Microsoft Excel and SigmaPlot v.13 were

used to plot analyzed data and for statistical assessments. For the input-output (I/O), and PPF analyses, amplitudes of the negative deflection of the population spikes were calculated to quantitate the size of evoked response. Amplitude calculations were performed using Igor Pro software. Data were represented as means with the standard error of the mean (SEM) and were statistically compared using either a one-way (PPF) or two-way (I/O) analysis of variance (ANOVA). A calculated *p*-value of less than 0.05 was considered significantly different. Post-hoc multiple comparison using the Holm-Sidak method was used to compare multiple experimental groups. All quantitation and statistical analysis as well as graphs were generated using Microsoft Excel and SigmaPlot v.13.

Necropsy

Following the conclusion of experimental exposures, rats were euthanized by pneumothorax under deep anesthesia, after intraperitoneal injection of 150 mg/kg Euthasol (pentobarbital sodium and phenytoin sodium). Blood was drawn via the inferior vena cava using a 22 gauge, 1/2" needle. This method of blood draw minimized the escape of volatile TMS chemical into the atmosphere from the blood when drawn from the vena cava, maximizing the accuracy of blood gas sampling.

Blood collection and Blood TMS Analysis

A total of 3-5 mL whole blood was taken post exposure from the inferior vena cava using a 10 mL syringe, then placed into 10 mL screw cap vials (Gerstel Inc., Linthicum Heights, MD) with 0.1 mL of 1000 USP units per mL of sodium heparin added for anticoagulation. Blood that was used for calibrations and blanks was taken from rats that were not exposed to the TMS.

Samples were prepared by weighing out approximately 0.2 g of sodium chloride (NaCl) (Fisher Scientific) and adding it into 10 mL screw cap vials (Gerstel Inc., Linthicum Heights, MD). Deionized water and blood were added to the vials totaling 1 mL, not to exceed more than 0.5 mL of blood. A known amount of internal standard and surrogate (Restek Corporation, Bellefonte, PA) were added and the vial was capped. The procedure up to this point was done as quickly as possible to eliminate loss of volatile compounds.

The analysis of TMS was performed by gas chromatography (GC) (Agilent 7890, Agilent Technologies, Wilmington, DE) with a mass selective detector (MS) (5975C, Agilent Technologies, Wilmington, DE) with introduction of the sample using solid phase microextraction (SPME) on a Multi Purpose Sampler (MPS) (Gerstel Inc., Linthicum Heights, MD)

The analytical conditions were as follows: The MPS incubated the sample at 70°C for 5 minutes with the agitator switching on and off as follows: on for 10 seconds then off for 1 second at a speed of 250 RPM. After the incubation was completed, the SPME fiber (Restek PDMS SPME fiber 30 µm fiber thickness, Restek Corp., Bellefonte, PA) moved to the vial and with a 21 mm vial penetration, extracted the sample for 5 minutes. The sample was then desorbed onto the GC column for 1 minute with an injection penetration of 54 mm. The SPME fiber had a 5 minute pre bake out and a 2 minute post bake out. GC conditions used helium as the carrier gas on an Rxi-5ms 60m × 0.32mm ID × 0.50 µm column (Restek Corp., Bellefonte, PA) with a pressure of 11.576 psi and initial flow of 2 mL/min. The initial oven temperature was 35°C with an initial hold time

of 0 minutes. The oven temperature program was as follows: 1°C/minute to 47°C, 20°C to 100°C followed by 30°C to 320°C. The run time was 21.983 minutes. The compounds were identified on the 5975C MS by single ion mode (SIM) by looking for three ions for each compound during specific times.

TMS reference standard was purchased from Fisher Chemical (Fisher Scientific). TMS was made to a concentration of 8.14 mg/dL in methanol. Dilutions were made from this standard at different concentrations for the calibration curve.

A calibration curve was generated and verified every 24 hours with a continuing calibration verification (CCV). To measure the extraction effectiveness, as well as to see matrix effects, each sample and quality control (QC) standard was spiked with a known amount of internal standard (IS) and surrogate. The internal standard used for TMS analysis was bromochloromethane from the 624 Internal Standard Mix (Restek) and the surrogates were pentafluorobenzene and fluorobenzene from the 624 Surrogate Standard (Restek). The calibration curve met 20% relative standard deviation or R² of >0.98 with five points minimum. The calibration curve for TMS ranged from 0.2442 mg/dL to 16.28 mg/dL. Each batch included a CCV (no blood), a laboratory control standard (LCS) and a method blank (MB). The LCS and MB had blood to account for any matrix effects, where the CCV did not. The CCV, LCS and surrogates were considered passing if they yielded 70-130% recovery. The IS was passing if it yielded 50-200% of the IS of the batch's CCV.

Data Analysis

Unless otherwise noted, all accelerometer performance and physiology data were compared at pre-exposure baseline, 90, and 110/115 minute timepoints during exposure using a within-subjects repeated measures analysis using the General Linear Model on Statistical Package for the Social Sciences (SPSS) software (International Business Machines, Armonk, NY). A Greenhouse-Geisser correction was performed for any tests not meeting sphericity assumption, and a Bonferroni correction was applied for multiple comparisons for each time point. Repeated measures ANOVA was also used to compare average changes from baseline between control groups, combination exposures, and the individual exposures that were used in the combination exposure for each dataset to determine if the responses of the combination exposures differed from control or individual exposure responses. Levene's test of homogeneity and the Shapiro-Wilk test for normality were used to determine if datasets met ANOVA assumptions. Concentration-response curves for accelerometer performance tests for CO and hypoxia were created using linear regression analysis. A p-value of $\alpha=0.05$ was used as a threshold for significance for all tests.

Combinations effects during the accelerometer performance experiment were analyzed for synergy/additivity/antagonism using the Bliss Independence model to predict combined responses from the individual exposures. Average percentage change in latency to fall was treated as a probability with values between zero and one, with zero representing no change (or positive change) in latency and one representing a 100% or greater decrease in latency. The equation $E_A + E_B - E_A * E_B$ was then used to predict the expected response for the combinations, with E_A representing the percent change in latency of one individual exposure and E_B representing the percent change in latency of the other exposure. The calculated value was then compared to the measured change

in latency of the combination using a one-sample t-test. If the measured value was significantly greater than the predicted value, it was determined that there was a synergistic effect, and if the measured value was significantly lower than the predicted value, it was determined that there was an antagonistic effect. If there was no difference between the measured and predicted values, then it was determined that the effect was additive.

Results

Accelerod Performance – CO and Hypoxia Test Atmosphere Characterization

The mean +/- standard deviation of the CO concentrations and environmental parameters of each of the eight exposures per target concentration are given in Table 4. The mean measured CO concentrations at each exposure level were within 10% of their target values, with standard deviations of no more than 5% of the mean. All mean values of environmental parameters, including chamber airflow, temperature, relative humidity, and static pressure, were within expected ranges and were acceptably consistent run-to-run within each exposure group. Exposure and environmental data for each of the individual CO exposure runs are presented in Appendix A1.

Table 4. Mean exposure concentration and environmental data at each exposure level for CO exposures.

Data presented as mean $\pm$ standard deviation of the four exposures (two animals per exposure) at each target CO concentration.

Target Conc. (ppm)	Measured Conc. (ppm)	Air Flow L/min	Temperature °C	RH %	Static Pressure Inches H ₂ O
0	4 $\pm$ 5	139 $\pm$ 1	24.8 $\pm$ 0.5	56.7 $\pm$ 3.0	-0.06 $\pm$ 0.01
250	259 $\pm$ 6	138 $\pm$ 1	24.7 $\pm$ 0.4	59.7 $\pm$ 3.3	-0.06 $\pm$ 0.01
375	383 $\pm$ 10	141 $\pm$ 3	22.7 $\pm$ 0.8	46.7 $\pm$ 3.0	-0.08 $\pm$ 0.01
500	513 $\pm$ 2	139 $\pm$ 1	24.2 $\pm$ 0.5	51.6 $\pm$ 2.5	-0.06 $\pm$ 0.01
625	651 $\pm$ 18	141 $\pm$ 2	22.7 $\pm$ 0.7	43.7 $\pm$ 3.2	-0.06 $\pm$ 0.04
750	790 $\pm$ 10	143 $\pm$ 3	22.4 $\pm$ 0.8	50.9 $\pm$ 3.0	-0.06 $\pm$ 0.02

The mean +/- standard deviation of the oxygen concentrations and environmental parameters of each of the eight exposures per target concentration are given in Table 5. The mean measured oxygen concentrations at each hypoxia exposure level were within 10% of their target values, with standard deviations of no more than 5% of the mean. Mean values of the environmental parameters of temperature and static pressure within expected ranges and were acceptably consistent run-to-run within each exposure group. The flow of air decreased with lower levels of oxygen, while a compensatory increase in nitrogen flow was used to generate lower oxygen concentrations while maintaining a comparable average total flow across between exposure groups. The relative humidity decreased with higher levels of nitrogen flow, likely due to the greater fraction of dry nitrogen in the chamber relative to the humidified air. Relative humidity values at the lowest two target concentrations were just below the minimum expected chamber humidity of 30% and not expected to cause any adverse effects on the animals during a two-hour exposure. Exposure and

environmental data for each of the individual hypoxia exposure runs are presented in Appendix A2.

Table 5. Average exposure concentration and environmental data at each level for hypoxia exposures.

Data presented as mean $\pm$ standard deviation of the four exposures (two animals per exposure) of each target oxygen concentration.

Target % O ₂	Measured % O ₂	Air Flow L/min	N ₂ Flow L/min	Temperature °C	RH %	Static Pressure Inches H ₂ O
21	20.9 $\pm$ 0.0	152 $\pm$ 3	0.0 $\pm$ 0.0	24.4 $\pm$ 0.7	52.6 $\pm$ 3.5	-0.04 $\pm$ 0.02
14	15.0 $\pm$ 0.1	105 $\pm$ 5	46.9 $\pm$ 0.2	24.1 $\pm$ 0.6	42.7 $\pm$ 4.7	-0.04 $\pm$ 0.00
13	13.8 $\pm$ 0.3	92 $\pm$ 3	58.8 $\pm$ 2.1	22.5 $\pm$ 0.5	31.7 $\pm$ 1.4	-0.08 $\pm$ 0.01
12	11.7 $\pm$ 0.2	79 $\pm$ 4	68.8 $\pm$ 0.6	24.1 $\pm$ 0.6	31.6 $\pm$ 2.0	-0.05 $\pm$ 0.01
11	11.1 $\pm$ 0.2	82 $\pm$ 3	81.1 $\pm$ 0.2	22.6 $\pm$ 0.7	28.9 $\pm$ 1.7	-0.05 $\pm$ 0.01
10	10.7 $\pm$ 0.3	82 $\pm$ 4	84.1 $\pm$ 0.2	22.3 $\pm$ 0.7	28.1 $\pm$ 3.5	-0.06 $\pm$ 0.01

Accelerod Performance – Body Weights

Rats were received at approximately 6-7 weeks of age and were exposed at approximately 8-9 weeks of age. Because each set of exposures took place over two weeks, there was approximately a 50-gram difference in body weights between rats that were exposed in the first week as opposed to the second week of each exposure set (Figure 8). This difference in week-to-week body weights was anticipated during the experimental planning, and it was expected that a one-week difference in age and a ~15-20% difference in body weight would not make a substantial difference in behavioral responses, especially with animals being compared to their own baseline.

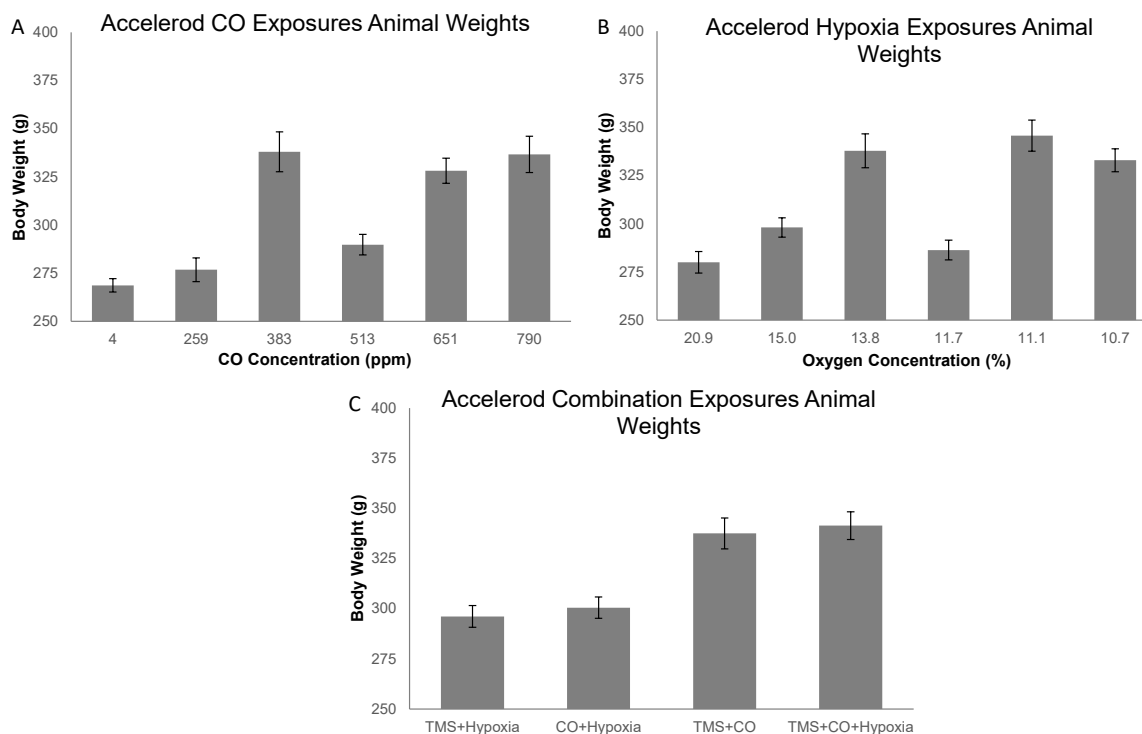


Figure 8. Average animal body weights for each exposure group during accelerrod testing.

Data represents mean + SEM of body weights for each exposure group during A) CO, B) hypoxia, and C) combination exposures.

Accelerrod Performance – Individual Exposures

Latency to fall during each five-minute accelerrod test was measured at 90-minutes and at 110-minutes during the exposure to CO for each of the eight animals at each exposure concentration. The mean +/- standard deviation of latency to fall for each exposure concentration is given in Figure 9A for the 90-minute timepoint and Figure 9B for the 110-minute timepoint. Higher CO concentrations were associated with a reduced latency to fall in comparison to baseline for both timepoints, although the 110-minute timepoint had a stronger association ($r^2=0.922$, $p<0.001$) than did the 90-minute timepoint ($r^2=0.683$, $p<0.001$). Individual data for each CO exposure run are presented in Appendix B1.

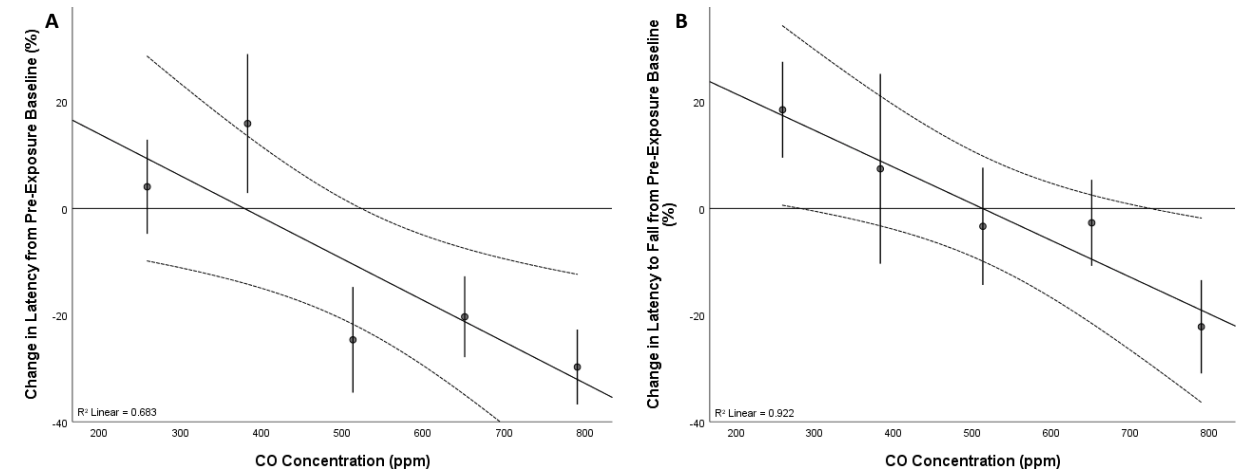


Figure 9. Mean latency to fall at each CO exposure concentration

A) 90- and B) 110-minute timepoints.

The mean latencies to fall at the 90- and 110-minute timepoints were compared to their baseline values for each exposure group. The mean latencies to fall of the 500 ppm ($p=0.015$) and 750 ppm ($p=0.02$) concentrations were significantly lower than the baseline values of the same exposure groups for the 90-minute timepoint (Table 6). No other exposure groups at the 90-minute timepoint and no exposure groups at the 110 minute timepoint had mean latencies to fall that were significantly different from their baseline values (Table 6).

Table 6. Mean latency to fall at each CO exposure concentration for 90- and 110-minute timepoints.

Shaded rows indicate a significant difference from pre-exposure baseline, with significant p-values listed in the rightmost column. Concentrations for which there were no difference in latency from pre-exposure baseline are unshaded and the p-value is listed as not significant (NS). Latency to fall data represented as the mean $\pm$ SEM of the latencies of eight animals per exposure concentration at each timepoint.

Exposure	Mean Concentration ppm	Time minutes	Mean Latency to Fall % Change from Baseline	P-value
CO	4	90	5.2 $\pm$ 4.7	NS
CO	259	90	4.1 $\pm$ 8.8	NS
CO	383	90	15.9 $\pm$ 13.0	NS
CO	513	90	-24.6 $\pm$ 9.9	0.015
CO	651	90	-20.3 $\pm$ 7.6	NS
CO	790	90	-29.7 $\pm$ 7.0	0.02
CO	4	110	8.1 $\pm$ 10.7	NS
CO	259	110	18.5 $\pm$ 9.0	NS
CO	383	110	7.4 $\pm$ 17.8	NS
CO	513	110	-3.3 $\pm$ 11.0	NS
CO	651	110	-2.7 $\pm$ 8.1	NS
CO	790	110	-22.2 $\pm$ 8.8	NS

Latency to fall during a five-minute accelerod test was measured at 90-minutes and at 110-minutes during the exposure to hypoxia for each of the eight animals at each exposure concentration. The mean $\pm$ SEM of latency to fall for each exposure concentration is given in Figure 10A for the 90-minute timepoint and Figure 10B for the 110-minute timepoint. Lower oxygen concentrations were associated with a reduced latency to fall in comparison to baseline for both timepoints, with both timepoints having a strong positive association (90 minutes: $r^2=0.830$, $p<0.001$, 110 minutes: $r^2=0.729$, $p<0.001$) between oxygen concentration and latency to fall.

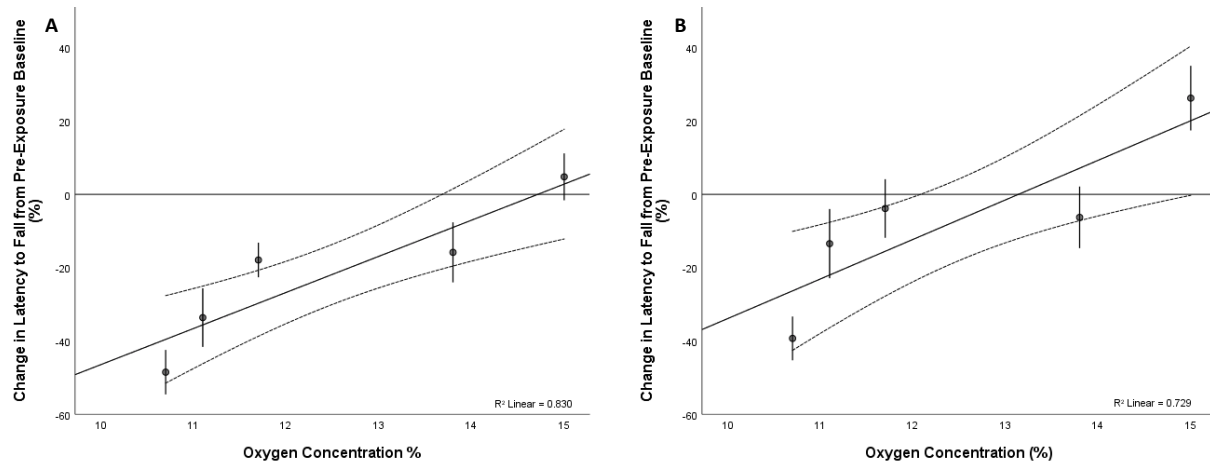


Figure 10. Mean latency to fall at each oxygen concentration
A) 90- and B) 110-minute timepoints.

The mean latencies to fall at the 90- and 110-minute timepoints were compared to their baseline values for each exposure group. The mean latencies to fall of the 10% ($p<0.001$), 11% ($p=0.024$), and 12% ($p=0.032$) oxygen concentrations at the 90-minute timepoint were significantly lower than the baseline values (Table 7). A significant decrease in latency to fall only occurred at the 10% oxygen concentration ($p=0.002$) for the 110-minute timepoint, while a significant increase in latency was associated with the 14% oxygen concentration ($p=0.031$) exposure at the same timepoint (Table 7). Individual data for each hypoxia exposure run are presented in Appendix B2.

Table 7. Mean latency to fall at each oxygen exposure concentration for 90- and 110-minute timepoints.

Shaded rows indicate a significant difference from pre-exposure baseline, with significant p-values listed in the rightmost column. Concentrations for which there was no difference in latency from pre-exposure baseline are unshaded and the p-value is listed as not significant (NS). Latency to fall data represented as the mean $\pm$ SEM of the latencies of eight animals per exposure concentration at each timepoint.

Exposure	Mean Concentration %O ₂	Time minutes	Mean Latency to Fall % Change from Baseline	P-value
Hypoxia	20.9	90	6.7 $\pm$ 7.7	NS
Hypoxia	15	90	4.8 $\pm$ 6.4	NS
Hypoxia	13.8	90	-15.8 $\pm$ 8.2	NS
Hypoxia	11.7	90	-17.9 $\pm$ 4.7	0.032
Hypoxia	11.1	90	-33.6 $\pm$ 8.0	0.024
Hypoxia	10.7	90	-48.5 $\pm$ 6.1	<0.001
Hypoxia	20.9	110	18.6 $\pm$ 7.9	NS
Hypoxia	15	110	26.2 $\pm$ 8.8	0.031
Hypoxia	13.8	110	-6.3 $\pm$ 8.4	NS
Hypoxia	11.7	110	-3.9 $\pm$ 8.0	NS
Hypoxia	11.1	110	-13.5 $\pm$ 9.4	NS
Hypoxia	10.7	110	-39.3 $\pm$ 6.0	0.002

The MECs for latency to fall in the accelerod performance test were determined to be 513 ppm for CO and 11.7% for oxygen, based on the lowest concentrations in each of the series of exposures that produced a significant reduction in latency to fall at either timepoint (Table 6 and Table 7). The MEC for latency to fall in the accelerod performance test during an exposure to TMS in a previous study using the same exposure paradigm was 750 ppm. These three MECs were used as the target concentrations to test for the combined effects of these exposures on latency to fall in the accelerod performance test.

Accelerod Performance – Combination Exposures

The mean $\pm$ standard deviation of the CO, TMS, and oxygen concentrations and environmental parameters of each of the four exposures (per target concentration) are provided in Table 8. The mean measured TMS and CO concentrations were within 10% of their target values of 750 ppm and 500 ppm, respectively, with standard deviations of no more than 5% of the mean. The mean measured oxygen concentration was slightly higher than 10% of the target value in the TMS+Hypoxia and TMS+CO+Hypoxia exposures, so these exposures were less hypoxic than the target value of 12% oxygen. The flows of air, nitrogen, and relative humidity varied with target oxygen concentration in the same manner as during the hypoxia exposures, with increased nitrogen flow and decreased relative humidity in the combination exposures with hypoxia. Mean values of temperature and static pressure within expected ranges and were acceptably consistent run-to-run within each exposure group. Exposure and environmental data for each of the combination exposure runs are presented in Appendix A3.

Table 8. Average exposure concentration and environmental data for each of the combination exposures.

Data presented as mean +/- standard deviation of the four exposures (2 animals per exposure) of each combination. The nitrogen flow for the TMS+Hypoxia exposure was not determined (ND) because of issues with data recording. C: CO, T:TMS, H: Hypoxia

Exposure	Measured CO (ppm)	Measured % O ₂	Measured TMS (ppm)	Air Flow L/min	N ₂ Flow L/min	Temperature °C	RH %	Static Pressure Inches H ₂ O
H, T	N/A	13.3 ± 0.3	767 ± 4	84 ± 5	ND	25.3 ± 0.5	36.0 ± 1.9	-0.05 ± 0.02
C, H	508 ± 4	12.9 ± 0.3	N/A	99 ± 8	68.0 ± 0.0	25.3 ± 0.6	43.5 ± 5.5	-0.03 ± 0.00
C, T	537 ± 20	N/A	766 ± 20	144 ± 2	N/A	24.6 ± 0.5	53.9 ± 3.4	-0.06 ± 0.00
C,H,T	546 ± 18	13.6 ± 0.5	793 ± 20	94 ± 3	68.0 ± 0.0	23.7 ± 0.5	26.1 ± 1.8	-0.07 ± 0.01

The mean latencies to fall at the 90- and 110-timepoints were compared to their baseline values for each exposure group. The mean latencies to fall of all combinations were significantly lower than the baseline values of the same exposure groups for the 90-minute timepoint, with all p-values for the comparisons less than 0.001 (Table 9). A significant decrease in latency to fall was present for all combinations except TMS+CO the 110-minute timepoint (Table 9).

Table 9. Mean latency to fall at each MEC combination exposure for 90- and 110-minute timepoints.

Shaded rows indicate a significant difference from pre-exposure baseline, with p-values less than 0.05 given in the rightmost column. Combinations for which there was no difference in latency from pre-exposure baseline are unshaded and the p-value is listed as not significant (NS). Latency to fall data represented as the mean + SEM of the latencies of eight animals per exposure combination at each timepoint.

Exposure	Time minutes	Mean Latency to Fall %Change from Baseline	P-value
TMS+Hypoxia	90	-32.7 ± 6.3	<0.001
CO+Hypoxia	90	-34.8 ± 4.7	<0.001
TMS+CO	90	-31.2 ± 7.9	<0.001
TMS+CO+Hypoxia	90	-34.0 ± 7.3	<0.001
TMS+Hypoxia	110	-28.4 ± 10.1	0.03
CO+Hypoxia	110	-26.4 ± 6.3	0.021
TMS+CO	110	-23.9 ± 12.0	NS
TMS+CO+Hypoxia	110	-47.8 ± 10.9	<0.001

The change in latency to fall from baseline for each combination was compared to the change in latency to fall for the individual exposures. There was no difference in latency to fall between any of the combination pairs and the individual exposures that made up the pair (Figure 11, A-C). There was a significant reduction in latency to fall at the 110-minute timepoint between the TMS+CO+Hypoxia exposure and the individual hypoxia and CO exposures, although that difference was not present at the 90-minute timepoint (Figure 11D). Individual data for each combination exposure run are presented in Appendix B3.

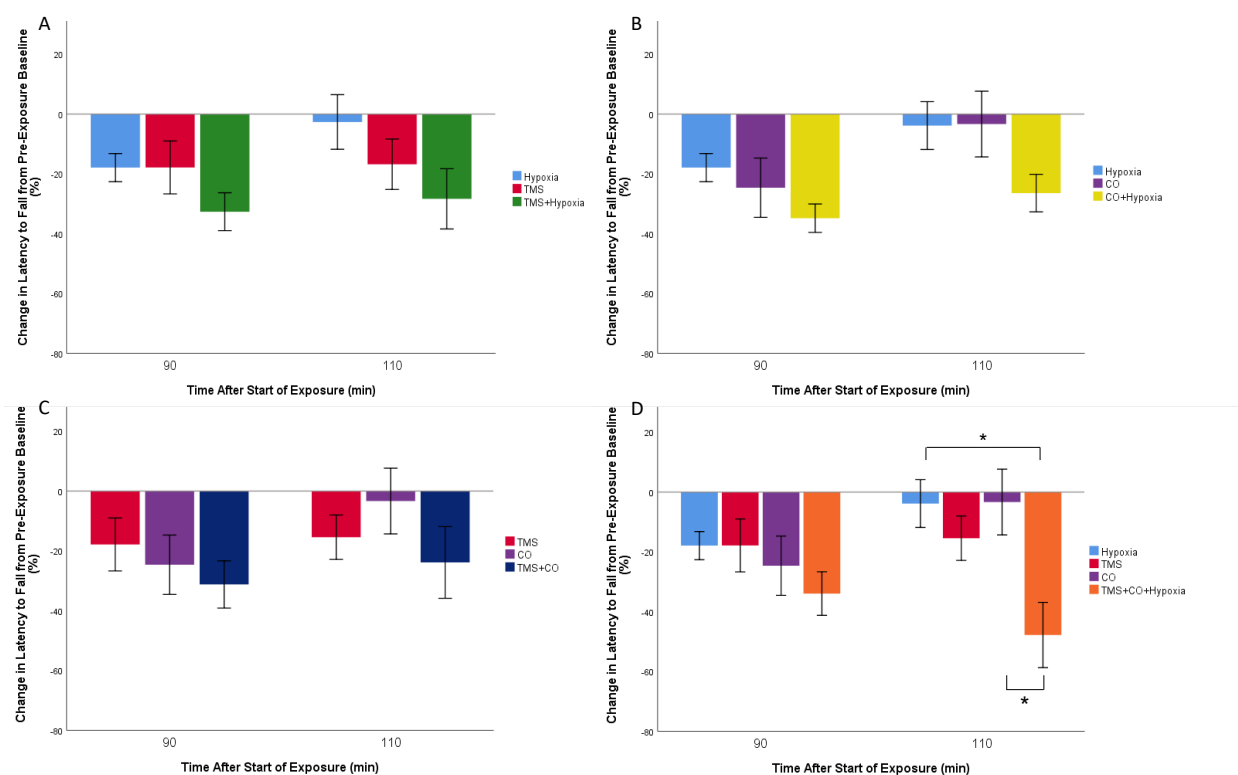


Figure 11. Mean latency to fall at for individual and combination MEC exposures.

Each graph represents exposure group means $\pm$ SEM of change in latency to fall during from pre-exposure baseline during accelerod testing performed at the 90- and 110-minute timepoints during the 120 minute exposure. * $p < 0.05$ for the indicated comparison. **A)** Mean change in latency during individual and combined TMS+Hypoxia exposures. **B)** Mean change in latency during individual and combined CO+Hypoxia exposures. **C)** Mean change in latency during individual and combined TMS+CO exposures. **D)** Mean change in latency during individual and combined TMS+CO+Hypoxia exposures.

Accelerod Performance – Combinations Analysis

Predictions of latency to fall in combined exposures were calculated from individual latencies using the Bliss Model of combinations. The prediction was then statistically compared to measured data using a one sample t-test to determine if there was a difference from the distribution of the measured latencies to fall in the combination exposures.

There was no significant difference between the Bliss Model prediction of latency and the average measured latency values for most of the exposures at each timepoint, indicating an additive effect of all combinations tested (Table 10). The 110-minute CO+Hypoxia average latency was significantly higher than the prediction from the individual hypoxia and CO exposures (Table 10, $p = 0.018$), indicating a synergistic response. An antagonistic response was determined for the exposure to all three conditions at 90-minutes, with the response being significantly lower than the Bliss model prediction (Table 10, $p = 0.009$). This analysis for both the latencies of 110-minute CO+Hypoxia and 90-minute TMS+CO+Hypoxia groups contrasts with standard statistical

comparisons (Figure 11), in which there was no difference between the individual and combined exposures.

Table 10. Combinations analysis for combined accelerated exposures at 90 and 110 minutes.

Individual and combined mean + SEM latencies to fall, presented as percent change from pre-exposure baseline, are listed for each combination. The Bliss Model was used to calculate predicted combined responses, and those predictions were statistically compared to the measured responses. Measured values that are significantly higher than predicted suggest a synergistic response, and values that are significantly lower than the prediction indicate an antagonistic response. NS, or measured values that are not significantly different from the prediction, suggest an additive response.

Exposure Group/Parameter	% Change in Latency to Fall from Baseline	
	90 Minutes	110 Minutes
Hypoxia	-17.9 ± 4.7	-3.9 ± 8.0
TMS	-17.8 ± 8.8	-15.4 ± 7.4
TMS+Hypoxia	-32.7 ± 6.3	-28.4 ± 10.1
Bliss Model Prediction	-32.6	-18.7
P-value Prediction vs. Measured	NS	NS
TMS	-17.8 ± 8.8	-15.4 ± 7.4
CO	-24.6 ± 9.9	-3.3 ± 11.0
TMS+CO	-31.2 ± 7.9	-23.9 ± 12.0
Bliss Model Prediction	-38.1	-18.3
P-value Prediction vs. Measured	NS	NS
Hypoxia	-17.9 ± 4.7	-3.9 ± 8.0
CO	-24.6 ± 9.9	-3.3 ± 11.0
CO+Hypoxia	-34.8 ± 4.7	-26.4 ± 6.3
Bliss Model Prediction	-38.1	-7.1
P-value Prediction vs. Measured	NS	0.018
Hypoxia	-17.9 ± 4.7	-3.9 ± 8.0
CO	-24.6 ± 9.9	-3.3 ± 11.0
TMS	-17.8 ± 8.8	-15.4 ± 7.4
TMS+CO+Hypoxia	-34.0 ± 7.3	-47.8 ± 10.9
Bliss Model Prediction	-59.6	-22.6
P-value Prediction vs. Measured	0.009	NS

Physiology Analysis – Test Atmosphere Characterization

The mean ± standard deviation of the chemical concentrations and environmental parameters for each exposure group are given in Table 11. The average CO, TMS, and oxygen concentrations of the exposures were within 10% of their target values of 500 ppm, 750 ppm, and 12%, respectively, for all groups receiving CO, TMS, and/or hypoxia exposures. The standard deviations of each chemical for each exposure group were also no more than 5% of the mean. All mean values of environmental parameters, including temperature, relative humidity, and static pressure, were within expected ranges and were acceptably consistent run-to-run within each exposure group. Exposure and environmental data for each of the physiology exposure runs are presented in Appendix C.

Table 11. Average exposure concentration and environmental data for each group during physiology testing.
Data presented as mean +/- standard deviation of each parameter for each exposure group.

Exposure	Measured Conc. CO (ppm)	Measured Conc. O ₂ (%)	Measured Conc. TMS (ppm)	Temperature °C	RH %	SP Inches H ₂ O
Control	0 ± 4	20.9 ± 0.1	-2 ± 3	21.5 ± 1.3	35.6 ± 8.5	-0.04 ± 0.00
CO	489 ± 4	20.9 ± 0.1	5 ± 4	22.5 ± 1.1	35.5 ± 10.0	-0.04 ± 0.01
Hypoxia	1 ± 2	12.0 ± 0.0	2 ± 6	21.8 ± 1.1	36.9 ± 9.4	-0.04 ± 0.01
TMS	0 ± 1	20.9 ± 0.2	776 ± 27	22.1 ± 1.3	36.4 ± 8.8	-0.04 ± 0.00
TMS+Hypoxia	0 ± 1	12.0 ± 0.1	747 ± 8	22.0 ± 1.3	36.4 ± 11.5	-0.03 ± 0.02
CO+Hypoxia	492 ± 3	12.1 ± 0.2	1 ± 7	22.5 ± 2.2	39.1 ± 10.0	-0.06 ± 0.02
TMS+CO	493 ± 2	20.9 ± 0.1	749 ± 11	22.9 ± 1.8	35.8 ± 10.6	-0.05 ± 0.01
TMS+CO+Hypoxia	496 ± 9	12.2 ± 0.1	743 ± 4	22.3 ± 1.5	32.9 ± 10.2	-0.05 ± 0.01

Physiology Analysis – Body Weights

Rats were received at approximately 6-7 weeks of age and were exposed at approximately 8-9 weeks of age. Average body weights for animals used in physiology testing exposures are presented in Figure 12. There was no difference in body weights between exposure groups. The average weight range of ~310-340g was consistent with the animals used during the second week of exposures during the accelerated performance experiments, suggesting that the animals were in good health and within expected weight range after surgery at the time of exposure.

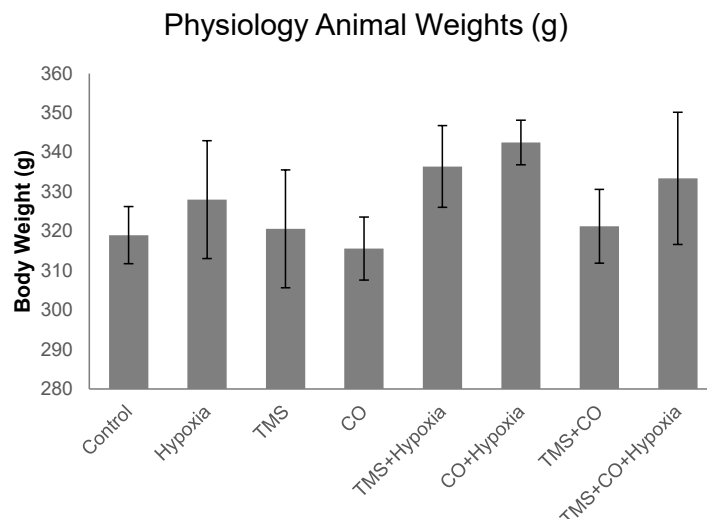


Figure 12. Average animal body weights for each exposure group during physiology experiments.
Weights were measured immediately prior to exposures.

Physiology Analysis – Physiology Data

Electrophysiology

The input-output (I/O) function of CA1 hippocampal neurons was determined by measuring the size of evoked field response (output) induced by varying stimulating input (10-100 μ A). The evoked response was measured by using the amplitude of the inward deflection. Stimulating currents were delivered to one of the 16 channels of the electrode array and channels showing an output amplitude response greater than 50 μ A were averaged together to yield the output data point.

Control rats did not exhibit any change in I/O before and at various time points (40-, 80- and 105-minutes) during exposure (Figure 13A). Two-way ANOVA performed in control I/O data with timepoints (baseline, 40-, 80-, 105-minutes) as one factor and stimulation intensity (10-100 μ A) as the second factor, yielded $F(3, 277)=0.77$ $p=0.51$ ($n=5$ rats, 1-2 recordings per rat). Exposure to CO, TMS or hypoxia alone did not result in significant effect on I/O function (Figure 13B-D). Combination exposure to CO+TMS also did not result in significant effect on I/O function (Figure 14A).

There was a statistically significant reduction in the evoked response, as reported by decreased I/O, in rats exposed to CO and hypoxia. Two-way ANOVA was performed on I/O data from rats exposed to CO and hypoxia with time points (baseline, 40-, 80-, 105-minutes) as one factor and stimulation intensity (10-100 μ A) as the second factor. There was a significant difference across time (baseline, 40-, 80-, 105-minutes) with $F(3, 259) = 11.2$ $p<0.001$ ($n=4$ rats at 2 recordings per rat) (Figure 14C). Post-hoc multiple comparison analysis using the Holm-Sidak Method showed significant difference between the 105-minute versus baseline data ($p<0.001$), 105-minute versus 40-minute data ($p<0.008$), 80-minute versus baseline data ($p=0.008$), and 105-minute versus 80-minute data ($p=0.032$). There was no significant difference within each individual stimulation and there was not a statistically significant interaction between groups and stimulation intensity.

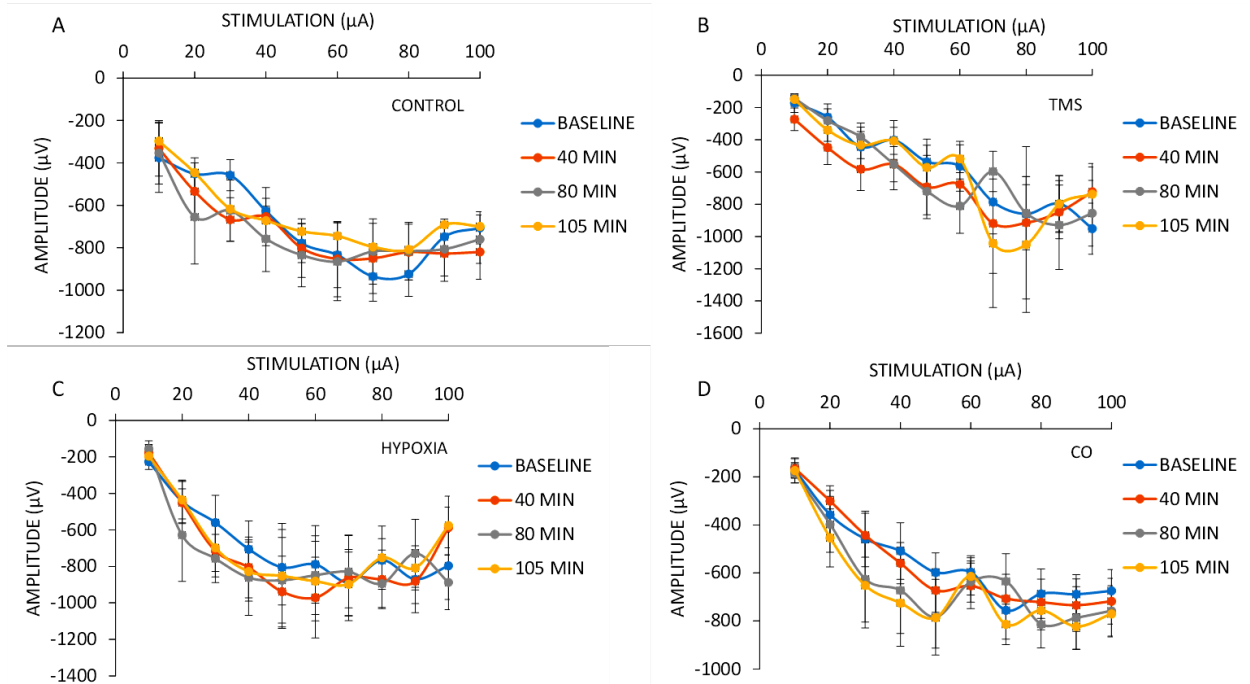


Figure 13. Input-output (I/O) function before and during exposure to individual chemicals.

Control rats exposed to ambient air (A), TMS-exposed (B), hypoxia-exposed (C), and CO-exposed (D) rats did not show statistically significant difference in I/O function.

There was a statistically significant decrease in I/O function in rats exposed to CO, TMS, and hypoxia (CO+TMS+Hypoxia). Two-way ANOVA was performed on I/O data from rats exposed to CO, TMS, and hypoxia with timepoints (baseline, 40-, 80-, 105-minutes) as one factor and stimulation intensity (10-100 μ A) as the second factor. There was a significant difference across time (baseline, 40-, 80-, 105-minutes) with $F(3, 268)=3.7$ $p=0.013$ ($n=4$ rats at 2 recordings per rat) (Figure 14B). Post-hoc multiple comparison analysis using the Holm-Sidak Method showed significant difference between the 105-minute vs 40-minute data ($p=0.045$). There was a trending decrease that was statistically significant between the 105-minute vs baseline data ($p=0.121$) and 80-minute vs 40-minute data ($p=0.073$). There was no significant difference within each individual stimulation and there was not a statistically significant interaction between groups and stimulation intensity.

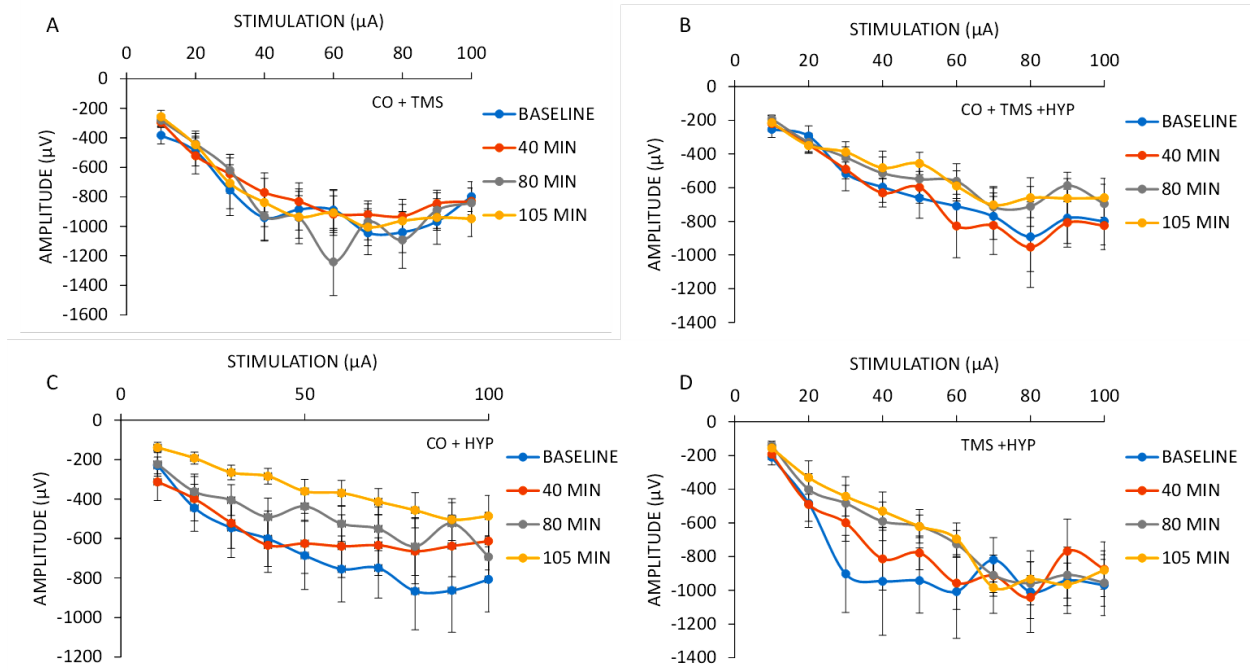
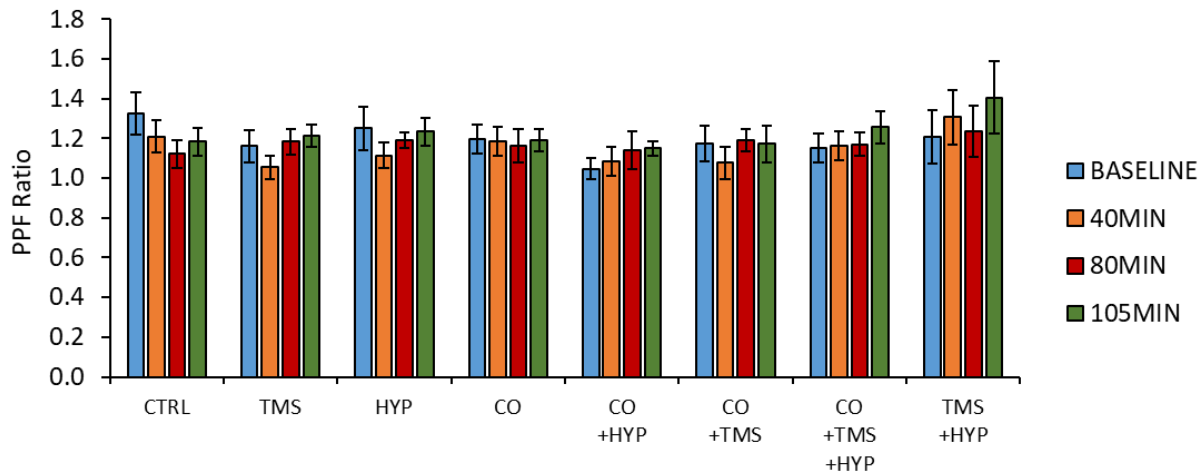


Figure 14. Input-output (I/O) function before and during exposure to combinations of chemicals.

A) Rats exposed to CO and TMS did not show statistically significant difference in I/O function. **B)** Rats exposed to CO, TMS and hypoxia showed statistically significant reduction at 80-minutes following initiation of exposure compared to data obtained at 40-minutes after exposure. **C)** Rats exposed to CO and hypoxia showed statistically significant reduction in I/O function at 105-minutes following initiation of exposure compared to baseline and 40-minutes data. Statistically significant reduction in I/O was also observed at 80-minutes after exposure compared to baseline. **D)** Rats exposed to TMS and hypoxia showed statistical difference between exposure groups but pairwise comparison analysis did not yield statistically significant differences.

There was a statistically significant decrease in I/O function in rats exposed to TMS and hypoxia. Two-way ANOVA was performed on I/O data from rats exposed to TMS and hypoxia with timepoints (baseline, 40-, 80-, 105-minutes) as one factor and stimulation intensity (10-100 μ A) as the second factor. There was a significant difference among groups (baseline, 40-, 80-, 105-minutes) with $F(3, 236)=2.7$ $p=0.049$ ($n=4$ rats at 2 recordings per rat) (Figure 14D). However, post-hoc multiple comparison analysis using the Holm-Sidak Method did not show significant difference between the 105-minute vs baseline or 40-minute data ($p=0.073, 0.56$). There was also no significant difference within each individual stimulation and there was not a statistically significant interaction between time and stimulation intensity.

There was no statistically difference in PPF in rats exposed to CO, TMS, hypoxia, CO+TMS, CO+TMS+Hypoxia, or TMS+Hypoxia (Figure 15).



PPF	BASELINE	40MIN	80MIN	105MIN
CONTROL	1.33 ± 0.11	1.21 ± 0.08	1.12 ± 0.07	1.18 ± 0.07
TMS	1.16 ± 0.08	1.05 ± 0.06	1.18 ± 0.07	1.21 ± 0.06
HYPOXIA	1.25 ± 0.11	1.11 ± 0.06	1.19 ± 0.04	1.23 ± 0.07
CO	1.19 ± 0.07	1.19 ± 0.07	1.16 ± 0.09	1.19 ± 0.05
CO+HYP	1.05 ± 0.05	1.08 ± 0.07	1.14 ± 0.09	1.15 ± 0.04
CO+TMS	1.17 ± 0.09	1.08 ± 0.08	1.19 ± 0.05	1.17 ± 0.09
CO+TMS+HYP	1.15 ± 0.07	1.16 ± 0.07	1.17 ± 0.06	1.26 ± 0.08
TMS+HYP	1.21 ± 0.13	1.31 ± 0.14	1.23 ± 0.13	1.41 ± 0.18

Figure 15. Paired pulse facilitation (PPF) before and during exposure.

A) Bar graph showing no significant differences in PPF during exposures to control, CO, TMS, Hypoxia, CO+TMS, CO+Hypoxia, TMS+Hypoxia, or CO+TMS+Hypoxia. **B)** Table showing averaged PPF data $\pm$ SEM from each exposure group at various time points.

Arterial Blood Gases and pH Analyses

Arterial blood samples were collected from the left common carotid artery of each rat at baseline, 90-, and 115-minutes into the exposure. Arterial blood samples were analyzed for pH, $p\text{CO}_2$, %COHb, and %O₂Hb. Blood oxygen ($p\text{O}_2$) could not be measured because of instrument errors that artificially elevated the value of the measurement that were not able to be resolved during the experiment.

The values of mean arterial pH for each exposure group were compared between the repeated measures taken during the time course. pH was significantly elevated over baseline values at both time points for the Hypoxia ($p < 0.05$) and CO+Hypoxia ($p < 0.001$) exposure groups. pH was also significantly higher than baseline for the CO exposure group ($p < 0.05$) at 115-minutes and the TMS+CO+Hypoxia group ($p < 0.001$) at 90-minutes (Figure 16).

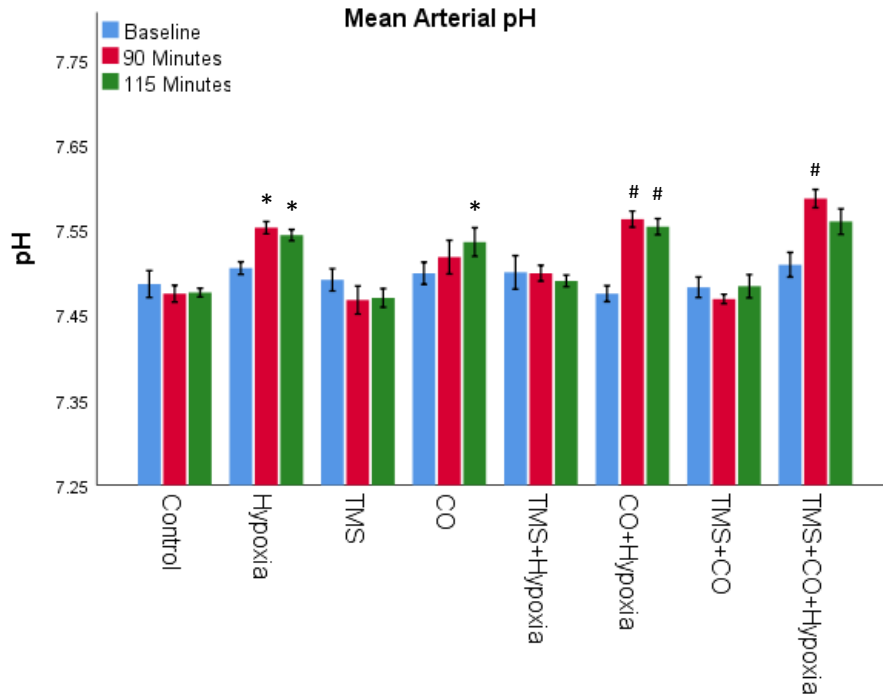


Figure 16. Mean change in arterial pH during pre-exposure baseline and after 90 and 115 minutes of exposure. Graph represents exposure group means $\pm$ SEM of pH for arterial blood draws taken at pre-exposure baseline, and at the 90- and 115-minute timepoints during the 120-minute exposure. * $p < 0.05$ compared to baseline. # $p < 0.001$ compared to baseline.

Change in mean arterial pH from baseline was compared between individual and combination exposure groups for each set of combinations. For the TMS+Hypoxia combination, the change in mean arterial pH of the Hypoxia group was significantly greater than the Control ($p < 0.05$) and TMS ($p < 0.001$) exposure groups, but pH change of the combination exposure did not differ from any other groups (Figure 17A). A significantly elevated change in mean arterial pH was present in the CO+Hypoxia group compared to the Control group at 90-minutes ($p < 0.05$) and 115-minutes ($p < 0.05$) of the exposure (Figure 17B). The change in arterial pH was also higher in the CO+Hypoxia exposure group than the CO exposure group at 90-minutes ($p < 0.05$, Figure 17B). There was no difference in pH change between TMS+CO and the Control or individual exposures (Figure 17C). The change in mean arterial pH during the TMS+CO+Hypoxia exposure was significantly greater than the TMS ($p < 0.001$) and Control ($p < 0.05$) exposures at 90-minutes, but there was no difference between exposure groups at 115 minutes (Figure 17D).

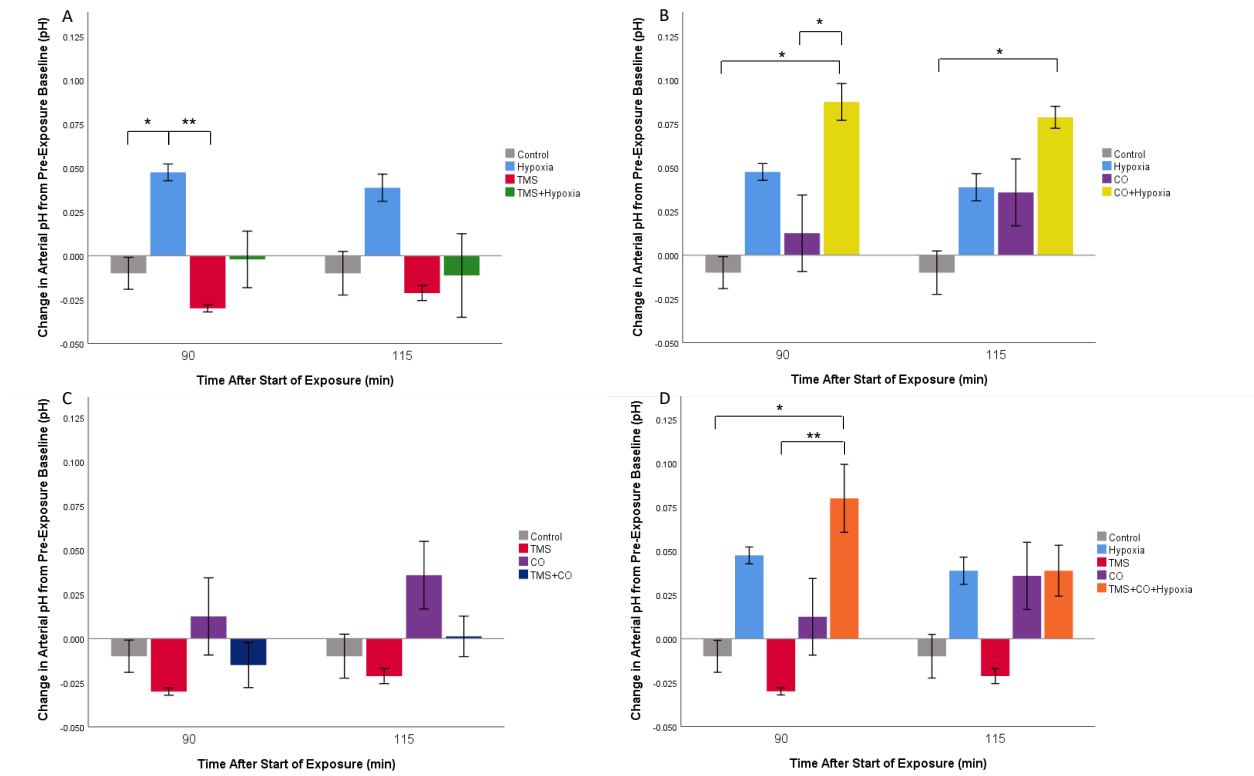


Figure 17. Mean change in arterial pH, comparison between individual and combination exposures.

Each graph represents exposure group means $\pm$ SEM of change in pH from pre-exposure baseline for arterial blood draws taken at the 90- and 115-minute timepoints during the 120-minute exposure. * $p < 0.05$ for the indicated comparison. ** $p < 0.001$ for the indicated comparison. **A)** Mean change in pH during individual and combined TMS+Hypoxia exposures. **B)** Mean change in pH during individual and combined CO+Hypoxia exposures. **C)** Mean change in pH during individual and combined TMS+CO exposures. **D)** Mean change in pH during individual and combined TMS+CO+Hypoxia exposures.

The values of mean arterial $p\text{CO}_2$ for each exposure group were compared between the repeated measures taken during the time course. $p\text{CO}_2$ was significantly diminished below baseline values at both time points for the CO+Hypoxia ($p < 0.001$) and TMS+CO+Hypoxia ($p < 0.001$) exposure groups. $p\text{CO}_2$ was also significantly lower than baseline for the Hypoxia exposure group ($p < 0.05$) at 90-minutes (Figure 18).

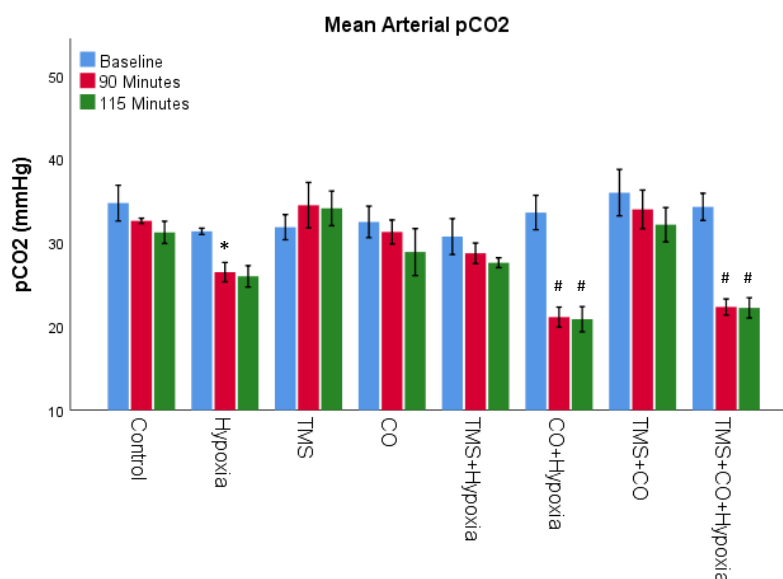


Figure 18. Mean arterial pCO₂ during pre-exposure baseline and after 90 and 115 minutes of exposure.

Graph represents exposure group means $\pm$ SEM of pCO₂ for arterial blood draws taken at pre-exposure baseline, and at the 90- and 115-minute timepoints during the 120-minute exposure. * $p < 0.05$ compared to baseline. # $p < 0.001$ compared to baseline.

Change in mean arterial pCO₂ from baseline was compared between individual and combination exposure groups for each set of combinations. For the TMS+Hypoxia combination, there was no difference between the individual exposures and the combination exposure (Figure 19A). A significant attenuation in mean arterial pCO₂ was present in the CO+Hypoxia group compared to the Control and CO exposure groups at 90-minutes ($p < 0.05$) of the exposure (Figure 19B). There was no difference in pCO₂ change between TMS+CO and the Control or individual exposures (Figure 19C). The decrease in mean arterial pCO₂ during the TMS+CO+Hypoxia exposure was significantly different than the TMS ($p < 0.05$) and CO ($p < 0.05$) responses at 90-minutes, and was also significantly different than the TMS exposure group ($p < 0.05$) at 115-minutes (Figure 19D).

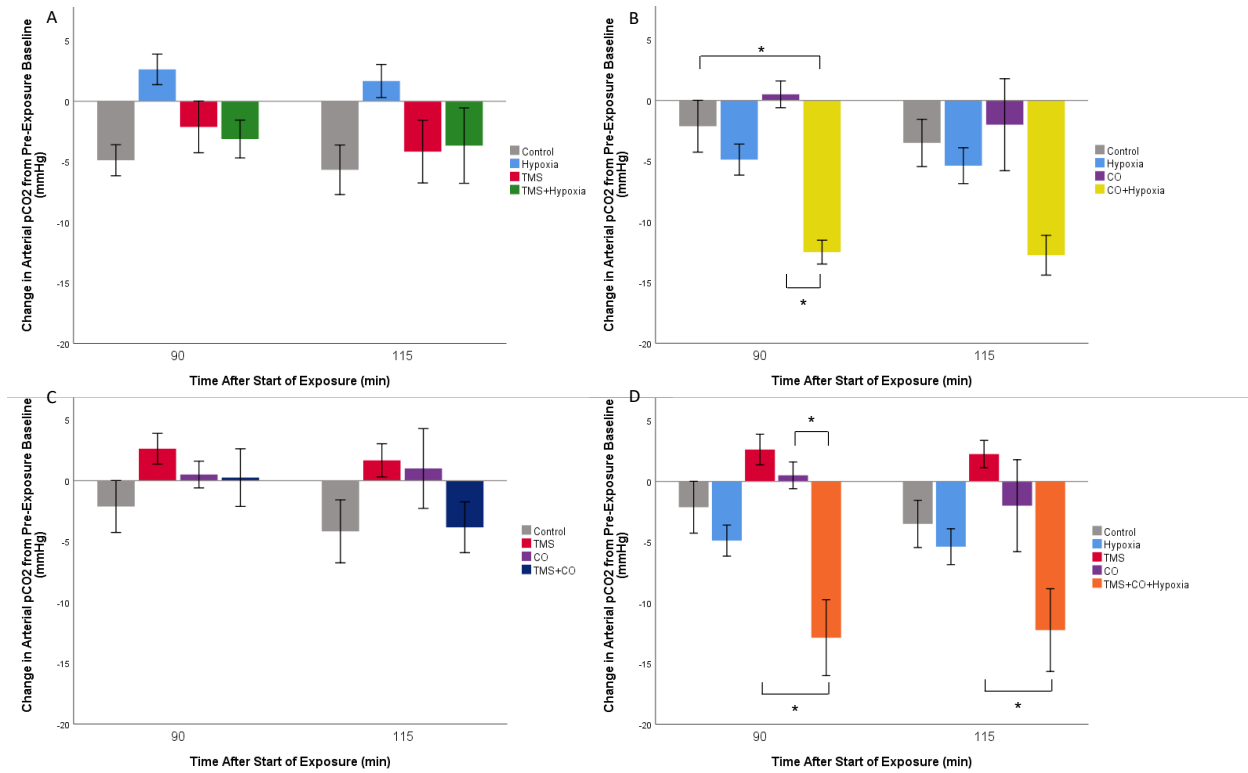


Figure 19. Mean change in arterial pCO₂, comparison between individual and combination exposures.

Each graph represents exposure group means $\pm$ SEM of change in pCO₂ from pre-exposure baseline for arterial blood draws taken at the 90- and 115-minute timepoints during the 120-minute exposure. * $p < 0.05$ for the indicated comparison. **A)** Mean change in pCO₂ during individual and combined TMS+Hypoxia exposures. **B)** Mean change in pCO₂ during individual and combined CO+Hypoxia exposures. **C)** Mean change in pCO₂ during individual and combined TMS+CO exposures. **D)** Mean change in pCO₂ during individual and combined TMS+CO+Hypoxia exposures.

The values of mean arterial %COHb for each exposure group were compared between the repeated measures taken during the time course. %COHb was significantly elevated above baseline values at both timepoints for the CO exposure group and all combinations with CO exposure ($p < 0.001$, Figure 20). %COHb was also significantly lower than baseline for the Hypoxia exposure group ($p < 0.05$) at 90- and 115-minutes, and for the TMS+Hypoxia group at 90-minutes ($p < 0.05$, Figure 20).

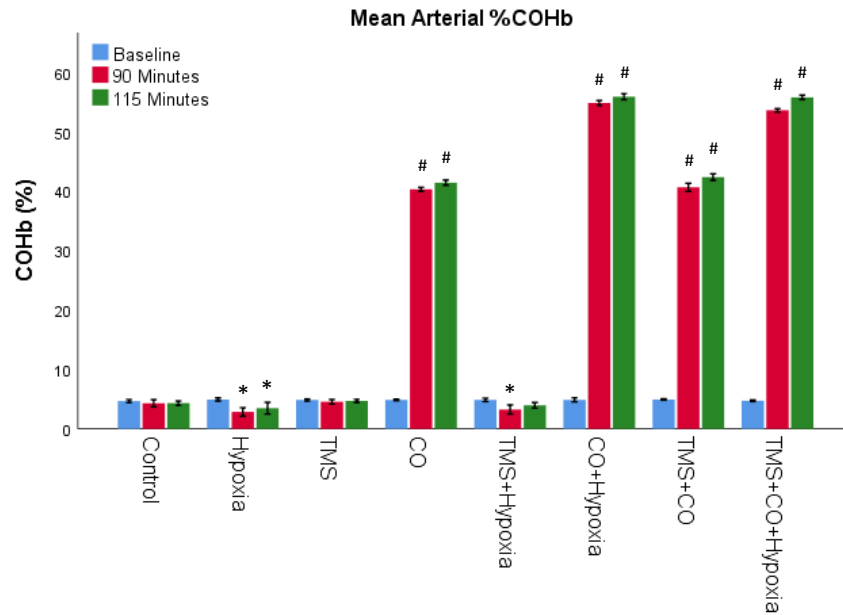


Figure 20. Mean arterial %COHb during pre-exposure baseline and after 90 and 115 minutes of exposure.

Graph represents exposure group means $\pm$ SEM of %COHb for arterial blood draws taken at pre-exposure baseline, and at the 90- and 115-minute timepoints during the 120-minute exposure. * $p < 0.05$ compared to baseline. # $p < 0.001$ compared to baseline.

Change in mean arterial %COHb from baseline was compared between individual and combination exposure groups for each set of combinations. For the TMS+Hypoxia combination, there was no difference between the individual exposures and the combination exposure (Figure 21A). A significant increase in mean arterial %COHb was present in the CO and CO+Hypoxia groups compared to the Control and Hypoxia exposure groups at both timepoints of the exposure ($p < 0.001$) (Figure 21B). The change in %COHb was also significantly greater in the CO+Hypoxia exposure group compared to the CO exposure group ($p < 0.001$, Figure 21B). The change in %COHb was significantly higher in the CO exposure group and the TMS+CO combination exposure than the TMS or Control exposure groups at both timepoints ($p < 0.001$, Figure 21C). However, there was no difference in %COHb between the CO and TMS+CO exposures (Figure 21C). The increase in mean arterial %COHb during the TMS+CO+Hypoxia exposure was significantly different than the CO ($p < 0.001$) %COHb response at both timepoints, and was also significantly different than the individual TMS, Hypoxia, and Control exposure groups ($p < 0.001$) at both timepoints (Figure 21D).

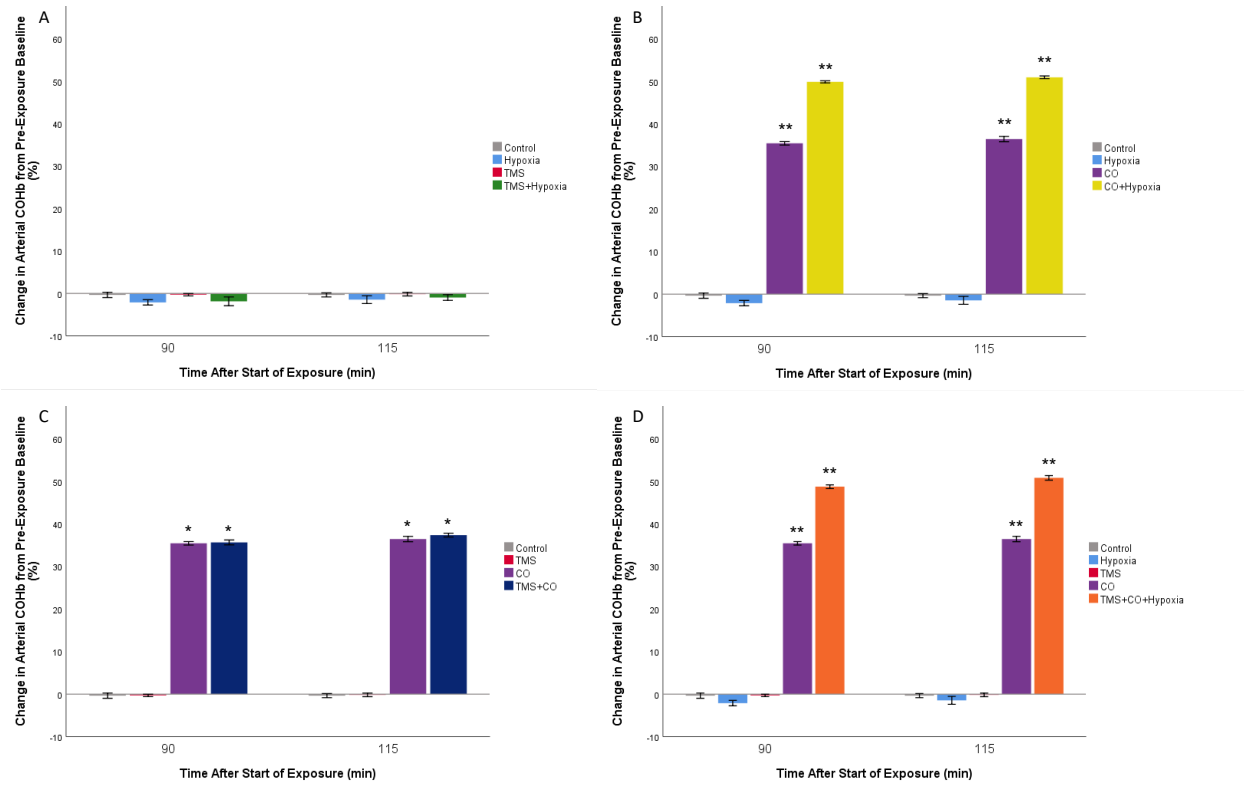


Figure 21. Mean change in arterial %COHb, comparison between individual and combination exposures.

Each graph represents exposure group means $\pm$ SEM of change in %COHb from pre-exposure baseline for arterial blood draws taken at the 90- and 115-minute timepoints during the 120-minute exposure. * $p < 0.001$ compared to TMS and control groups. ** $p < 0.001$ compared to all other exposure groups. **A)** Mean change in %COHb during individual and combined TMS+Hypoxia exposures. **B)** Mean change in %COHb during individual and combined CO+Hypoxia exposures. **C)** Mean change in %COHb during individual and combined TMS+CO exposures. **D)** Mean change in %COHb during individual and combined TMS+CO+Hypoxia exposures.

The values of mean arterial %O₂Hb for each exposure group were compared between the repeated measures taken during the time course. %O₂Hb was significantly decreased below baseline values at both timepoints for the CO exposure group and all combinations with CO exposure ($p < 0.001$, Figure 22). %O₂Hb was also significantly lower than baseline values at both timepoints for the hypoxia exposure group and all combinations with hypoxia exposure ($p < 0.001$, Figure 22).

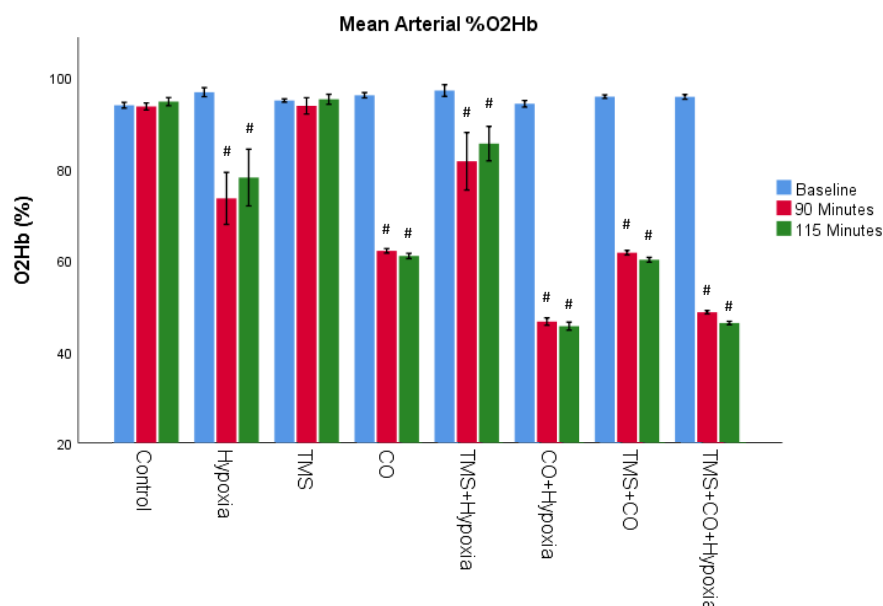


Figure 22. Mean arterial %O₂Hb during pre-exposure baseline and after 90 and 115 minutes of exposure.

Graph represents exposure group means $\pm$ SEM of % O₂Hb for arterial blood draws taken at pre-exposure baseline, and at the 90- and 115-minute timepoints during the 120-minute exposure. * $p < 0.05$ compared to baseline. # $p < 0.001$ compared to baseline.

Change in mean arterial %O₂Hb from baseline was compared between individual and combination exposure groups for each set of combinations. For the TMS+Hypoxia combination, there was significantly more reduction in %O₂Hb in the Hypoxia and TMS+Hypoxia exposures than the Control or TMS exposures at both timepoints ($p < 0.05$, Figure 23A). A significant decrease in %O₂Hb was present in the CO, CO+Hypoxia, and Hypoxia groups compared to the Control exposure groups at both timepoints of the exposure ($p < 0.05$, Figure 23B). The reduction in %O₂Hb was significantly greater in the CO exposure group and the TMS+CO combination exposure than the TMS or Control exposure groups at both timepoints ($p < 0.05$, Figure 23C). However, there was no difference in %O₂Hb between the CO and TMS+CO exposures (Figure 23C). The decrease in %O₂Hb during the TMS+CO+Hypoxia exposure was significantly different than the control and TMS ($p < 0.05$) %O₂Hb response at both timepoints ($p < 0.05$, Figure 23D).

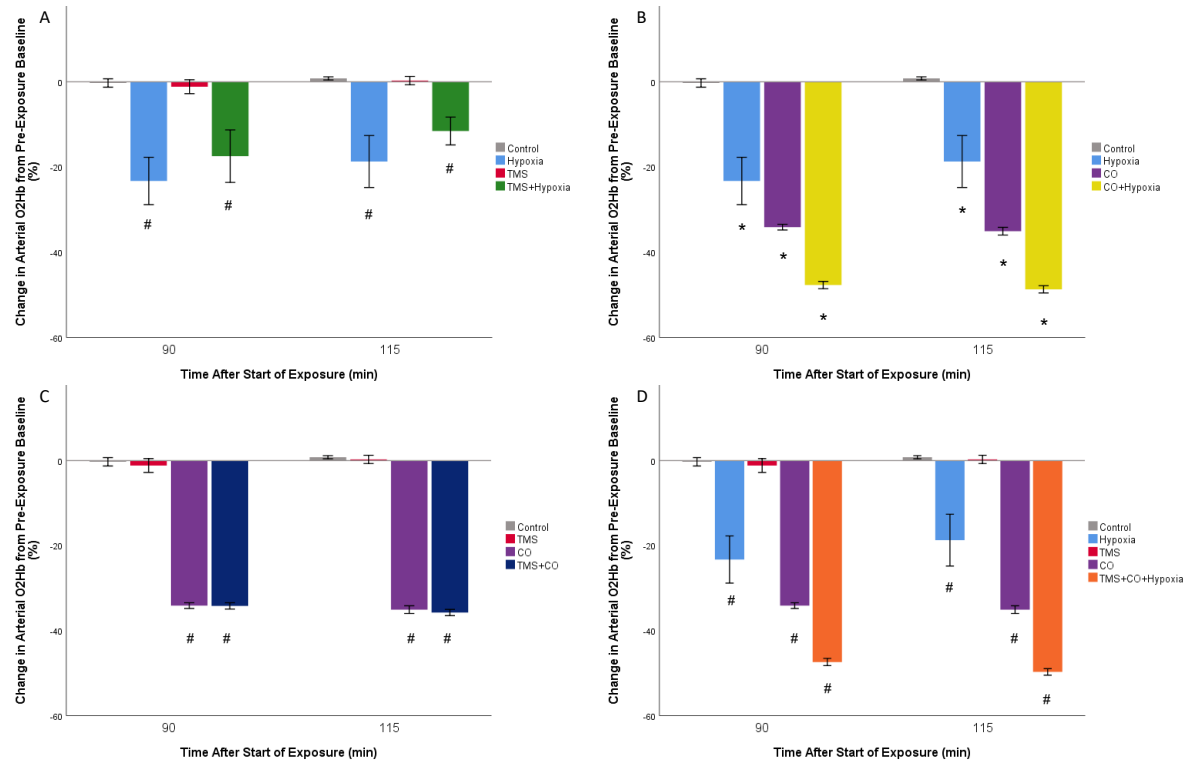


Figure 23. Mean change in arterial %O₂Hb, comparison between individual and combination exposures.

Each graph represents exposure group means $\pm$ SEM of change in %O₂Hb from pre-exposure baseline for arterial blood draws taken at the 90- and 115-minute timepoints during the 120-minute exposure. # p <0.05 compared to TMS and control groups. * p <0.05 compared to control group. **A)** Mean change in %O₂Hb during individual and combined TMS+Hypoxia exposures. **B)** Mean change in %O₂Hb during individual and combined CO+Hypoxia exposures. **C)** Mean change in %O₂Hb during individual and combined TMS+CO exposures. **D)** Mean change in %O₂Hb during individual and combined TMS+CO+Hypoxia exposures.

Blood Pressure

Mean arterial blood pressure was measured from the left common carotid artery of each rat during the pre-exposure baseline period and throughout the 120-minutes of exposure. Mean arterial blood pressure values were averaged every 15 minutes of exposure for each animal. The mean arterial blood pressure values that were averaged during the baseline period, 75-90 minutes, and 105-120 minutes during exposure were compared between time periods for each exposure group. Generally, blood pressure decreased from baseline for all groups that were exposed to either CO or Hypoxia. There was no change in blood pressure at either timepoint for control nor TMS exposure groups (Figure 24). Hypoxia and CO+Hypoxia groups both had decreases from baseline at both timepoints at the p <0.05 significance level, while the CO and TMS+Hypoxia groups had lower blood pressure values than baseline at the p <0.05 significance level for the 90-minute timepoint, and p <0.001 significance level for the 120-minute timepoint (Figure 24). TMS+CO and TMS+CO+Hypoxia exposures both had diminished blood pressure values at both timepoints at the p <0.001 significance level (Figure 24).

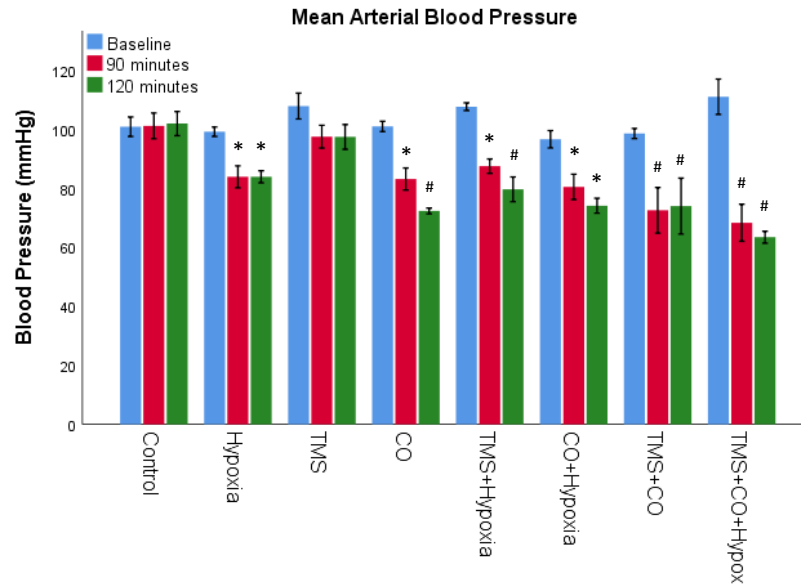


Figure 24. Mean arterial blood pressure during pre-exposure baseline and after 90 and 115 minutes of exposure.

Graph represents exposure group means $\pm$ SEM of blood pressure during the ~10-minute pre-exposure baseline period (Baseline), and at the 75-90 minute (90 minute) and 105-120-minute (120-minute) time periods during the 120-minute exposure. * $p < 0.05$ compared to baseline. # $p < 0.001$ compared to baseline.

The overall change in blood pressure was compared between exposure groups. The decrease in blood pressure associated with the TMS+CO+Hypoxia exposure was significantly different from all other exposure groups ($p < 0.05$, Figure 25). There were no other differences between exposure groups, although there was an apparent downward trend in blood pressure over time in all groups except for the control.

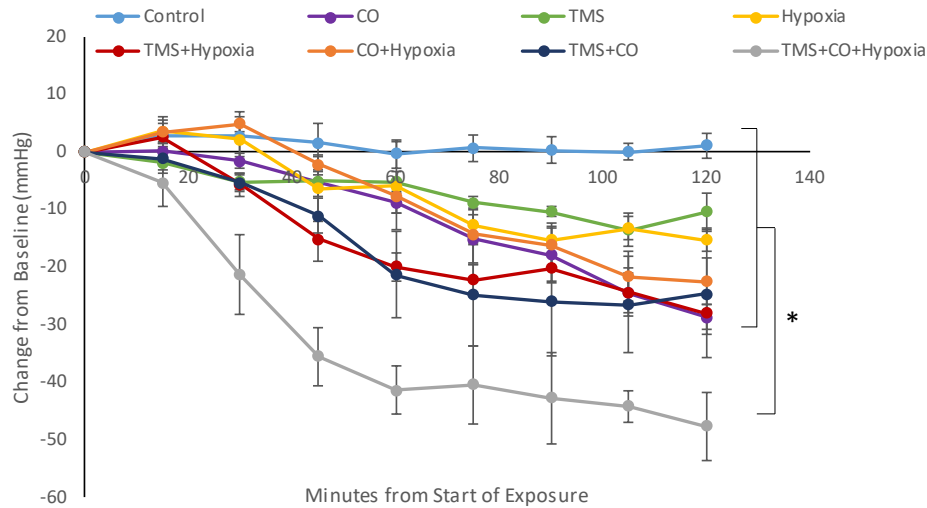


Figure 25. Mean change in arterial blood pressure, comparison between individual and combination exposures. Graph represents exposure group means $\pm$ SEM of change in blood pressure from pre-exposure baseline averaged over 15-minute time intervals.

Heart Rate

Mean heart rate was measured from the left common carotid artery of each rat during the pre-exposure baseline period and throughout the 120-minutes of exposure. Mean heart rate values were averaged every 15-minutes of exposure for each animal. The mean heart rate values that were averaged during the baseline period and during the 75-90 minute and 105-120-minute periods during exposure were compared between time periods for each exposure group.

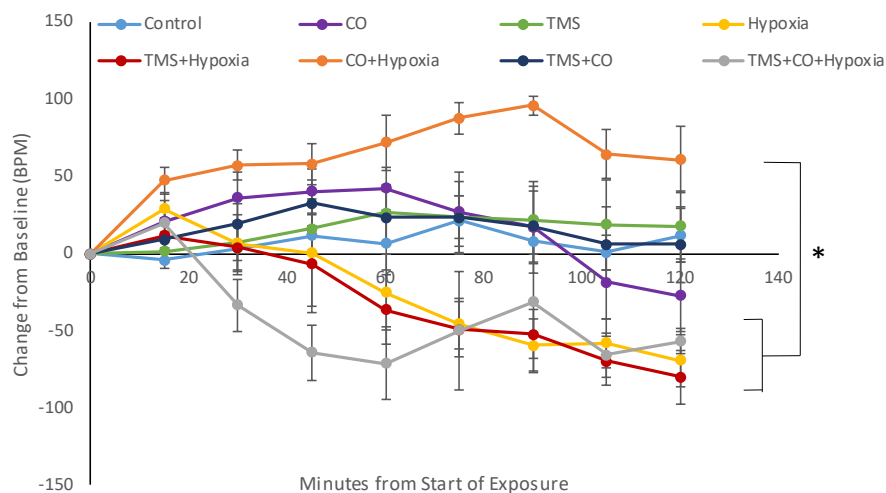


Figure 26. Mean change in heart rate, comparison between individual and combination exposures. Graph represents exposure group means $\pm$ SEM of change in blood pressure from pre-exposure baseline averaged over 15-minute time intervals.

The overall change in heart rate was compared between exposure groups. The increase in heart rate associated with the CO+Hypoxia exposure was significantly different from the Hypoxia, TMS+Hypoxia, and TMS+CO+Hypoxia groups for which the heart rate slowed ($p<0.05$, Figure 26). There were no other differences between exposure groups, with no exposure groups differing from the control group.

Plethysmography

Mean respiratory rate, tidal volume, and minute volume were measured during the exposure and during a pre-exposure baseline period using whole-body plethysmography. Averages of each respiratory parameter were calculated for the pre-exposure baseline period and every 15 minutes of exposure for each animal. The average values of each parameter were compared between three time periods for each exposure group: baseline, 75-90-minutes during exposure, 105-120-minutes during exposure. Mean respiratory rate increased from baseline for the Hypoxia, CO+Hypoxia, and TMS+CO+Hypoxia exposure groups at both timepoints ($p<0.001$ for each, except for TMS+CO+Hypoxia at 115-minutes was $p<0.05$, Figure 27). Respiratory rate also decreased with exposure to TMS at both timepoints ($p<0.05$, Figure 27), but this decrease was not observed in any of the TMS-exposed combinations.

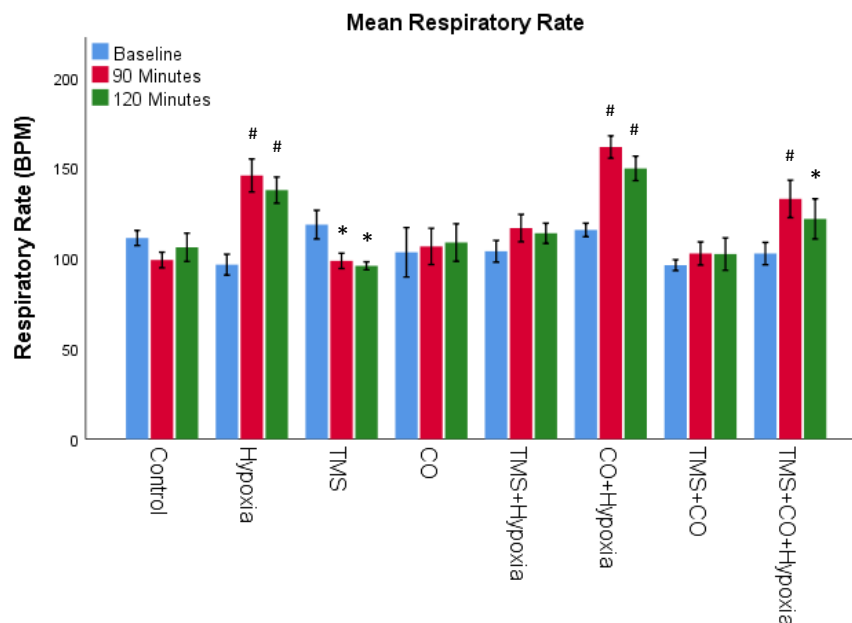


Figure 27. Mean respiratory rate during pre-exposure baseline and after 90 and 120 minutes of exposure.

Graph represents exposure group means $\pm$ SEM of respiratory rate during the 8-10 minute pre-exposure baseline period (Baseline), and at the 75-90-minute (90-minute) and 105-120-minute (120-minute) time periods during the 120-minute exposure. * $p<0.05$ compared to baseline. # $p<0.001$ compared to baseline.

Change in respiratory rate from baseline was compared between individual and combination exposure groups for each set of combinations. For the TMS+Hypoxia combination, Hypoxia and TMS+Hypoxia exposure significantly increased respiratory rate compared to the Control and TMS exposure group respiratory rate responses ($p<0.05$ for both comparisons, Figure 28A). However, the TMS+Hypoxia respiratory rate increase was attenuated from the increase due to hypoxia exposure alone ($p<0.05$, Figure 28A). A significant increase in respiratory rate was present in the Hypoxia and CO+Hypoxia groups compared to the Control and CO exposure groups ($p<0.05$, Figure 28B). It appeared that the addition of CO to the Hypoxia exposure did not impact the respiratory rate response. There was no difference in respiratory rate change between TMS+CO and the Control, CO, or TMS exposures (Figure 28C). Similar to the CO+Hypoxia combination, the TMS+CO+Hypoxia respiratory rate change did not differ from the Hypoxia respiratory rate response, however, the respiratory rate increase for both exposures was greater than the changes produced by the Control, CO, and TMS exposures (Figure 28D). Although they did not significantly differ, there was a trend of attenuation of the respiratory rate increase with the TMS+CO+Hypoxia exposure group relative to the Hypoxia exposure group that mimicked the difference in respiratory rate response displayed by the Hypoxia and TMS+Hypoxia exposure groups in Figure 28A.

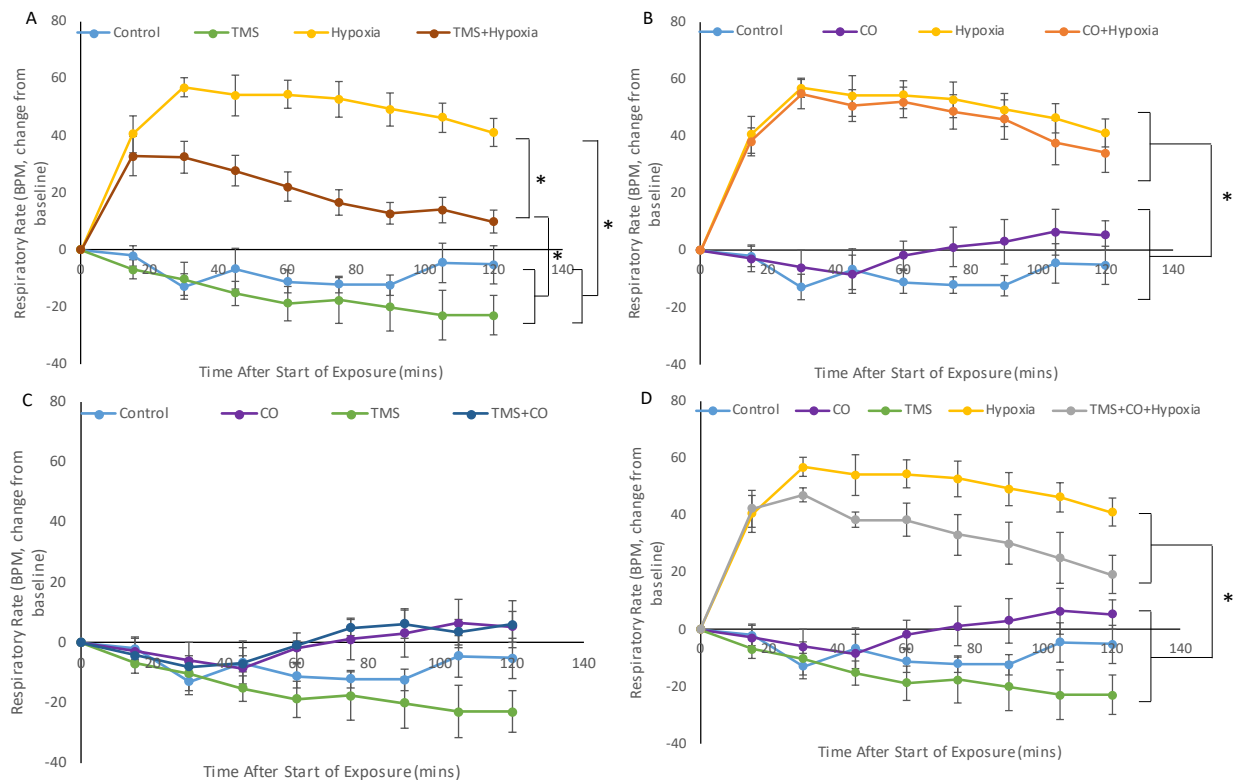


Figure 28. Mean change in respiratory rate, comparison between individual and combination exposures. Each graph represents exposure group means $\pm$ SEM of change in respiratory rate from pre-exposure baseline averaged over 15-minute time intervals. * $p<0.05$ for the indicated comparisons

Mean tidal volume was reduced from baseline at 90 minutes for the TMS+Hypoxia and TMS+CO+Hypoxia exposure groups ($p<0.05$, Figure 29). Tidal volume was also lowered for TMS+Hypoxia ($p<0.001$) and TMS+CO+Hypoxia ($p<0.05$) exposure groups at 120-minutes of the exposure, along with the TMS+CO exposure group ($p<0.05$, Figure 29). There was no significant change from baseline for any other exposure group.

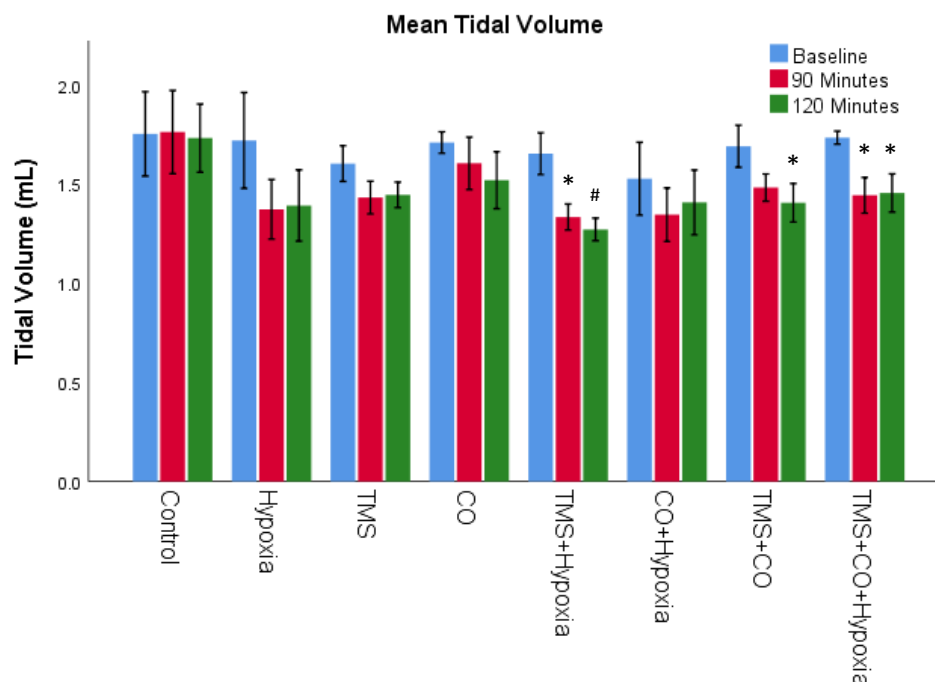


Figure 29. Mean tidal volume during pre-exposure baseline and after 90 and 120 minutes of exposure.

Graph represents exposure group means $\pm$ SEM of tidal volume during the ~10-minute pre-exposure baseline period (Baseline), at the 75-90-minute (90-minute) and 105-120-minute (120-minute) time periods during the 120-minute exposure. * $p<0.05$ compared to baseline. # $p<0.001$ compared to baseline.

Average change in tidal volume from baseline was compared between individual and combination exposure groups for each set of combinations. Although all exposure groups, with the exception of the Control group, had a decreasing tidal volume over the time course of the exposure, there was no difference in the average change in tidal volume between any exposure groups (Figure 30A-D).

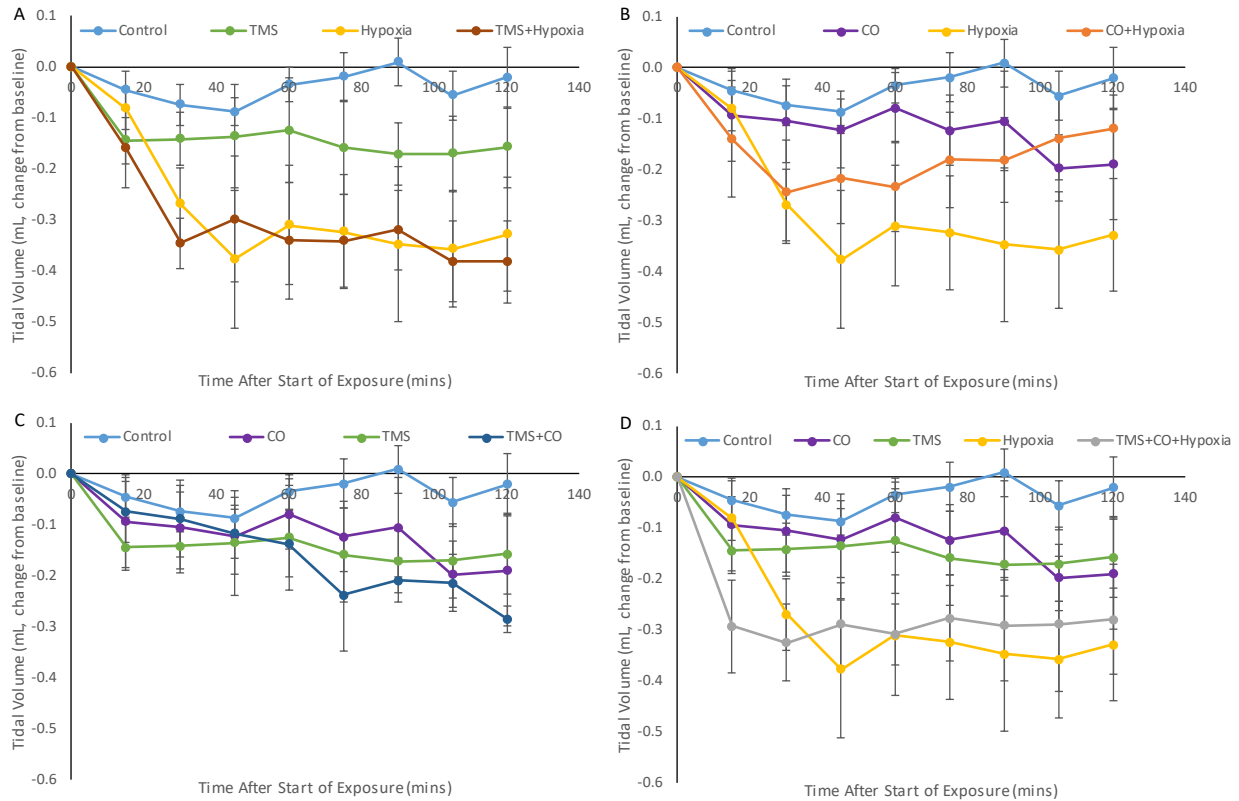


Figure 30. Mean change in tidal volume, comparison between individual and combination exposures.

Each graph represents exposure group means $\pm$ SEM of change in tidal volume from pre-exposure baseline averaged over 15-minute time intervals.

Mean minute volume increased from baseline at 90-minutes for the Hypoxia and CO+Hypoxia exposure groups ($p < 0.05$, Figure 31), a change that was similar to the increased respiratory rate present in the same exposure groups. However, unlike the respiratory rate response this increase did not persist to the 120-minute timepoint. The minute volume of TMS-exposed group was reduced compared to baseline at both timepoints ($p < 0.05$, Figure 31), which was again similar to the respiratory rate response for this group. There was no significant change from baseline for any other exposure group.

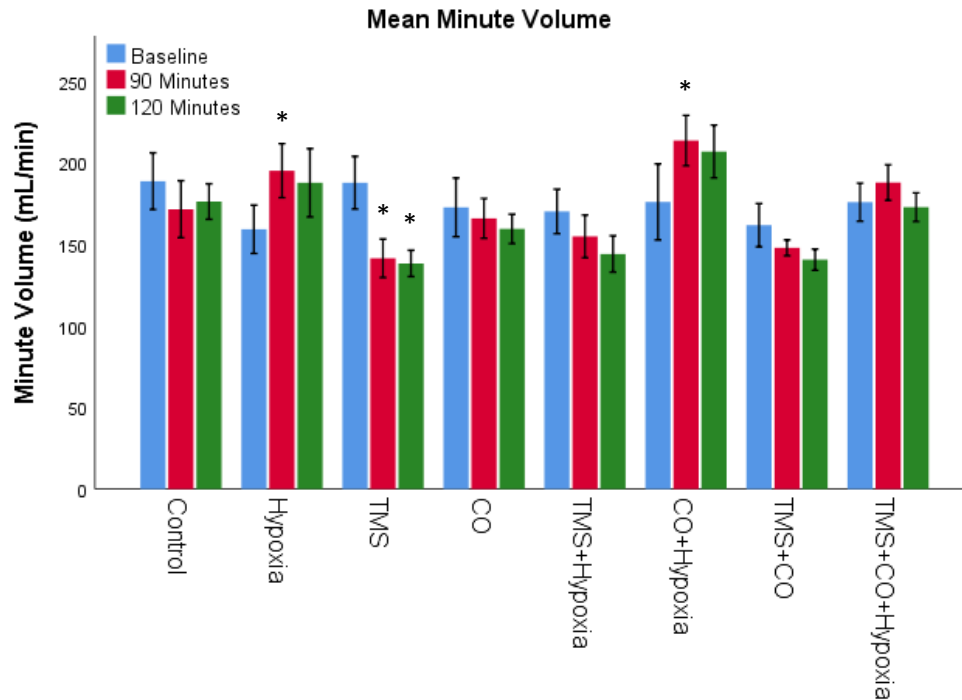


Figure 31. Mean minute volume during pre-exposure baseline and after 90 and 120 minutes of exposure.

Graph represents exposure group means $\pm$ SEM of minute volume during the ~10-minute pre-exposure baseline period (Baseline), at the 75-90 -minute (90-minute), and at the 105-120-minute (120-minute) time periods during the 120-minute exposure. * $p < 0.05$ compared to baseline. # $p < 0.001$ compared to baseline.

Change in minute volume from baseline was compared between individual and combination exposure groups for each set of combinations. For the TMS+Hypoxia combination, Hypoxia exposure significantly increased respiratory rate compared to the Control, TMS, and TMS+Hypoxia exposure group respiratory rate responses ($p < 0.05$ for all comparisons, Figure 32A). Even though the respiratory rate increase of TMS+Hypoxia was elevated over the Control and TMS exposure groups, the decrease in tidal volume was sufficient to blunt the minute volume for the TMS+Hypoxia exposure group so that it was not different from either group. A significant increase in minute volume was present in the Hypoxia and CO+Hypoxia groups compared to the control and CO exposure groups ($p < 0.05$, Figure 32B). It appeared that the addition of CO to the Hypoxia exposure did not impact the minute volume response, the same result that occurred in the respiratory rate data for both groups. There was no difference in minute volume change between TMS+CO and the control, CO, or TMS exposures (Figure 32C). Similar to the CO+Hypoxia combination, the TMS+CO+Hypoxia minute volume change did not differ from the hypoxia minute volume response, however, the CO+Hypoxia group had an increase in minute volume that was elevated over the other three exposure groups (control, TMS, CO) while the TMS+CO+Hypoxia minute volume change was only greater than the TMS exposure group (Figure 32D). Although they did not significantly differ, there was a trend of attenuation of the minute

volume increase with the TMS+CO+Hypoxia exposure group relative to the Hypoxia exposure group that mimicked the pattern of respiratory rate response between these two exposure groups.

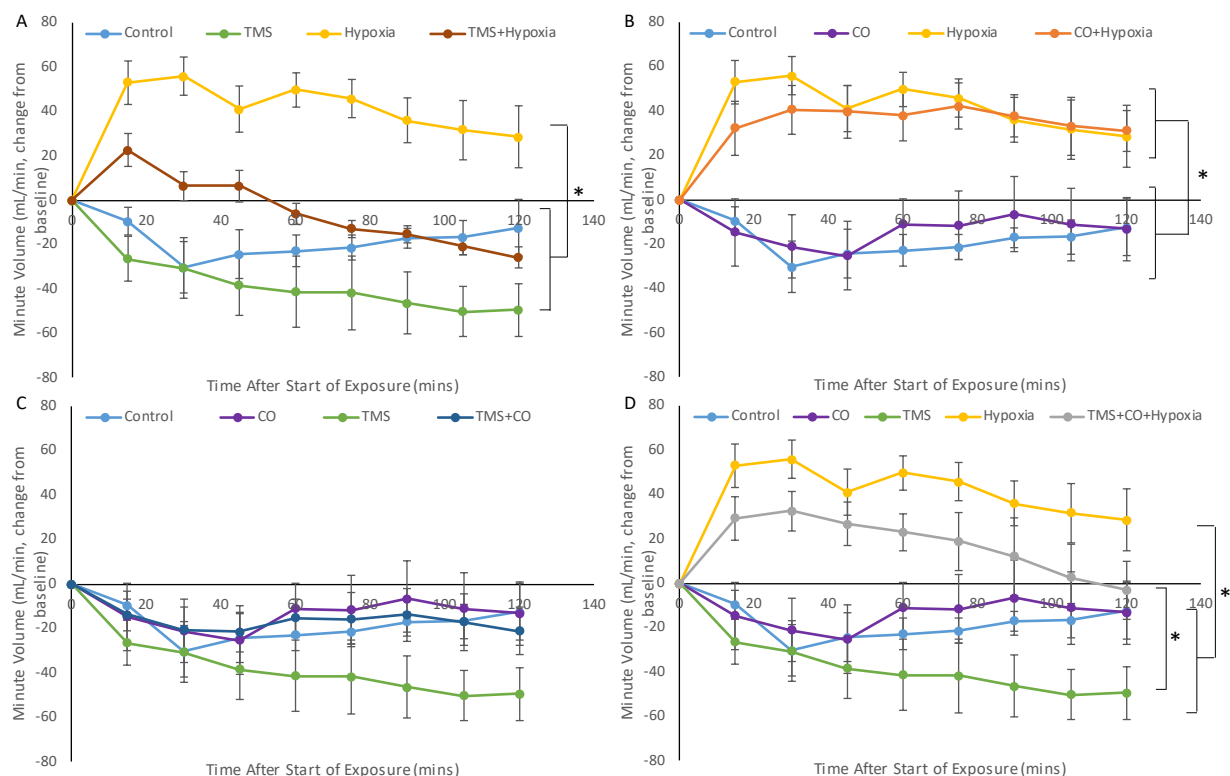


Figure 32. Mean change in tidal volume, comparison between individual and combination exposures. Each graph represents exposure group means $\pm$ SEM of change in minute volume from pre-exposure baseline averaged over 15-minute time intervals. * $p < 0.05$ for the indicated comparisons

Blood TMS Levels

Whole blood was drawn from the vena cava within 30 minutes following the cessation of exposure and analyzed for TMS concentration using gas chromatography-mass spectrometry. TMS concentrations for all samples from the control group were below the limit of detection (<0.05 mg/dL). Average TMS blood concentrations in the TMS, TMS+Hypoxia, and TMS+CO exposure groups were all very similar, each averaging 5.9 ± 0.6 mg/dL, 6.0 ± 0.5 mg/dL, and 6.0 ± 0.5 mg/dL, respectively (Figure 33). The average TMS blood concentration from the TMS+CO+Hypoxia exposure group trended higher at 7.6 ± 0.6 mg/dL (Figure 33), but there was no statistical difference between exposure groups ($p = 0.15$). Individual blood TMS levels for each animal are presented in Appendix D.

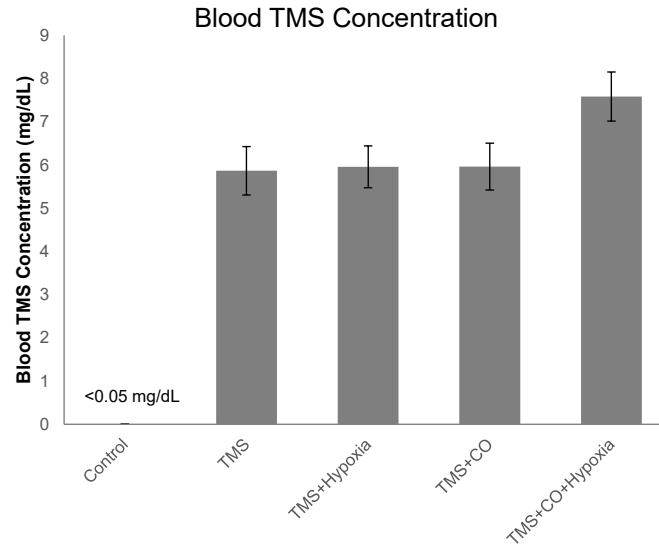


Figure 33. Average blood TMS concentration for each TMS-exposed group.

Control group also shown, all control animals had blood TMS levels below the limit of detection of 0.05 mg/dL.

Physiology Summary

The data in Table 12 summarizes the findings of the physiology experiment by listing the direction of change in each parameter for responses from each exposure group that were significantly different from the control group. The CO+Hypoxia exposure group had significant changes in all but four parameters, including an attenuated hippocampal electrophysiology input/output response and decreased arterial pCO₂ level that were not present in either of the individual CO or Hypoxia exposure groups. The changes in input/output, pCO₂, and minute volume were not present in the TMS+CO+Hypoxia exposure group, despite only differing from the CO+Hypoxia exposure in the addition of a simultaneous TMS exposure. TMS+CO+Hypoxia exposed animals did, on average, have a significant drop in blood pressure that was greater than all other exposure groups, including CO+Hypoxia.

The TMS exposure alone did not significantly impact any physiological parameters relative to the control responses. Similarly, exposure to CO and TMS+CO only increased %COHb, with a concurrent decrease in %O₂Hb, but impacted no other physiological parameters. Hypoxia exposure by itself was associated with an increase in arterial pH, increased respiratory rate and increased minute volume. It appeared that the addition of TMS to the hypoxia exposure (TMS+Hypoxia) diminished the pH and minute volume increases.

Table 12. Directions of significant changes in physiological parameters of each exposure group.

Parameter	Hypoxia	TMS	CO	TMS+Hypoxia	CO+Hypoxia	TMS+CO	TMS+CO+Hypoxia
Electrophysiology							
Input/Output	---	---	---	---	↓	---	---
Paired-Pulse Facilitation	---	---	---	---	---	---	---
Arterial Blood Measures							
pH	↑	---	---	---	↑	---	↑
pCO ₂	---	---	---	---	↓	---	---
%COHb	---	---	↑	---	↑	↑	↑
%O ₂ Hb	↓	---	↓	↓	↓	↓	↓
Mean Arterial Blood Pressure							
	---	---	---	---	---	---	↓
Heart Rate							
	---	---	---	---	---	---	---
Plethysmography							
Respiratory Rate	↑	---	---	↑	↑	---	↑
Tidal Volume	---	---	---	---	---	---	---
Minute Volume	↑	---	---	---	↑	---	---

Discussion

The objective of this study was to determine if there was evidence of additivity or synergy for combined inhalation exposures to TMS, CO, and hypoxia to provide evidence as to whether additional considerations need to be made for aircrew protection from combinations of exposures to these and similar chemicals in the aviation environment. These chemicals were first tested individually in a rodent model of exposure with an initial goal of finding individual MECs for each chemical on impairment of performance on a complex motor coordination task that was tested “real-time” while the exposure was occurring. In separate groups of rats, the same testing was performed during exposures to all pairs of combinations of the three chemicals at their MEC and an exposure to all three chemicals in combination at their MEC.

The results of this experimentation demonstrated some evidence of additivity, but weak to no evidence of synergy when comparing the performance on a complex motor coordination task between individual and combined exposures. The combination exposures were more detrimental to performance than the individual exposures as evidenced by the clear difference from baseline in accelerated latency to fall during the combination exposures. When compared to the individual exposures barely meeting the threshold for significance, this result suggests an additive effect of the combined exposures. However, when average changes in performance were compared directly between the individual and combined exposures, the only significant difference occurred between the TMS+CO+Hypoxia exposure and two of the individual exposures. Despite being the only combination that was determined to be statistically significant from its individual exposures, the average effect of TMS+CO+Hypoxia combination was found to be antagonistic, rather than additive or synergistic, when compared to the level of effect predicted by the Bliss model. Furthermore, using the Bliss Independence model of synergy, the only synergistic effect was found to be between a combination, CO+Hypoxia, that did not have a statistically significant difference

in latency to fall from the individual exposures, making it difficult to conclude that this was truly a synergistic effect.

The case for additivity was stronger than for synergy. As stated above, there was a clear difference from baseline in the accelerod performance testing for the combination exposures, while there was only a borderline statistically significant difference from baseline in the individual exposures, suggesting an additive effect of the combination. The case for additivity would be strengthened had there been a significant difference between the direct comparison of the change from baseline responses on the accelerod testing between individual and combined exposure groups, however, the fact that there was a difference present in the within-subjects responses is sufficient to support the conclusion that an additive response occurred in the combination exposures. There was even evidence of antagonism with the TMS+CO+Hypoxia combination, suggesting that there is a blunting of the effect with the combined exposures compared to what would be expected from independently additive individual responses. In summary, there was little evidence in this testing to suggest that there is an additional risk of effects of combined exposures to TMS, CO, and hypoxia on complex motor tasks that would be greater than the response predicted by straightforward additivity of the individual exposure risks.

Results of neurobehavioral testing were indicative of CNS disruption at multiple concentrations across all three exposure conditions. Accelerod is a commonly used test to assess neurological and motor deficits induced by a wide range of insults such as traumatic brain injury, neurodegeneration, or drugs that disrupt the CNS. However, this type of assessment lacks specificity with regards to the neurobiology correlating with CNS and/or motor disruption. Given the foundational data provided from this project, further analyses could use this information in more nuanced cognitive tests that would provide specific outcomes in regards to spatial reasoning, learning and memory, as well as attentional processing. The impact of low-level inhaled contaminants on these aspects of CNS function would provide information relevant not only to the ongoing research behind physiological episodes, but aviator performance as a whole. An operant learning task, such as the Five Choice Serial Test, would provide a range of a data in regards to audio-visual cues, latency and accuracy, and motivational performance in a real-time environment across a range of concentrations. Additional tasks such as the Radial Arm Maze or Morris Water Maze would identify potential deficits in long-term memory consolidation and spatial orientation, however similar to operant tasks, these tests require weeks of training for a single experimental condition. The concentration and baseline performance data provided in the study presented in this report provide sufficient foundational data to efficiently design and execute further studies requiring a greater training investment, however yielding more specific cognitive outcomes. While this study provided an initial first screening for the effects of these chemicals using a general motor coordination task, the use of one of these other more targeted tests could provide a more sensitive result and be more easily translatable to the military aviator.

The challenge with the aforementioned tests is adapting them for “real-time” testing inside of the exposure chamber to determine effects during the exposure. Although effects could linger post-exposure, the optimal time for testing that is most relevant to the real-life exposure scenario in the

aircraft is while the exposure is occurring. The present study demonstrated that this type of “real-time” testing can be performed, as evidenced by the statistically significant association between exposure concentration and decreased latency to fall on the accelerod test for the exposures tested. Other tests can be adapted using these chambers to test more specific outcomes; however, test selection will be critical because of timing or space limitations of some tests that hinder the feasibility of “real-time” testing.

Further supporting the potential for cognitive effects are the findings of the physiology experiments that were performed in this study. There was an effect of exposure to CO and Hypoxia in combination on hippocampal responses to input stimuli at a series of intensities that was not present in either of the individual CO or Hypoxia exposures. This result suggests that there is a combined effect of the CO+Hypoxia on hippocampal function that needed the contribution of both the CO and Hypoxia exposures to present at the exposure levels tested. The hippocampus is a region of the CNS that has been shown to be particularly susceptible to hypoxia exposure (Hossmann, 1999), which is consistent with the findings in this study. The combined CO and hypoxia exposure that was predicted to induce the greatest level of tissue hypoxia, due to the combination of hypoxic hypoxia and hypemic hypoxia, had the most pronounced effect on hippocampal function as measured by electrophysiology. Although no behavioral testing was performed during this study that specifically assessed changes in learning or memory associated with hippocampal function, these findings suggest that such testing would be warranted in future studies, and it is possible that those cognitive functions may be more susceptible to the combined CO and hypoxia exposure than the coordination and motor function assessed during the behavioral testing in the present study.

There were other physiological responses that were only observed with the CO+Hypoxia exposure group. Arterial pCO₂ was significantly reduced in the CO+Hypoxia group during the exposure, and the average drop in pCO₂ in the CO+Hypoxia group was over double the hypoxia exposure group, which had a pCO₂ reduction that was not significantly different from the Control group. Mean arterial pCO₂ values in the TMS+CO+Hypoxia group had a comparable magnitude of decrease to the CO+Hypoxia group, however a significant difference was not attained due to elevated variability of pCO₂ values within that group. Despite the differences in pCO₂, all three groups (Hypoxia, CO+Hypoxia, and TMS+CO+Hypoxia) had an increase in pH, although the pH increase was only 0.05 in the Hypoxia group while it was approximately 0.08 for the CO+Hypoxia and TMS+CO+Hypoxia groups.

Arterial pCO₂ plays an important homeostatic function in maintaining proper acid-base balance in the blood by its involvement in the bicarbonate buffering system (Eichenholz, 1965). Hyperventilation, during which more CO₂ is exhaled than inhaled, can cause a reduction in arterial pCO₂, which can then in turn cause an increase in pH due to a corresponding increase in bicarbonate, and is known as respiratory alkalosis (Eichenholz, 1965; Swenson, 2016). Changes in pCO₂ have been linked to alterations of cerebral blood flow independently of arterial blood pH, both through changes in pH in the cerebral spinal fluid due to rapid equilibration between CO₂ in CSF and arterial blood, and potentially through direct effects of pCO₂ on the cerebral vascular

endothelium (Yoon et al., 2012). The effect on pCO₂ in the CO+Hypoxia group corresponded to the reduction in hippocampal activity in the same animals, suggesting a correlation of the two effects that would be consistent with the effect of a reduction in pCO₂ on cerebral blood flow. Therefore, this potential link between pCO₂ reduction, respiratory alkalosis and alterations in hippocampal function as a result of the combined CO+Hypoxia exposure is an intriguing target for future study. In particular, it would be useful to determine what individual concentrations of CO and Hypoxia need to be present in the combination to induce an effect, and what, if any, cognitive functional consequences on the subject may present that could impact performance.

The %COHb values measured in this study were consistent with literature values of arterial %COHb at which behavioral effects are present. The average %COHb in the CO-exposed group increased approximately 35% from the average baseline value of 5%. Values of 20-30% COHb have been consistently associated with behavioral deficiencies in exposure studies in both rodents and humans (Benignus, 1994; Raub and Benignus, 2002), although in some cases a much lower threshold of %COHb has been associated with neurobehavioral alterations and visual sensitivity reduction (Raub and Benignus, 2002). While our %COHb value for the CO-exposed animals was slightly higher than this range at the MEC for our behavioral testing, coordination and motor function are just one metric of performance and it is possible that other more sensitive motor activity tests or cognitive-based behavioral tasks would have an effect threshold at a lower %COHb. Additionally, with even control animals having baseline values of approximately 5% COHb, and assuming there was no CO exposure in the animal's housing area, the zero calibration of the CO-oximeter used was called into question. A typical smoker's average %COHb would be around 3-5% (Hampson, et al., 2012), so the baseline value determined in the present study appears to be artificially high, and adjusting for this discrepancy would put the 40% COHb value measured during exposure closer to 35%.

The %COHb was approximately 15% higher, about 50% above baseline, in the CO+Hypoxia and TMS+CO+Hypoxia groups than it was in the group exposed to CO alone. Values of 50-70% COHb have been associated with mortality in the literature (Committee on Acute Exposure Guideline Levels, 2010), so this increase between the CO and CO+Hypoxia/TMS+CO+Hypoxia groups is significant in the added potential risk from the elevated %COHb level. Additionally, although there was no significant difference, there was also a trend of higher blood TMS levels associated with the TMS+CO+Hypoxia group. One possible explanation for these increases was the heightened minute volume of 25-35% above baseline levels that was present in these groups, in particular during the first half of the exposure. There was no change in minute volume for the group exposed to only CO. An increased minute volume would cause an intake of more CO into the lungs and increase the available amount of CO that could be absorbed into the bloodstream, consequently leading to more CO available to bind to hemoglobin. Although the minute volume of the CO+Hypoxia group stayed elevated throughout the exposure, the minute volume of the TMS+CO+Hypoxia group decreased during the second half of the exposure. Despite this difference, the %COHb levels were very similar between the two groups at both the 90- and 115-minute timepoints. This finding suggests that either the increase in respiratory rate was sufficient

in both groups throughout the exposure to maintain a steady state of elevated %COHb compared to the group receiving only CO, or that other mechanisms were responsible for this increase.

The combination exposure of all three chemicals produced a drop in blood pressure to approximately half of the baseline value, which was a significantly greater reduction than all other exposure groups. Blood pressure was reduced from baseline with CO and hypoxia exposures, which have been shown to decrease blood pressure in rats in acute exposures (Cowburn et al., 2017; Penney, 1988), and there was no evidence of an effect of TMS exposure on blood pressure. Despite the lack of effect of TMS by itself, the TMS+CO+Hypoxia exposure still reduced blood pressure more than the CO+Hypoxia exposure. A sustained reduction in blood pressure, and no compensating increase in heart rate, would over time limit perfusion to the tissues and, along with the lower oxygen intake and reduced oxygen carrying capacity, could severely limit oxygen delivery. This pathway would potentially explain why, although there was not much of a difference between the TMS+CO+Hypoxia exposure and the other combinations at 90 minutes, the persistence of the reduced oxygen delivery may have made it difficult for oxygen-starved tissues to perform in the second run. Other than the rats receiving TMS+CO+Hypoxia exposures, all other groups appeared to perform better at the 110-minute timepoint, suggesting that they adapted to the impairment to mitigate their reduced ability to stay on the rod at the 90-minute timepoint. The TMS+CO+Hypoxia rats, however, might not have been able to overcome the impairment of all three exposures and could not adapt their performance because they were physiologically incapable, potentially due to severely impaired oxygen delivery to the tissues.

TMS did not have a significant effect on any physiological parameters in the individual exposure. This result was inconsistent with previous work at NAMRU-Dayton, which demonstrated a reduction in tidal volume and minute volume with TMS exposure at concentrations from 100, 500, and 1000 ppm (unpublished data). The reduction in breathing volume was determined to be characteristic of a CNS-mediated respiratory depression, and supported the hypothesis that TMS exposure would have CNS depressant effects. In the present study, although TMS had the largest decrease in minute volume compared to baseline, it was not significantly lower than the minute volume change measured from control animals and appeared to be driven more by a respiratory rate decrease than a reduction in tidal volume. It is possible that with the additional measurements being taken, such as blood draws and electrophysiology, and consequently more interaction with the animals during the exposure compared to the previous study, that the impact on tidal volume for these animals may have been blunted.

Although the individual TMS exposure did not alter any physiological parameters, it did appear to have an impact when combined with hypoxia. The average respiratory rates in the TMS+Hypoxia and the TMS+CO+Hypoxia groups were blunted compared to the individual Hypoxia exposure, suggesting that the hypoxic ventilatory response of increased respiratory rate was being suppressed by TMS exposure. Despite the difference in ventilation, there was no difference in the reduction in %O₂Hb, with the hypoxia exposure group trending toward a larger decrease than the TMS+Hypoxia group. However, this difference in minute volume may have accounted for the difference in pH and pCO₂ changes between the Hypoxia and TMS+Hypoxia groups. The hypoxia

exposure group had an increase in pH compared to baseline greater than what was observed in the control group, along with a trend of decreased pCO₂. When TMS was added to the hypoxia exposure these changes did not occur, which would follow the logic that the less pCO₂ is being blown off by less hyperventilation, the less increase in pH would occur.

There were several limitations with this study. As other authors have stated (Foucquier and Guedj, 2015; Roell et al., 2017), the inconsistencies between synergy analysis and traditional statistical testing underlie the challenges with performing analyses of combination synergy. The Bliss Independence model was determined to be the best fit for the design of the study because of the different mechanisms of action of the chemicals and the substantially different exposure-response curves, however, the basic form of this model does not consider the variability of the individual exposures and essentially produces a point prediction of the combined effect. Therefore, as is the case with other models that predict combination effects, the variability in the individual exposures is lost, and a result can be obtained as with the CO+Hypoxia combination where synergy is found even though there was no statistical difference between the individual and combined exposure groups. But given lack of statistical difference with this combination and the criticism that the Bliss model gives a lower threshold for synergy than others (Foucquier and Guedj, 2015), it can be concluded that there is very weak to no evidence of synergy for this combination.

Measures of arterial pO₂ were planned to be obtained during this study, however, an error with instrumentation cause pO₂ readings to be unreliable with high intrasample variability and therefore the data was not used. The level of pO₂ represents the amount of oxygen dissolved in the blood and would have given another metric of hypoxic resulting from lower oxygen levels as potentially altered ventilation.

The timing of the analyses was also a limitation, especially for the physiology experiments. Only three blood draws could be accomplished per animal without overdrawing, so in addition to a baseline timepoint, the other two timepoints were chosen to match the timepoints used in the behavioral testing at 90 and 115 minutes into the exposure. Timepoints earlier in the exposure at 30 and 60 minutes would have helped track the progressive change in some of the parameters from baseline and helped determine if and when a steady-state was reached in arterial blood parameters. The electrophysiology measurements were also limited to be performed prior to, and not simultaneously with, the timepoints used for the blood draws because of the concern that interaction with the chambers necessary to draw blood would influence the animal's activity and consequently electrophysiological responses.

It was also desired to monitor TMS levels during the exposure, however, there was not sufficient sample per blood draw to perform TMS and arterial blood parameter analysis. Consequently, the TMS analysis was performed as an endpoint measure at necropsy. The half-life of TMS in the blood is expected to be similar to tert-butanol, which is on the order of 5-10 hours (Amberg, et al., 1999; James, 1996), so levels present at necropsy are expected to be representative of exposure. However, it is possible that significantly higher TMS levels would have been obtained had blood draws been performed during the exposure, and consequently there may have been a difference

between the TMS+CO+Hypoxia and other TMS exposure groups in this scenario, but this will need to be tested in a future study.

Exposure levels used in this testing were comparable to some environmentally relevant concentrations, however, were still substantially higher than average concentrations that aviators would be expected to be exposed to over the course of a typical flight. The 12% oxygen concentration used in this study is a level of hypoxia that corresponds to roughly 15,000 feet. Most aviators are required to use supplemental oxygen at these altitudes that would mitigate this reduction in oxygen provided that the equipment is functioning properly. Therefore, although 12% oxygen levels would not be expected to persist, in aircraft operating at lower altitudes (where supplemental oxygen isn't required) or in situations where insufficient oxygen was being provided by life support equipment, it is possible that moderate or intermittent hypoxia exposures could be present.

The 500 ppm concentration used in this study is comparable to the amount of CO exposure resulting from smoking a cigarette, and although aviation-relevant spikes in CO concentration to this level are possible, only CO concentrations averaging approximately 11 ppm have been measured in aircraft breathing air (Mummy, 2018). So, although there is a gap between minimum effect concentrations in the present two-hour exposures and common real-life exposures, once factoring in uncertainty in extrapolating between human and animal effects (which can be a factor of 3-10x), these MECs are sufficiently low with the additivity that was present that what are individually thought of as "safe" exposure levels might sum to elicit observable effects when present in combination. Additionally, there are other military-relevant scenarios where more persistent CO and hypoxia exposures would be present in combination, in particular high-altitude mountainous operations in which low oxygen levels would be combined with CO exposures present as a result of combustion from heating equipment in enclosed spaces like tents or buildings. Additional experimentation would be necessary to specifically identify the minimum individual concentrations of CO and hypoxia needed to induce an effect in a combination exposure, but the present study has identified this combination exposure as a potential target for such research.

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Appendix

Appendix A – Test Atmosphere Characterization for Accelerod Performance Individual Exposures

Table A1. CO exposures accelerod individual run exposure data.

Animal IDs	Run	Chamber	Exposure	Target Conc. ppm	Measured Conc. ppm	Airflow L/min	Temperature °C	RH %	SP Inches H ₂ O
1, 15	1	3	CO	0	-3 ± 3	140 ± 3	24.2 ± 0.2	60.1 ± 2.6	-0.07 ± 0.06
21, 29	2	3	CO	0	7 ± 3	139 ± 2	24.9 ± 0.2	58.6 ± 1.9	-0.06 ± 0.05
36, 38	1	4	CO	0	3 ± 4	138 ± 3	24.8 ± 0.2	54.3 ± 2.6	-0.06 ± 0.04
45, 48	2	4	CO	0	8 ± 2	138 ± 3	25.4 ± 0.2	54.1 ± 2.2	-0.06 ± 0.04
Average of Runs			CO	0	4 ± 5	139 ± 1	24.8 ± 0.5	56.7 ± 3.0	-0.06 ± 0.01
2, 3	1	3	CO	250	253 ± 14	139 ± 2	24.2 ± 0.2	59.6 ± 0.6	-0.06 ± 0.03
18, 19	2	3	CO	250	261 ± 6.7	139 ± 2	24.9 ± 0.2	63.5 ± 2.1	-0.07 ± 0.03
22, 32	1	4	CO	250	266 ± 2	138 ± 2	24.6 ± 0.2	55.5 ± 0.5	-0.06 ± 0.03
41, 47	2	4	CO	250	256 ± 4	138 ± 2	25.2 ± 0.1	60.2 ± 1.9	-0.05 ± 0.02
Average of Runs			CO	250	259 ± 6	138 ± 1	24.7 ± 0.4	59.7 ± 3.3	-0.06 ± 0.01
10, 12	1	3	CO	375	395 ± 13	144 ± 6	21.8 ± 0.2	50.1 ± 0.8	-0.10 ± 0.10
13, 14	2	3	CO	375	378 ± 11	ND	22.3 ± 0.2	ND	ND
17, 24	1	4	CO	375	372 ± 10	140 ± 2	23.0 ± 0.2	45.8 ± 0.2	-0.08 ± 0.05
33, 37	2	4	CO	375	387 ± 8	139 ± 1	23.6 ± 0.2	44.3 ± 0.5	-0.08 ± 0.01
Average of Runs			CO	375	383 ± 10	141 ± 3	22.7 ± 0.8	46.7 ± 3.0	-0.08 ± 0.01
9, 16	1	3	CO	500	513 ± 13	139 ± 2	23.7 ± 0.2	54.7 ± 0.7	-0.06 ± 0.04
23, 27	2	3	CO	500	510 ± 9	140 ± 2	24.4 ± 0.2	52.3 ± 0.5	-0.07 ± 0.04
28, 35	1	4	CO	500	516 ± 9	139 ± 2	24.0 ± 0.2	50.9 ± 0.9	-0.06 ± 0.03
40, 44	2	4	CO	500	513 ± 8	139 ± 2	24.7 ± 0.2	48.8 ± 0.5	-0.06 ± 0.03
Average of Runs			CO	500	513 ± 2	139 ± 1	24.2 ± 0.5	51.6 ± 2.5	-0.06 ± 0.01
4, 11	1	3	CO	625	664 ± 15	144 ± 1	22.0 ± 0.2	47.1 ± 1.6	-0.01 ± 0.03
30, 31	2	3	CO	625	670 ± 14	ND	22.3 ± 0.2	ND	ND
34, 39	1	4	CO	625	636 ± 8	140 ± 2	23.1 ± 0.2	43.3 ± 0.5	-0.09 ± 0.06
42, 46	2	4	CO	625	636 ± 9	140 ± 2	23.5 ± 0.2	40.7 ± 0.6	-0.08 ± 0.05
Average of Runs			CO	625	651 ± 18	141 ± 2	22.7 ± 0.7	43.7 ± 3.2	-0.06 ± 0.04
5, 6	1	3	CO	750	779 ± 16	145 ± 1	21.5 ± 0.3	54.2 ± 0.4	-0.04 ± 0.02
7, 8	2	3	CO	750	784 ± 16	146 ± 2	22.4 ± 0.2	52.7 ± 1.0	-0.05 ± 0.06
20, 25	1	4	CO	750	800 ± 8	140 ± 2	22.5 ± 0.3	49.1 ± 0.4	-0.08 ± 0.05
26, 43	2	4	CO	750	796 ± 9	140 ± 2	23.3 ± 0.2	47.7 ± 1.0	-0.08 ± 0.04
Average of Runs			CO	750	790 ± 10	143 ± 3	22.4 ± 0.8	50.9 ± 3.0	-0.06 ± 0.02

Table A2. Hypoxia exposures accelero individual run exposure data.

Animal IDs	Run	Chamber	Exposure	Target Conc. ppm	Measured Conc. ppm	Airflow L/min	N2 Flow L/min	Temperature °C	RH %	SP Inches H ₂ O
59, 64	1	3	Hypoxia	21	20.9 ± 0.0	156 ± 2	0.0 ± 0.0	23.7 ± 0.4	56.1 ± 2.8	-0.02 ± 0.03
77, 79	2	3	Hypoxia	21	20.9 ± 0.0	ND	0.0 ± 0.0	24.0 ± 0.4	ND	ND
81, 87	1	4	Hypoxia	21	20.8 ± 0.0	150 ± 3	0.0 ± 0.0	24.6 ± 0.2	49.2 ± 0.8	-0.05 ± 0.06
90, 94	2	4	Hypoxia	21	20.8 ± 0.0	150 ± 3	0.0 ± 0.0	25.2 ± 0.2	52.6 ± 0.5	-0.05 ± 0.06
Average of Runs			Hypoxia	21	20.9 ± 0.0	152 ± 3	0.0 ± 0.0	24.4 ± 0.7	52.6 ± 3.5	-0.04 ± 0.02
49, 60	1	3	Hypoxia	14	15.1 ± 0.8	111 ± 19	46.6 ± 1.4	23.4 ± 0.2	47.9 ± 8.3	-0.04 ± 0.04
63, 70	2	3	Hypoxia	14	15.0 ± 0.6	ND	47.0 ± 0.0	24.0 ± 0.3	ND	ND
71, 72	1	4	Hypoxia	14	14.9 ± 0.6	101 ± 14	47.0 ± 0.1	24.2 ± 0.2	38.7 ± 5.5	-0.03 ± 0.04
80, 91	2	4	Hypoxia	14	14.9 ± 0.5	102 ± 16	47.0 ± 0.0	24.9 ± 0.1	41.5 ± 6.1	-0.04 ± 0.06
Average of Runs			Hypoxia	14	15.0 ± 0.1	105 ± 5	46.9 ± 0.2	24.1 ± 0.6	42.7 ± 4.7	-0.04 ± 0.00
53, 54	1	3	Hypoxia	13	14.0 ± 0.7	93 ± 22	60.4 ± 1.3	21.9 ± 0.2	33.6 ± 6.8	-0.07 ± 0.03
58, 65	2	3	Hypoxia	13	14.1 ± 0.8	88 ± 25	61.0 ± 0.1	22.3 ± 0.1	31.7 ± 6.5	-0.09 ± 0.04
69, 85	1	4	Hypoxia	13	13.5 ± 0.7	93 ± 21	56.9 ± 0.2	22.6 ± 0.1	31.2 ± 5.7	-0.09 ± 0.04
86, 95	2	4	Hypoxia	13	13.5 ± 0.9	95 ± 19	57.0 ± 0.1	23.0 ± 0.1	30.4 ± 5.5	-0.08 ± 0.06
Average of Runs			Hypoxia	13	13.8 ± 0.3	92 ± 3	58.8 ± 2.1	22.5 ± 0.5	31.7 ± 1.4	-0.08 ± 0.01
50, 74	1	3	Hypoxia	12	11.8 ± 0.4	80 ± 27	68.7 ± 1.6	23.3 ± 0.2	34.1 ± 9.0	-0.05 ± 0.04
76, 78	2	3	Hypoxia	12	11.9 ± 1.1	76 ± 17	68.0 ± 0.1	24.3 ± 0.2	32.3 ± 6.3	-0.04 ± 0.05
82, 83	1	4	Hypoxia	12	11.5 ± 0.3	84 ± 28	69.6 ± 1.2	24.3 ± 0.0	30.6 ± 7.8	-0.06 ± 0.08
88, 93	2	4	Hypoxia	12	11.7 ± 1.0	77 ± 16	68.9 ± 0.1	24.8 ± 0.2	29.5 ± 6.1	-0.04 ± 0.09
Average of Runs			Hypoxia	12	11.7 ± 0.2	79 ± 4	68.8 ± 0.6	24.1 ± 0.6	31.6 ± 2.0	-0.05 ± 0.01
55, 56	1	3	Hypoxia	11	11.3 ± 1.2	83 ± 26	81.1 ± 0.1	21.7 ± 0.2	30.6 ± 9.4	-0.04 ± 0.04
62, 66	2	3	Hypoxia	11	11.2 ± 1.2	78 ± 30	80.9 ± 0.2	22.5 ± 0.2	30.1 ± 9.8	-0.07 ± 0.05
68, 89	1	4	Hypoxia	11	11.0 ± 1.1	85 ± 28	81.4 ± 1.3	22.6 ± 0.2	27.7 ± 8.6	-0.04 ± 0.05
92, 96	2	4	Hypoxia	11	10.8 ± 1.2	81 ± 29	80.9 ± 0.2	23.4 ± 0.2	27.4 ± 8.7	-0.06 ± 0.09
Average of Runs			Hypoxia	11	11.1 ± 0.2	82 ± 3	81.1 ± 0.2	22.6 ± 0.7	28.9 ± 1.7	-0.05 ± 0.01
51, 52	1	3	Hypoxia	10	10.9 ± 1.0	87 ± 37	84.2 ± 0.4	21.7 ± 0.2	32.2 ± 12.1	-0.05 ± 0.05
57, 61	2	3	Hypoxia	10	11.1 ± 1.3	ND	83.8 ± 0.2	21.8 ± 0.4	ND	ND
67, 73	1	4	Hypoxia	10	10.5 ± 1.0	78 ± 32	84.2 ± 0.4	22.8 ± 0.1	26.1 ± 8.7	-0.07 ± 0.05
75, 84	2	4	Hypoxia	10	10.5 ± 1.3	82 ± 28	84.0 ± 0.2	23.0 ± 0.1	26.1 ± 8.6	-0.05 ± 0.05
Average of Runs			Hypoxia	10	10.7 ± 0.3	82 ± 4	84.1 ± 0.2	22.3 ± 0.7	28.1 ± 3.5	-0.06 ± 0.01

Table A3. Combination exposures accelero individual run exposure data.

Animal IDs	Run	Chamber	Exposure	Measured Conc. CO (ppm)	Measured Conc. O ₂ (%)	Measured Conc. TMS (ppm)	Airflow L/min	N ₂ Flow L/min	Temperature °C	RH %	SP Inches H ₂ O
100, 105	1	3	H, T	N/A	13.4 ± 0.9	772 ± 13	79 ± 29	ND	24.7 ± 0.1	37.0 ± 10.0	-0.05 ± 0.05
110, 114	2	3	H, T	N/A	13.7 ± 0.8	769 ± 27	85 ± 31	ND	25.3 ± 0.2	38.1 ± 9.9	-0.07 ± 0.06
117, 118	1	4	H, T	N/A	12.9 ± 1.0	761 ± 9	83 ± 25	ND	25.3 ± 0.1	34.0 ± 9.0	-0.02 ± 0.05
124, 128	2	4	H, T	N/A	13.2 ± 0.8	768 ± 21	90 ± 33	ND	25.8 ± 0.1	34.8 ± 9.4	-0.05 ± 0.05
Average of Runs			H, T	N/A	13.3 ± 0.3	767 ± 4	84 ± 5	ND	25.3 ± 0.5	36.0 ± 1.9	-0.05 ± 0.02
101, 102	1	3	C, H	514 ± 7	13.2 ± 1.0	N/A	108 ± 31	68.0 ± 0.1	24.6 ± 0.3	49.9 ± 12.6	-0.03 ± 0.03
111, 116	2	3	C, H	505 ± 9	13.1 ± 0.9	N/A	ND	68.0 ± 0.1	25.3 ± 0.4	ND	ND
120, 122	1	4	C, H	507 ± 3	12.7 ± 1.0	N/A	94 ± 23	68.0 ± 0.2	25.2 ± 0.2	40.5 ± 9.8	-0.03 ± 0.06
125, 127	2	4	C, H	505 ± 25	12.6 ± 0.8	N/A	95 ± 24	67.9 ± 0.1	26.0 ± 0.2	40.1 ± 10.0	-0.04 ± 0.05
Average of Runs			C, H	508 ± 4	12.9 ± 0.3	N/A	99 ± 8	68.0 ± 0.0	25.3 ± 0.6	43.5 ± 5.5	-0.03 ± 0.00
99, 107	1	3	C, T	540 ± 50	N/A	752 ± 16	142 ± 3	N/A	24.1 ± 0.1	54.3 ± 0.5	-0.06 ± 0.04
108, 113	2	3	C, T	539 ± 64	N/A	792 ± 11	142 ± 2	N/A	24.4 ± 0.2	58.1 ± 0.4	-0.06 ± 0.06
115, 119	1	4	C, T	511 ± 48	N/A	749 ± 16	146 ± 3	N/A	24.9 ± 0.2	49.9 ± 0.2	-0.06 ± 0.04
121, 123	2	4	C, T	561 ± 76	N/A	770 ± 12	146 ± 3	N/A	25.2 ± 0.1	53.5 ± 0.4	-0.05 ± 0.05
Average of Runs			C, T	537 ± 20	N/A	766 ± 20	144 ± 2	N/A	24.6 ± 0.5	53.9 ± 3.4	-0.06 ± 0.00
97, 98	1	3	C, H, T	542 ± 8	14.0 ± 0.9	812 ± 11	92 ± 26	68.0 ± 0.1	23.2 ± 0.1	28.2 ± 7.1	-0.06 ± 0.06
103, 104	2	3	C, H, T	535 ± 7	14.0 ± 0.8	807 ± 11	92 ± 26	68.0 ± 0.1	23.3 ± 0.1	26.7 ± 6.3	-0.05 ± 0.04
106, 109	1	4	C, H, T	572 ± 21	13.2 ± 0.9	783 ± 12	96 ± 27	68.0 ± 0.2	24.0 ± 0.1	25.6 ± 6.4	-0.08 ± 0.09
113, 126	2	4	C, H, T	534 ± 8	13.2 ± 0.8	770 ± 11	97 ± 25	68.0 ± 0.2	24.2 ± 0.1	24.0 ± 5.8	-0.07 ± 0.06
Average of Runs			C, H, T	546 ± 18	13.6 ± 0.5	793 ± 20	94 ± 3	68.0 ± 0.0	23.7 ± 0.5	26.1 ± 1.8	-0.07 ± 0.01

Appendix B – Individual Animal Weights and Accelerod Performance Results

Table B1. CO exposures individual animal body weights and accelerod latency to fall (total seconds and percent change from baseline) at baseline, 90 minute and 110 minute timepoints.

ID	Weight (g)	CO Concentration ppm	Baseline seconds	90 Min. seconds	Latency to Fall		
					90 Min. Change %	110 Min. seconds	110 Min. Change %
1	284	4	150	198	32.0	256	70.7
15	269	4	251	272	8.4	207	-17.5
21	261	4	205	215	4.9	251	22.4
29	262	4	223	205	-8.1	169	-24.2
36	263	4	238	272	14.3	221	-7.1
38	264	4	234	217	-7.3	212	-9.4
45	284	4	126	123	-2.4	144	14.3
48	262	4	208	208	0.0	241	15.9
Group Average		4	204 ± 16	214 ± 17	5.2 ± 4.7	213 ± 14	8.1 ± 10.7
2	269	259	255	236	-7.5	300	17.6
3	303	259	174	175	0.6	206	18.4
18	299	259	203	300	47.8	300	47.8
19	271	259	199	214	7.5	278	39.7
22	279	259	232	300	29.3	300	29.3
32	250	259	190	160	-15.8	215	13.2
41	265	259	260	178	-31.5	162	-37.7
47	278	259	189	193	2.1	225	19.0
Group Average		259	213 ± 11	220 ± 19	4.1 ± 8.8	248 ± 19	18.4 ± 9.1
10	355	383	168	181	7.7	203	20.8
12	344	383	193	300	55.4	300	55.4
13	333	383	233	133	-42.9	102	-56.2
14	371	383	162	272	67.9	203	25.3
17	356	383	221	300	35.7	300	35.7
24	327	383	204	204	0.0	139	-31.9
33	344	383	181	151	-16.6	72	-60.2
37	274	383	115	138	20.0	196	70.4
Group Average		383	185 ± 13	210 ± 25	15.9 ± 13.0	189 ± 30	7.4 ± 17.8
9	302	513	285	213	-25.3	227	-20.4
16	277	513	221	176	-20.4	154	-30.3
23	294	513	230	129	-43.9	152	-33.9
27	263	513	180	229	27.2	257	42.8
28	280	513	215	106	-50.7	174	-19.1
35	305	513	193	83	-57.0	206	6.7
40	304	513	207	148	-28.5	174	-15.9
44	293	513	201	204	1.5	288	43.3
Group Average		513	217 ± 11	161 ± 19	-24.6 ± 9.9	204 ± 18	-3.3 ± 11.0
4	333	651	178	116	-34.8	141	-20.8
11	344	651	109	74	-32.1	100	-8.3
30	318	651	222	91	-59.0	165	-25.7
31	310	651	158	145	-8.2	147	-7.0
34	300	651	194	178	-8.2	270	39.2
39	352	651	201	163	-18.9	225	11.9
42	345	651	140	122	-12.9	105	-25.0
46	323	651	171	191	11.7	195	14.0
Group Average		651	172 ± 13	135 ± 15	-20.3 ± 7.6	169 ± 21	-2.7 ± 8.1
5	295	790	246	126	-48.8	169	-31.3
6	348	790	241	216	-10.4	250	3.7
7	325	790	213	134	-37.1	117	-45.1
8	349	790	256	92	-64.1	146	-43.0
20	313	790	250	172	-31.2	244	-2.4
25	377	790	134	127	-5.2	151	12.7
26	326	790	218	181	-17.0	102	-53.2
43	360	790	211	160	-24.2	171	-19.0
Group Average		790	221 ± 14	151 ± 14	-29.7 ± 7.0	169 ± 19	-22.2 ± 8.8

Table B2. Hypoxia exposures individual animal body weights and accelerod latency to fall (total seconds and percent change from baseline) at baseline, 90 minute and 110 minute timepoints.

ID	Weight (g)	O ₂ Concentration %	Latency to Fall				
			Baseline seconds	90 Min. seconds	90 Min. Change %	110 Min. seconds	110 Min. Change %
59	277	20.9	193	275	42.5	254	31.6
64	283	20.9	202	208	3.0	171	-15.3
77	295	20.9	225	221	-1.8	229	1.8
79	278	20.9	201	213	6.0	246	22.4
81	264	20.9	172	186	8.1	194	12.8
87	283	20.9	160	160	0.0	194	21.3
90	305	20.9	129	164	27.1	208	61.2
94	255	20.9	201	138	-31.3	228	13.4
Group Average		20.9	185 ± 11	196 ± 15	6.7 ± 7.7	216 ± 10	18.6 ± 7.9
49	308	15.0	157	196	24.8	200	27.4
60	282	15.0	187	150	-19.8	170	-9.1
63	308	15.0	143	155	8.4	195	36.4
70	284	15.0	135	181	34.1	200	48.1
71	282	15.0	288	292	1.4	300	4.2
72	320	15.0	194	202	4.1	248	27.8
80	299	15.0	217	185	-14.7	234	7.8
91	302	15.0	101	101	0.0	169	67.3
Group Average		15.0	178 ± 20	183 ± 19	4.8 ± 6.4	215 ± 16	26.2 ± 8.8
53	384	13.8	188	150	-20.2	170	-9.6
54	361	13.8	169	210	24.3	238	40.8
58	324	13.8	219	106	-51.6	219	0.0
65	311	13.8	266	193	-27.4	181	-32.0
69	341	13.8	209	174	-16.7	139	-33.5
85	314	13.8	244	173	-29.1	214	-12.3
86	325	13.8	254	221	-13.0	223	-12.2
95	343	13.8	234	251	7.3	254	8.5
Group Average		13.8	223 ± 12	185 ± 16	-15.8 ± 8.2	205 ± 14	-6.3 ± 8.4
50	293	11.7	228	212	-7.0	200	-12.3
74	281	11.7	199	165	-17.1	224	12.6
76	267	11.7	194	109	-43.8	140	-27.8
78	298	11.7	268	196	-26.9	188	-29.9
82	288	11.7	198	155	-21.7	173	-12.6
83	279	11.7	236	235	-0.4	275	16.5
88	312	11.7	193	171	-11.4	171	-11.4
93	273	11.7	94	80	-14.9	126	34.0
Group Average		11.7	201 ± 18	165 ± 18	-17.9 ± 4.7	187 ± 17	-3.9 ± 8.0
55	349	11.1	114	118	3.5	151	32.5
56	342	11.1	232	105	-54.7	124	-46.6
62	369	11.1	166	97	-41.6	148	-10.8
66	297	11.1	218	95	-56.4	142	-34.9
68	333	11.1	123	89	-27.6	111	-9.8
89	365	11.1	169	160	-5.3	192	13.6
92	357	11.1	197	95	-51.8	169	-14.2
96	354	11.1	163	106	-35.0	102	-37.4
Group Average		11.1	173 ± 15	108 ± 8	-33.6 ± 8.0	142 ± 11	-13.5 ± 9.4
51	361	10.7	271	75	-72.3	80	-70.5
52	332	10.7	263	172	-34.6	180	-31.6
57	342	10.7	228	159	-30.3	119	-47.8
61	335	10.7	221	136	-38.5	159	-28.1
67	316	10.7	219	91	-58.4	104	-52.5
73	314	10.7	272	163	-40.1	228	-16.2
75	347	10.7	238	142	-40.3	153	-35.7
84	317	10.7	212	56	-73.6	144	-32.1
Group Average		10.7	241 ± 9	124 ± 16	-48.5 ± 6.1	146 ± 16	-39.3 ± 6.0

Table B3. Combination exposures individual animal body weights and accelerod latency to fall (total seconds and percent change from baseline) at baseline, 90 minute and 110 minute timepoints.

ID	Weight (g)	Exposure Group	Latency to Fall				
			Baseline seconds	90 Min. seconds	90 Min. Change %	110 Min. seconds	110 Min. Change %
101	312	CO+Hypoxia	187	85	-54.5	131	-29.9
102	302	CO+Hypoxia	208	162	-22.1	153	-26.4
111	305	CO+Hypoxia	209	122	-41.6	117	-44.0
116	325	CO+Hypoxia	196	119	-39.3	123	-37.2
120	295	CO+Hypoxia	143	86	-39.9	118	-17.5
122	291	CO+Hypoxia	228	133	-41.7	128	-43.9
125	274	CO+Hypoxia	189	137	-27.5	145	-23.3
127	300	CO+Hypoxia	158	139	-12.0	175	10.8
Group Average		CO+Hypoxia	190 ± 10	123 ± 9	-34.8 ± 4.7	136 ± 7	-26.4 ± 6.3
99	333	TMS+CO	223	178	-20.2	281	26.0
107	362	TMS+CO	183	171	-6.6	148	-19.1
108	298	TMS+CO	175	71	-59.4	193	10.3
113	340	TMS+CO	175	92	-47.4	23	-86.9
115	321	TMS+CO	168	113	-32.7	43	-74.4
119	333	TMS+CO	239	214	-10.5	206	-13.8
121	364	TMS+CO	162	142	-12.3	91	-43.8
123	349	TMS+CO	119	45	-62.2	48	-59.7
Group Average		TMS+CO	181 ± 13	128 ± 21	-31.4 ± 7.9	129 ± 33	-32.7 ± 14.2
100	287	TMS+Hypoxia	241	111	-53.9	67	-72.2
105	289	TMS+Hypoxia	201	115	-42.8	115	-42.8
110	322	TMS+Hypoxia	260	140	-46.2	140	-46.2
114	312	TMS+Hypoxia	246	147	-40.2	153	-37.8
117	299	TMS+Hypoxia	166	170	2.4	58	-65.1
118	301	TMS+Hypoxia	204	132	-35.3	215	5.4
124	279	TMS+Hypoxia	253	189	-25.3	261	3.2
128	280	TMS+Hypoxia	210	168	-20.0	178	-15.2
Group Average		TMS+Hypoxia	223 ± 11	147 ± 10	-32.7 ± 6.3	148 ± 25	-33.8 ± 10.3
97	326	TMS+CO+Hypoxia	178	177	-0.6	116	-34.8
98	309	TMS+CO+Hypoxia	213	172	-19.2	181	-15.0
103	335	TMS+CO+Hypoxia	198	139	-29.8	75	-62.1
104	355	TMS+CO+Hypoxia	177	99	-44.1	19	-89.3
106	363	TMS+CO+Hypoxia	124	37	-70.2	55	-55.6
109	362	TMS+CO+Hypoxia	170	120	-29.4	157	-7.6
112	352	TMS+CO+Hypoxia	183	99	-45.9	153	-16.4
126	329	TMS+CO+Hypoxia	184	127	-31.0	127	-31.0
Group Average		TMS+CO+Hypoxia	178 ± 9	121 ± 16	-33.8 ± 7.2	110 ± 20	-39.0 ± 9.9

Appendix C – Test Atmosphere Characterization for Physiology Analysis Individual Exposures

Table C1. Single exposures physiology individual run exposure data.

Animal ID	Exposure	Measured Conc. CO (ppm)	Measured Conc. O2 (%)	Measured Conc. TMS (ppm)	Temperature °C	RH %	SP Inches H2O
204	Control	5 ± 1	20.8 ± 0.0	0 ± 0	21.8 ± 0.3	40.0 ± 0.6	-0.04 ± 0.00
232	Control	0 ± 0	20.9 ± 0.0	0 ± 8	22.3 ± 0.1	24.9 ± 0.6	-0.04 ± 0.00
235	Control	0 ± 0	20.9 ± 0.0	0 ± 10	23.4 ± 0.1	47.7 ± 1.2	-0.04 ± 0.00
215	Control	-7 ± 1	21.0 ± 0.0	0 ± 8	20.1 ± 0.2	39.9 ± 1.2	-0.05 ± 0.00
224	Control	1 ± 0	20.9 ± 0.0	-4 ± 6	20.0 ± 0.1	32.6 ± 0.3	-0.04 ± 0.00
225	Control	4 ± 0	20.9 ± 0.0	-6 ± 10	21.2 ± 0.2	28.3 ± 2.1	-0.04 ± 0.00
Average of Runs	Control	0 ± 4	20.9 ± 0.1	-2 ± 3	21.5 ± 1.3	35.6 ± 8.5	-0.04 ± 0.00
210	CO	488 ± 30	20.7 ± 0.0	0 ± 0	22.4 ± 0.1	40.5 ± 0.3	-0.05 ± 0.00
223	CO	488 ± 31	20.9 ± 0.0	5 ± 8	21.7 ± 0.1	26.6 ± 0.6	-0.02 ± 0.03
211	CO	495 ± 28	20.9 ± 0.0	9 ± 6	23.7 ± 0.2	46.6 ± 0.4	-0.05 ± 0.00
212	CO	490 ± 29	20.9 ± 0.0	8 ± 7	21.1 ± 0.2	40.5 ± 0.9	-0.04 ± 0.00
708	CO	484 ± 28	20.9 ± 0.0	1 ± 9	23.4 ± 0.2	23.4 ± 0.5	-0.05 ± 0.00
Average of Runs	CO	489 ± 4	20.9 ± 0.1	5 ± 4	22.5 ± 1.1	35.5 ± 10.0	-0.04 ± 0.01
208	Hypoxia	4 ± 1	12.0 ± 0.5	0 ± 0	22.0 ± 0.1	42.3 ± 0.6	-0.04 ± 0.01
216	Hypoxia	-1 ± 0	12.1 ± 0.5	0 ± 6	21.6 ± 0.3	27.1 ± 0.4	-0.04 ± 0.00
211	Hypoxia	0 ± 0	12.0 ± 0.5	11 ± 11	23.6 ± 0.4	48.0 ± 0.8	-0.04 ± 0.00
233	Hypoxia	-2 ± 0	12.1 ± 0.5	5 ± 7	20.8 ± 0.2	40.0 ± 0.4	-0.05 ± 0.00
227	Hypoxia	1 ± 0	12.0 ± 0.5	-5 ± 6	21.1 ± 0.1	27.1 ± 1.6	-0.05 ± 0.00
Average of Runs	Hypoxia	1 ± 2	12.0 ± 0.0	2 ± 6	21.8 ± 1.1	36.9 ± 9.4	-0.04 ± 0.01
206	TMS	1 ± 0	20.8 ± 0.0	821 ± 66	22.0 ± 0.2	40.5 ± 0.9	-0.04 ± 0.00
229	TMS	2 ± 0	20.9 ± 0.0	771 ± 53	22.2 ± 0.1	27.5 ± 0.7	-0.04 ± 0.00
226	TMS	-1 ± 0	21.0 ± 0.0	760 ± 62	24.1 ± 0.2	47.4 ± 0.4	-0.03 ± 0.00
239	TMS	-1 ± 0	20.6 ± 0.0	775 ± 67	20.9 ± 0.2	39.4 ± 0.4	-0.05 ± 0.00
234	TMS	0 ± 0	21.1 ± 0.0	751 ± 60	21.3 ± 0.1	27.0 ± 1.5	-0.04 ± 0.00
Average of Runs	TMS	0 ± 1	20.9 ± 0.2	776 ± 27	22.1 ± 1.3	36.4 ± 8.8	-0.04 ± 0.00

Table C2. Combined exposures physiology individual run exposure data.

Animal ID	Exposure	Measured Conc. CO (ppm)	Measured Conc. O2 (%)	Measured Conc. TMS (ppm)	Temperature °C	RH %	SP Inches H2O
207	TMS+Hypoxia	2 ± 0	12.1 ± 0.6	745 ± 68	21.8 ± 0.1	26.1 ± 1.4	-0.04 ± 0.00
230	TMS+Hypoxia	0 ± 0	12.0 ± 0.6	740 ± 63	23.2 ± 0.1	52.4 ± 0.7	-0.04 ± 0.00
218	TMS+Hypoxia	-1 ± 0	12.1 ± 0.6	742 ± 62	20.6 ± 0.1	38.0 ± 0.4	-0.05 ± 0.00
222	TMS+Hypoxia	0 ± 0	12.0 ± 0.6	761 ± 57	20.9 ± 0.1	40.9 ± 0.4	-0.04 ± 0.00
807	TMS+Hypoxia	1 ± 0	11.9 ± 0.5	746 ± 47	23.3 ± 0.1	24.5 ± 0.4	0.00 ± 0.00
Average of Runs	TMS+Hypoxia	0 ± 1	12.0 ± 0.1	747 ± 8	22.0 ± 1.3	36.4 ± 11.5	-0.03 ± 0.02
202	CO+Hypoxia	490 ± 27	11.9 ± 0.5	-5 ± 5	20.6 ± 0.4	30.2 ± 1.7	-0.04 ± 0.00
240	CO+Hypoxia	495 ± 31	12.2 ± 0.5	10 ± 6	24.0 ± 0.0	45.8 ± 1.1	-0.05 ± 0.00
228	CO+Hypoxia	490 ± 24	12.0 ± 0.4	2 ± 5	24.8 ± 0.2	49.5 ± 0.4	-0.04 ± 0.00
236	CO+Hypoxia	493 ± 26	12.3 ± 0.4	-4 ± 6	20.4 ± 0.2	31.0 ± 0.3	-0.08 ± 0.04
Average of Runs	CO+Hypoxia	492 ± 3	12.1 ± 0.2	1 ± 7	22.5 ± 2.2	39.1 ± 10.0	-0.06 ± 0.02
203	TMS+CO	493 ± 26	20.9 ± 0.0	741 ± 48	21.5 ± 0.4	30.0 ± 1.5	-0.06 ± 0.02
237	TMS+CO	491 ± 26	20.8 ± 0.1	739 ± 52	22.7 ± 0.1	25.0 ± 0.7	-0.04 ± 0.00
231	TMS+CO	494 ± 26	20.8 ± 0.0	762 ± 56	25.5 ± 0.2	48.9 ± 0.4	-0.04 ± 0.00
214	TMS+CO	495 ± 29	21.0 ± 0.0	756 ± 61	21.7 ± 0.2	39.5 ± 0.6	-0.04 ± 0.00
Average of Runs	TMS+CO	493 ± 2	20.9 ± 0.1	749 ± 11	22.9 ± 1.8	35.8 ± 10.6	-0.05 ± 0.01
205	TMS+CO+Hypoxia	490 ± 30	12.3 ± 0.5	740 ± 64	22.1 ± 0.1	24.6 ± 1.1	-0.07 ± 0.00
213	TMS+CO+Hypoxia	491 ± 25	12.2 ± 0.4	741 ± 57	23.6 ± 0.1	48.1 ± 0.6	-0.04 ± 0.00
217	TMS+CO+Hypoxia	512 ± 30	12.2 ± 0.5	741 ± 63	20.7 ± 0.1	37.8 ± 0.4	-0.05 ± 0.00
238	TMS+CO+Hypoxia	493 ± 27	12.3 ± 0.5	746 ± 58	21.0 ± 0.2	30.8 ± 0.4	-0.05 ± 0.01
711	TMS+CO+Hypoxia	495 ± 26	12.2 ± 0.5	749 ± 58	24.2 ± 0.3	23.3 ± 0.6	-0.04 ± 0.00
Average of Runs	TMS+CO+Hypoxia	496 ± 9	12.2 ± 0.1	743 ± 4	22.3 ± 1.5	32.9 ± 10.2	-0.05 ± 0.01

Appendix D – Individual Animal Blood TMS Levels

ID	Exposure Group	[Blood TMS] µg/mL	[Blood TMS] mg/dL
204	Control	<0.5	<0.05
225	Control	<0.5	<0.05
224	Control	<0.5	<0.05
232	Control	<0.5	<0.05
235	Control	<0.5	<0.05
206	TMS	44.0	4.4
226	TMS	63.2	6.3
234	TMS	73.8	7.4
239	TMS	65.0	6.5
229	TMS	47.4	4.7
222	TMS+Hypoxia	53.8	5.4
207	TMS+Hypoxia	48.6	4.9
218	TMS+Hypoxia	53.4	5.3
230	TMS+Hypoxia	73.6	7.4
807	TMS+Hypoxia	68.6	6.9
203	TMS+CO	60.6	6.1
214	TMS+CO	46.0	4.6
231	TMS+CO	72.6	7.3
237	TMS+CO	59.4	5.9
205	TMS+CO+Hypoxia	73.0	7.3
217	TMS+CO+Hypoxia	63.6	6.4
711	TMS+CO+Hypoxia	91.0	9.1
213	TMS+CO+Hypoxia	75.8	7.6