REAL-TIME NEUROBEHAVIORAL EFFECTS OF SINGLE AND COMBINED CHEMICAL EXPOSURES VIA INHALATION IN RATS

Taj D. King, Ph.D.
Shawn M. McInturf
Karen L. Mumy, Ph.D., DABT
Naval Medical Research Unit Dayton
Wright-Patterson AFB, OH 45433

Richard Arden James
Elizabeth Phillips
Brian Sharits
Joshua Stricker
Kathleen Frondorf
Parsons Corporation
Naval Medical Research Unit Dayton
Wright-Patterson AFB, OH 45433

David Holtzapple
Odyssey Systems Consulting Group
Naval Medical Research Unit Dayton
Wright-Patterson AFB, OH 45433

Erin Roberts
Oak Ridge Institute for Science and Education
Naval Medical Research Unit Dayton
Wright-Patterson AFB, OH 45433

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ANIMAL CARE AND USE DISCLAIMER

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

Individuals who experience Physiological Episodes (PEs) may present with a broad range of symptoms including the following: tingling and/or weakness of the limbs and extremities, headaches, dizziness, and in some cases, loss of consciousness. A number of chemical contaminants have been identified in the breathing gases onboard U.S. Navy T-45 and F-18 family of aircraft. Two of those chemicals included trimethylsilanol (TMS) and trichloromethane (TCM)/chloroform. TMS and TCM have been shown to cause anesthetic effects; therefore, concern has been raised that their presence, or combined presence, may be a factor in some of the recent PEs that have been experienced by Navy aircrews.

The purpose of this study was to determine if exposure to these two different compounds in combination could cause short-term effects on the brain during exposure (i.e., in real-time) using a rat model. The reason that this is important is because it was determined that low concentrations of chemical contaminants were being detected in cockpit environments, including in the breathing air supplied by the Onboard Oxygen Generating System (OBOGS). Although many of the chemicals were found at very low levels (i.e. lower than what would be expected to cause an effect), it was unclear if a short exposure (i.e. 30 minutes) to a combination of the chemicals, each at a low level, can contribute to the cognitive effects and symptoms observed during PEs. This study determined the exposure-dependent effects of the two chemicals on male Sprague-Dawley rats. In Experiment 1, rats were exposed to low, middle, or high concentrations of TMS and TCM individually for 30 minutes while the animals were undergoing real-time behavioral testing. This process was necessary to determine the effective concentration for each chemical. Secondly, the two chemicals were combined based on the no observed adverse effect level or lowest observed adverse effect level (NOAEL/LOAEL) of each chemical, and rats were exposed to a combination of the two chemicals, which was hypothesized to cause additive/synergistic effects. The control group was exposed to filtered air only. In Experiment 2, rats were exposed to low, middle, or high concentrations of TMS and TCM for 30 minutes using single animal whole body (SAWB) exposure chambers and blood was drawn after 30 minutes to measure the blood concentrations of each chemical. The purpose of this was to establish a concentration response for each chemical in the blood that were correlated with any observed effects in Experiment 1. The original parent compounds of TMS and TCM were measured using gas chromatography mass spectrometry. All groups that were exposed to the two chemicals had blood drawn immediately before the start of the exposures, which served as the baseline. For the control group, which was exposed to filtered air only, the blood was also drawn at 0 and 30 minutes. A total of 332 male Sprague-Dawley rats (12 training animals and 320 test animals) were used. All animals used in Experiment 1 and Experiment 2 of the study were humanely euthanized by an American Veterinary Medical Association (AVMA) method approved by the Wright-Patterson Air Force Base Institutional Animal Care and Use Committee (WPAFB IACUC).

In this study, exposure of rats to increasing concentrations of TMS and TCM impaired spontaneous motor activity or the animals' natural ability to explore a novel environment. Furthermore, TMS and TCM impaired motor coordination concentration-dependently. When the two chemicals were combined at their lowest concentrations, motor activity but not motor coordination was impaired compared to the control group, thus suggesting that the combination of low level chemical contaminants can cause differential neurocognitive effects. Post-behavioral analysis of the blood and various parts of the brain revealed that blood levels of each parent compound increased with their

corresponding exposure concentrations but there were no significant effects on the levels of neurotransmitters, with the exception of Substance P, in response to increasing concentrations of TMS. In conclusion, results from this study can be used to set new exposure limits to mitigate the occurrence of PEs induced by exposure to a combination of low level chemical contaminants.

Introduction

In 2017 and 2018, PEs were Navy aviation's primary safety concern. As a result of this, the U.S. Navy Aviation Environmental Scientific Advisory Board (AESAB) recommended further evaluation of some of the chemicals discovered onboard T-45 and F-18 family of aircraft. Hundreds of chemicals were identified in and around Navy aircraft, including in their breathing gas, to which aviators may have been exposed (Mumy, 2018). While these chemicals were all found at very low concentrations (in the parts per billion [ppb]; (Mumy, 2018)), there remains the question as to whether low levels of chemicals are able to induce cognitive or neurological effects when found in combination (i.e., act additively or synergistically to induce an effect that they would not otherwise cause on their own). In order to investigate this, two of the chemicals that were chosen were found in the cockpits of these aircraft at low levels and are historically known to individually induce cognitive and neurological effects at given concentrations (Mumy, 2018). Rats were exposed to these chemicals at concentrations higher than those found in naval aircraft, but well below those that are anticipated to cause effects. This study examined whether exposure to a combination of chemicals at a low concentration is able to cause effects that the individual chemicals at those low concentrations would not be able to cause.

The following two chemicals, TMS and TCM, were tested in combination (and individually for comparison) in this study. TMS and TCM were the only two chemicals from a list of nearly 800 chemicals collected and identified through the use of various sampling media, including air canisters, sorbent tubes, swabs, and flight line health monitors, with all sampling being performed by the U.S. Navy Air Systems Command (NAVAIR). While any number of different chemicals from the list (not able to be disseminated) could have been investigated, two out of 17 chemicals that were known to contribute to CNS effects were chosen for evaluation. The following sections provide brief summaries of what is known regarding the chosen two chemicals.

Trimethylsilanol

TMS is an organosilicon compound that is produced largely as a result of the degradation of silicone products that are commonly used by the military. It was found to be a central nervous system (CNS) depressant in a variety of animal models (Dow Corning Corp., 1991). Previously, TMS was discovered onboard spacecraft, and as a result of its presence in these types of complicated environments, NASA determined the Spacecraft Maximum Allowable Concentration (SMAC) for a one hour exposure to TMS to be 15 ppm to avoid causing adverse CNS effects to astronauts (James, 2008). However, this value was based on studies where animals were administered TMS orally or via injection (Dow Corning Corp., 1991), as opposed to the more likely inhalation route. The only data available with regard to inhalation exposure indicate that for an acute four hour inhalation study in a rat model, the lethal concentration 50 (LC_{50}) was determined to be 3151 ppm (OECD, 2014);however, a separate calculation based on the results of the same study determined an LC_{50} of 3205 ppm

(unpublished data). Currently, there is no published inhalation data that examines the effects of TMS on the neurocognitive effects using animal models.

Trichloromethane

TCM, commonly known as chloroform, is an organic compound that consists of a carbon atom that is covalently bound to three chlorine atoms and one hydrogen atom. It is a clear, volatile liquid with a sweet odor and taste (IARC, 1999; WHO, 2004). TCM was previously used as an anesthetic but was discontinued due to its toxicity (Brown, 2012). TCM is still widely used as a solvent during the synthesis of fluorocarbons, drugs, and insecticides and is also produced by the chlorination of drinking water (IARC, 1999). Furthermore, TCM can be produced as a by-product of the "off-gassing" of plastics and adhesives and has been found onboard submarines (National Research Council, 1970 and 1984). The primary routes of exposure to TCM include ingestion, inhalation, and dermal exposure through contact with chlorinated water. In rat models of TCM-induced neurocognitive effects, rats experienced CNS depression after being exposed to 16,000 ppm TCM vapors for 10 min (Clark and Tinston, 1982). Based on a four hour exposure, the LC_{50} in rats was determined to be 9,617 ppm (NIOSH, 1994).

TCM was identified in air canister samples on T-45 aircraft on numerous occasions. The maximum concentration of TCM was 1.4 ppb, which was significantly lower than concentrations that were shown to cause physiological effects (Mumy, 2018). Furthermore, the SMAC for TCM was set at 2 ppm based on a one hour exposure due to its potential to cause adverse CNS depressive effects (James, 2008). Therefore, since TCM can cause neurological effects, it was recommended that additional research be conducted to determine the allowable concentrations for the flight environment.

Since the exact concentrations of TMS and TCM that could cause acute physiological and neurobehavioral effects when combined are not known, the goal of this study was to determine the concentration of each chemical that could induce real-time neurobehavioral effects in rats during a 30 minute exposure. Neurobehavioral effects were assessed in real-time during exposure to TMS and TCM individually and in combination using the open field motor activity and rotarod tests, which were used to determine the no or low observed adverse effect level (NOAEL, LOAEL) for each chemical based on measured and observed effects. Establishment of a broad concentration-response curve covered a range of concentrations based on the LC_{50} for each chemical with the high, middle, and low concentrations being 2/3, 1/3, and 1/24 that of the LC_{50} for each chemical, respectively. Rats were exposed to TMS and TCM in combination using the NOAEL or LOAEL of each chemical. Subsequently, single animal whole body (SAWB) exposure chambers were used to expose animals to TMS and TCM individually and in combination, and the chemicals were extracted from the blood of the exposed animals to correlate their measured levels with observed neurobehavioral effects. Furthermore, to determine whether exposure of the animals to TMS and TCM alters the levels of several neurotransmitters, post-mortem neurotransmitter concentrations were measured in rat brains.

Objective

The objectives consists of two experiments:

1) Experiment 1: The rotarod test was used to evaluate motor coordination, endurance, and balance while the animals walked on a rotating cylinder at pre-determined speeds for 30 minutes. The motor activity test measured the activity of the rats based on the time the animals were active versus the time they were inactive, the total distance traveled, animal speed, time spent in the center versus the perimeter and total rears on hind limbs.

2) Experiment 2: The blood concentrations of TMS, TCM, and a combination of the two chemicals were measured following exposure in SAWB exposure chambers to correlate their levels with measured and observed neurobehavioral effects in the animals.

Materials and Methods

Study Design

The subjects were placed individually inside exposure chambers and simultaneously exposed to filtered air and low, middle, and high concentrations of test chemical vapor mixed with air for 30 minutes. The test chemical vapors consisted of two sets of exposures with different single test chemicals and a set of exposures that combined the two individual test chemicals. For the combined test chemical runs there was only a control and a test chemical exposure subject. All subjects were given an adequate flow of air without restraint inside the chamber before, during, and after the exposure period. After the 30 minute exposure period, the test chemicals concentrations were allowed to return to safe levels before the chambers were opened and the subjects were removed. The subjects were then placed back in their respective housing cages for an observation period before being returned to the vivarium for immediate euthanization. Experiment 2 called for pre and post exposure blood draws to be performed to measure chemical levels in the blood of the subjects. The first two sets of single chemical exposures were intended as range finding runs to determine the target concentrations for the combination chemical runs, which were intended to look for additive or synergistic effects of exposing the subjects to multiple chemicals at the same time.

Animals and Animal Husbandry

A total of 332 male Sprague-Dawley (Rattus norvegicus) rats (12 training animals and 320 test animals) at approximately 6 – 7 weeks old were purchased from Charles River Laboratories (Wilmington, MA). Food and water were made available *ad libitum* during periods of non-exposure. Rats were quarantined for at least 7 days prior to the exposures.

Animals were delivered from the vivarium inside housing cages and under a tarp to the exposure laboratory at least 30 minutes prior to the exposures and returned to the vivarium under a tarp after completion of the exposures. The 30 minute time period was used as an acclimation period prior to the exposures. The exposures were carried out in three week blocks with a week in between for the

inhalation team to changeover the system to the next test chemical. Experiment 1 testing was performed during the first and third weeks of each block and Experiment 2 testing was performed during the second week of each block. See Table 1: Exposure Schedule. The single chemical exposures included four animals per exposure run with one animal serving as a control and the other three as low, middle, and high concentrations. The combo chemical exposures included two animals per exposure run with one animal serving as a control and the other being dosed with the combination of chemicals. The Experiment 2 animals had blood drawn immediately before and after the exposures.

Table 1: Exposure Schedule

Week Number	Exposure Chemical(s)	Number of Animals Experiment 1	Number of Animals Experiment 2	Exposure Apparatus
1	TMS	40	-	Motor Activity
2	TMS	-	32	SAWB
3	TMS	56	-	Rotarod
4	TCM	40	-	Motor Activity
5	TCM	-	32	SAWB
6	TCM	56	-	Rotarod
7	TMS & TCM	20	-	Motor Activity
8	TMS & TCM	-	16	SAWB
9	TMS & TCM	28	-	Rotarod
		240	80	
	Total	**320**		

Materials

Trimethylsilanol (TMS) at ≥ 97.5% purity (Sigma-Aldrich, Saint Louis, MO) was obtained for the first and third parts of the study (see Table 2). The material was stored in a chemical closet with ventilation according to recommended storage parameters.

Table 2: Trimethylsilanol Properties

Substance Name:	≥ 97.5% Trimethylsilanol
Other Names:	TMS Hydroxytrimethylsilane
CAS #:	1066-40-6
Chemical Formula:	$C_3H_{10}OSi$
Molecular Weight:	90.2 g/mol
Description:	Colorless liquid
Test Article Category:	Liquid
Storage:	Store in a well-ventilated place. Flammable liquid.
Stability:	Stable under recommended storage conditions
Supplier:	Sigma-Aldrich
Additional Information:	Vapor pressure: 20.8 hPa @ 20°C

Trichloromethane (TCM) at ≥ 99.8% purity (Alfa Aesar, Tewksbury, MA) was obtained for the second and third parts of the study (see Table 3). The material was stored in a chemical closet with ventilation according to recommended storage parameters.

Table 3: Trichloromethane Properties

Substance Name:	≥ 99.8% Trichloromethane
Other Names:	Methane trichloride Methenyl trichloride Formyl trichloride
CAS #:	67-66-3
Chemical Formula:	$CHCl_3$
Molecular Weight:	119.37 g/mol
Description:	Colorless liquid, aromatic sweet odor
Test Article Category:	Liquid
Storage:	Protect from sunlight. Protect from moisture. Store in a well-ventilated place.
Stability:	Stable under recommended storage conditions
Supplier:	Alfa Aesar
Additional Information:	Vapor pressure: 207 hPa @ 20°C

Generation System

The chemical vapor generation system was a vapor head-space type generator and consisted of triplets of 5-gallon pressure vessels (Alloy Products Corp, Waukesha, WI) containing the test chemicals within ventilated hoods in the exposure lab (Figures 1 and 2). 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 three exposure hoods. One triplet of pressure vessels was dedicated to each chemical being generated. The pressure vessels were pressurized to approximately 10 pounds per square inch gauge (PSIG) with filtered compressed air supplied through ¼" linear low density polyethylene (LLDPE) tubing (McMaster-Carr, Aurora, OH) from a common air line to keep the pressure even across them. They were also wrapped in heat tape (Brisk Heat 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 converter 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 head-space and compressed air mixture from each of the three pressure vessels then passed through ¼" polytetrafluoroethylene (PTFE) tubing (Cole-Parmer, Vernon Hills, IL) and was recombined at a cross before entering the delivery line through a ½" 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 for the single chemical exposures. For the combo chemical exposures, both branches of the tee supplied one of the chemicals from their respective pressure vessels so the flows of the individual test chemical gas streams could be adjusted in proportion to each other. The test chemical laden exposure gas then passed through a 3-way valve that allowed either that or filtered compressed air to be selected to flow to the delivery line.

The delivery line 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 ¼” 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 each of the exposure chambers. Excess exposure gas flow from the delivery line passed through a ½” stainless steel needle valve and a variable area flow meter (Dwyer Instruments Inc., South Bend, IN) at the end of the delivery line, then into the exposure hood exhaust. The variable area flow meters in each of the 3 exposure hoods were configured to deliver different amounts of exposure gas based on high, middle, and low exposure concentrations. For the combination chemical exposures, the flow meters allowed adjustment of the proportionally diluted combined chemical vapor stream to the desired target concentration for both chemicals simultaneously. The exposure gas from the outlet of each rotameter flowed through ¼” PTFE tubing to a tee where it was diluted to the final exposure concentration by compressed air supplied from a mass flow controller (Alicat Scientific, Tucson, AZ).

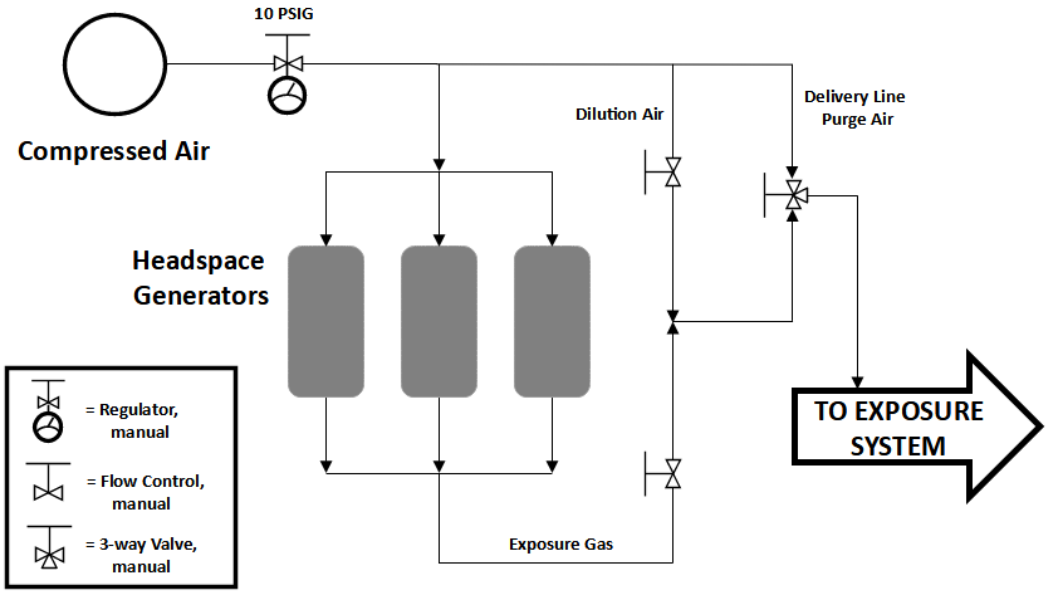

Figure 1: Diagram of Single Chemical Generation System

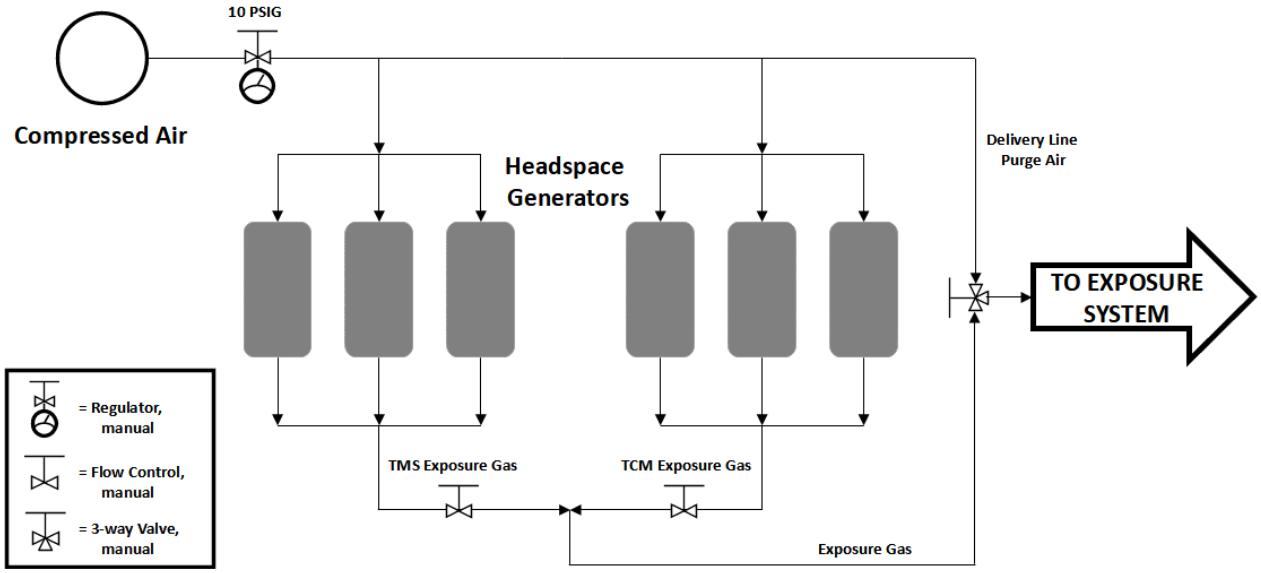

Figure 2: Diagram of Combo Chemical Generation System

Exposure System

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 (Figures 3 and 4). When off exposure, filtered compressed air was directed to the exposure chambers and the test chemical laden exposure gas was directed to the hood exhaust. When the solenoid valve switch was flipped the filtered compressed air flow was cut off and the exposure gas was directed to the exposure chambers. Once exposures concluded, the process was reversed to go off exposure by flipping the switch back and removing power from the solenoid valves. This design had the added benefit of allowing the system to drop back to filtered compressed air flow automatically in the event of power loss to the system.

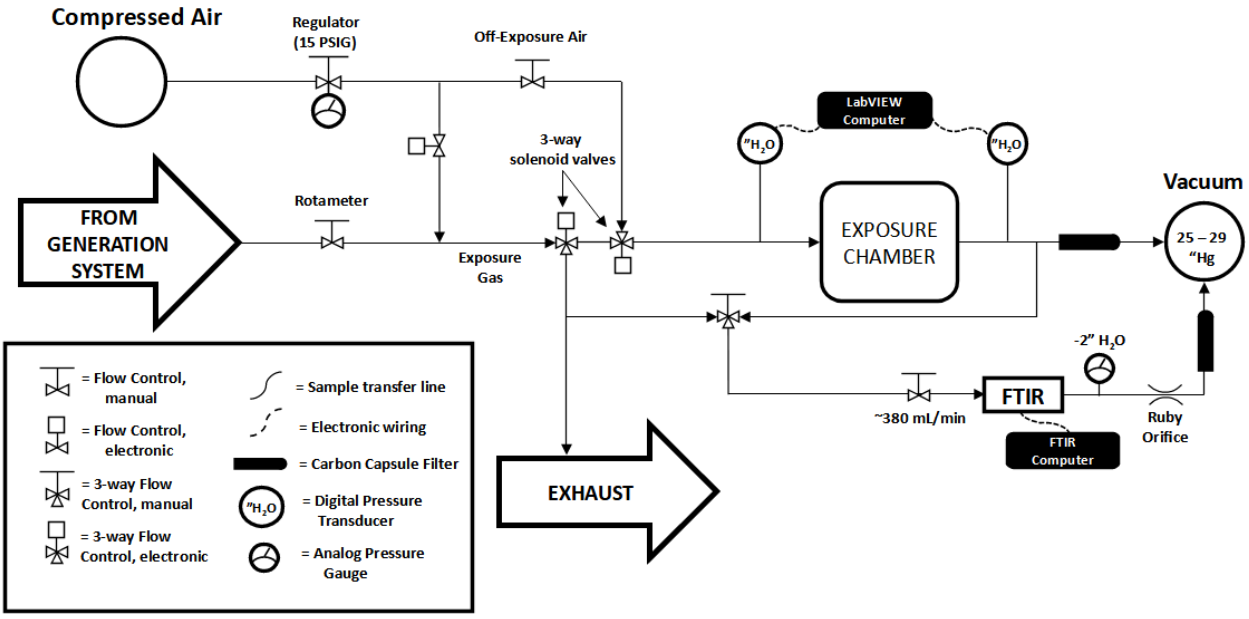

Figure 3: Diagram of the Exposure System

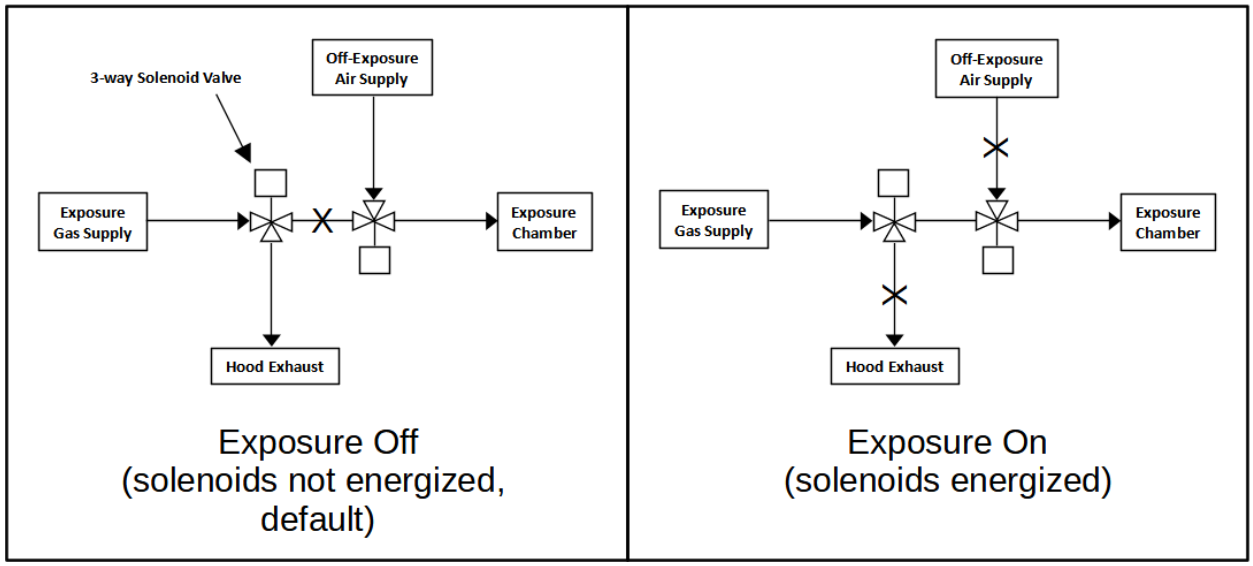

Figure 4: 3-way solenoid valve configuration

Experiment 1

The first experiment involved exposing the animals to TMS, TCM, or a combination of the two chemicals while they were inside either an open field motor activity box or a rotarod activity box. The behavioral apparatuses were used to establish a level of neurological impairment in the animals. There was also a control exposure for each of the tests in which the animals were only supplied with filtered compressed air.

For the open field motor activity box (Figure 5) (Omnitech Electronics, Inc., Columbus, OH) tests, one subject was placed inside an acrylic activity box inside each of the four exposure hoods. The activity boxes were 16.00” on each side at the base and 13.00” in height. The activity boxes sat inside an aluminum frame with opposing photo beam sensors set one inch apart. The sensors sent a signal to the software provided by the manufacturer of the activity boxes when a beam was broken, thereby tracking the movement of the animals. There were two rows of detection beams (Omnitech Electronics, Inc. model Super Flex Sensor, version 4.6, Columbus, OH). The first row was approximately 1” above the floor of the activity box and was able to provide x and y axis movement detection. The second row of sensors was 5.5” above the first row and provided z axis detection for when the animals reared up on their hind legs. The system was capable of measuring time active vs inactive, total distance traveled, animal speed, time spent in the center vs perimeter, and total rears on hind limbs (exploratory measure).

The off exposure filtered compressed air and exposure gases were delivered to each open field activity box at a minimum flow rate of 10.9 L/min to ensure at least 12 air changes per hour within the exposure chamber. The breathing gas was delivered to a central port in the lid of the activity box, then dispersed into a plenum in the lid before passing through several sets of holes to enter the main box and the breathing area of the animals. Exposure gases were exhausted through a central port in the lid and passed through a combination of HEPA and activated charcoal filters (GE Healthcare UK Ltd, Whatman Carbon Cap) before being exhausted into the house vacuum system.

Figure 5: Open Field Activity Box

The rotarod activity boxes (Figure 6) (Omnitech Electronics, Inc. model AccuRotor EzRod, version 4.6, Columbus, OH) utilized an adjustable revolutions per minute (RPM) rotating wheel that the animals walked on and an optical fall detection sensor in the bottom to detect when the animals fell off the wheel. The wheel was set to rotate at approximately 12 RPM until the animal fell off at which point it stopped rotating. The upper area of the rotarod activity boxes were modified to be enclosed exposure chambers with a latching polycarbonate top that was fabricated in-house. The polycarbonate top featured a skirt around the rotating wheel to prevent any body parts of the animals from being pinched in the apparatus as it rotated.

The rotarod activity boxes were modified to allow exposure gas delivery to the bottom of the main animal compartment as well as compressed air to the rear electronics and motor compartment. The off exposure filtered compressed air and exposure gases were delivered at a minimum flow rate of 6.35 L/min to ensure at least 12 air changes per hour within the exposure chamber. The compressed air flow to the rear electronics compartment was also delivered such that it produced at least 12 air changes per hour with a minimum calculated flow rate of at least 1.74 L/min. The exposure gas in the main exposure chamber was exhausted to the vacuum system through a port in the polycarbonate lid behind the animal. The exhaust gas passed through a combination of HEPA and activated charcoal filters before entering the vacuum system. The compressed air from the electronics and motor compartment was allowed to passively exhaust into the exposure hood through a second port in the compartment at the rear of the rotarod apparatus.

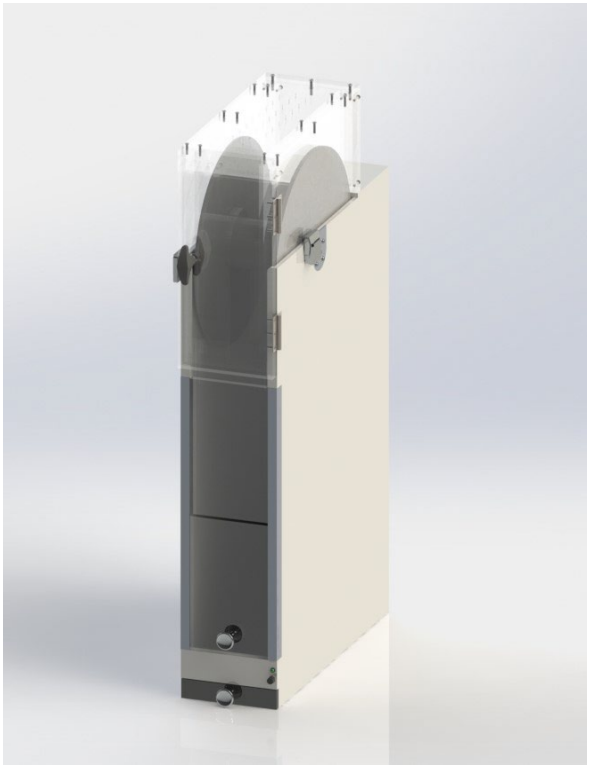

Figure 6: Rotarod Activity Box

Experiment 2

The second experiment involved placing animals inside SAWB chambers (Figure 7) and exposing them to the same combinations and levels of chemicals from Experiment 1. The off exposure filtered compressed air and on exposure test chemical laden air were each delivered at a minimum flow rate of 0.818 L/min to ensure at least 12 air changes per hour within the exposure chambers. The SAWB tubes were constructed from glass and had perforated stainless steel floor inserts that allowed animal waste to pass through and accumulate in the bottom area of the tubes. The ends of the tubes had knurled knobs that caused the O-rings on them to press against the inside of the glass tubes and create a seal when tightened. The ends of the tubes also had perforated steel plates that helped diffuse the supplied gases as they entered the SAWB tubes.

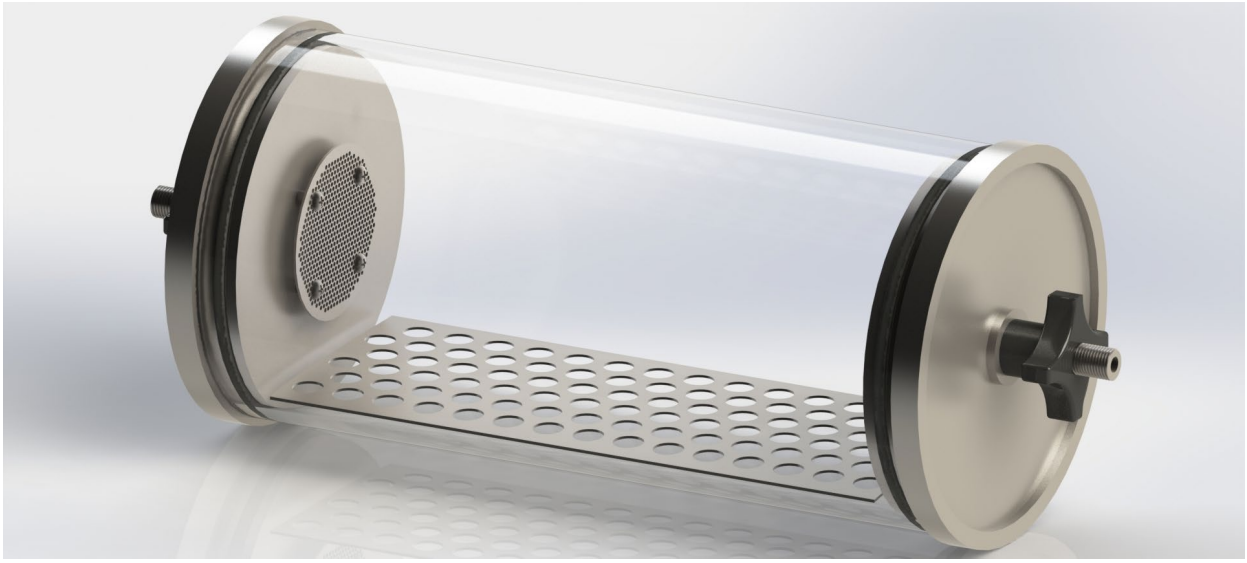

Figure 7: Single Animal Whole Body (SAWB) Exposure Chamber

Table 4: Single Chemical Exposure Target Concentrations

Test Substance	LC_{50}	High Concentration $(2/3)*LC_{50}$	Middle Concentration $(1/3)*LC_{50}$	Low Concentration $(1/24)*LC_{50}$
TMS	3205 ppm	2137 ppm	1068 ppm	134 ppm
TCM	9617 ppm	6411 ppm	3206 pm	401 ppm

Table 5: Combination Chemical Exposure Target Concentrations

Test Substance	Low Concentration (1/24)*LC_{50}
TMS	134 ppm
TCM	401 ppm

Test Atmosphere Monitoring

LabVIEW

Data collection and mass flow controller (MFC) flow set point control were performed by LabVIEW software (National Instruments, Austin TX). The LabVIEW software ran on a custom-built, uninterruptible power supply connected personal computer and interfaced with the data logging equipment via a Universal Serial Bus (USB) connected data acquisition (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) per hood 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 a USB connection. LabVIEW was able to log the data from the MFCs as well as set the target flow rates. The Fourier Transform Infrared (FTIR) spectrophotometer data was also captured and recorded in LabVIEW by reading from the text files that the FTIRs recorded data to over the local area network connection in the lab. The test chemical concentrations were reported by the FTIRs in mg/m^3 then converted to parts per million by volume (PPMV) by LabVIEW before being recorded.

Fourier Transform Infrared Spectrophotometer

An FTIR spectrophotometer (Thermo Scientific, model Nicolet Si10, Waltham, MA) fitted with a short path 2 meter gas cell was used for the analysis of vapor concentrations. A manual 3-way valve was placed in-line to allow the FTIR to sample from either the exposure chamber exhaust or the off exposure exhaust tube where the exposure gas flowed into the hood exhaust plenum while the system was off exposure. All FTIR sample gas flowed through ¼" PTFE tube. The sample flow for the 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 was attached just after the gas cell to monitor the pressure in the cell which was set to -0.2 "H_2O at all times. The cell pressure was regulated with a needle valve on the inlet side of the FTIR gas cell.

Characterization of the FTIR began 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 for each chemical. TCM was calibrated through a range of 739 to 51,758 mg/m^3 (179 to 12,557 PPMV at 20 °C). TMS was calibrated through a range of 407 to 13,845 mg/m^3 (131 to 4,445 PPMV at 20 °C). The wavenumbers for TMS = 2996.7 cm^{-1} and TCM = 2408.7cm^{-1} were chosen based on their intra-assay precision and accuracy statistics.

Each of these test articles in environment would influence the opposing wavenumber. A combination environment was a target of the protocol. As the wavenumber ratios on the FTIR spectrum have a constant relationship, the calibration of influence was identical across all four FTIR instruments. For every 1 atomic unit (AU) of 2408.7 cm^{-1}, a correction of 0.225 AU for 2996.7 cm^{-1} was used. For every 1 AU of 2996.7 cm^{-1}, a correction of -0.07552 AU for 2408.7 cm^{-1} was used. This calibration curve correction was only used during combination atmospheres and targeted a specific ratio of test articles at different concentrations (ex: 1x 1:2::TCM:TMS; 2x 1:2::TCM:TMS; and 5x 1:2::TCM:TMS). This gave an accuracy within 10% of true concentrations, where a 20% to 350% accuracy would be given without this correction.

The FTIR data for both chemicals was logged using a macro running in Omnic that used the calibration formulas calculated for each of the peaks. The macro ran on 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 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.

Table 6: FTIR Calibration Data

FTIR	Material	Path Length	Wavenumber	per AU	σ	Unit	R^2
Golf	TCM	Short	771.5	4,956	± 7	mg/m^3	0.5059
Golf	TCM	Short	1219.1	6,429	± 2	mg/m^3	0.9112
Golf	TCM	Short	2408.7	313,580	± 10	mg/m^3	0.9988
Golf	TCM	Short	3033.5	534,706	± 264	mg/m^3	0.9967
Golf	TMS	Short	1541	-270,722	± 138	mg/m^3	0.9591
Golf	TMS	Short	2908.7	2,440	± 14	mg/m^3	0.9551
Golf	TMS	Short	2996.7	120,216	± 726	mg/m^3	0.9962
Golf	TMS	Short	3769.0	4,485	± 1	mg/m^3	0.9909
Hotel	TCM	Short	771.5	4,716	± 14	mg/m^3	0.5094
Hotel	TCM	Short	1219.1	10,585	± 5	mg/m^3	0.9236
Hotel	TCM	Short	2408.7	314,090	± 21	mg/m^3	0.9992
Hotel	TCM	Short	3033.5	535,696	± 282	mg/m^3	0.9972
Hotel	TMS	Short	1541	14,707	± 270	mg/m^3	0.5827
Hotel	TMS	Short	2908.7	17,375	± 2	mg/m^3	0.5529
Hotel	TMS	Short	2996.7	128,941	± 1,444	mg/m^3	0.9978
Hotel	TMS	Short	3769.0	6,979	± 1	mg/m^3	0.9943
India	TCM	Short	771.5	4,175	± 14	mg/m^3	0.6751
India	TCM	Short	1219.1	6,438	± 5	mg/m^3	0.9115
India	TCM	Short	2408.7	313,346	± 14	mg/m^3	0.9991
India	TCM	Short	3033.5	541,473	± 200	mg/m^3	0.9972
India	TMS	Short	1541	48,822	± 828	mg/m^3	0.7113
India	TMS	Short	2908.7	17,030	± 8	mg/m^3	0.9860
India	TMS	Short	2996.7	120,424	± 1,260	mg/m^3	0.9981
India	TMS	Short	3769.0	7,116	± 121	mg/m^3	0.9966
Juliet	TCM	Short	771.5	4,090	± 11	mg/m^3	0.6648
Juliet	TCM	Short	1219.1	6,777	± 5	mg/m^3	0.8354
Juliet	TCM	Short	2408.7	317,079	± 22	mg/m^3	0.9989
Juliet	TCM	Short	3033.5	543,901	± 433	mg/m^3	0.9977
Juliet	TMS	Short	1541	49,574	± 9,217	mg/m^3	0.8144
Juliet	TMS	Short	2908.7	37,216	± 11	mg/m^3	0.9979
Juliet	TMS	Short	2996.7	120,643	± 12,975	mg/m^3	0.9975
Juliet	TMS	Short	3769.0	7,097	± 1	mg/m^3	0.9970

Temperature and humidity

A temperature and relative humidity (RH) detecting probe attached to a transmitter with a 4-20 mA analog output (Rotronic Instrument Corp. model HygroFlex5 – HF5, Hauppauge, NY) was positioned near the top of each exposure hood. The temperature and RH were recorded at a rate of approximately 1 time per second by the LabVIEW software. The probe came calibrated from the manufacturer for a range of 0 – 100% RH and 0 – 100 °F temperature. The following tables show the overall average temperature and RH statistics for the hoods, across all of the exposure days, and broken down by exposure apparatus.

Table 7: Motor Activity Overall Temperature and Relative Humidity

Temperature (°F)	Hood 1	Hood 2	Hood 3	Hood 4
Average	74.5	74.7	75.1	75.0
Min	72.9	73.5	74.2	74.2
Max	76.5	76.3	76.8	76.5
Std. Dev.	0.93	0.90	0.71	0.70
Count	25	25	25	25

Relative Humidity (%)	Hood 1	Hood 2	Hood 3	Hood 4
Average	55.5	54.4	55.3	55.2
Min	48.6	47.5	48.6	48.3
Max	60.8	60.0	62.1	62.1
Std. Dev.	3.58	3.58	3.79	4.08
Count	25	25	25	25

Table 8: SAWB Overall Temperature and Relative Humidity

Temperature (°F)	Hood 1	Hood 2	Hood 3	Hood 4
Average	74.6	74.7	75.0	74.5
Min	72.0	72.2	71.7	71.5
Max	78.2	78.3	78.4	77.9
Std. Dev.	2.04	2.02	2.04	2.00
Count	20	20	20	20

Relative Humidity (%)	Hood 1	Hood 2	Hood 3	Hood 4
Average	53.7	52.8	53.7	54.4
Min	44.5	43.8	44.3	44.5
Max	61.7	60.9	60.9	60.7
Std. Dev.	5.03	5.15	5.09	5.36
Count	20	20	20	20

Table 9: Rotarod Overall Temperature and Relative Humidity

Temperature (°F)	Hood 1	Hood 2	Hood 3	Hood 4
Average	74.8	75.1	75.6	75.4
Min	70.6	70.7	71.9	71.8
Max	77.2	77.6	77.4	77.0
Std. Dev.	1.51	1.56	1.38	1.29
Count	35	35	35	35

Relative Humidity (%)	Hood 1	Hood 2	Hood 3	Hood 4
Average	53.6	52.6	53.7	54.0
Min	47.4	46.3	45.9	45.7
Max	63.4	60.4	60.9	61.3
Std. Dev.	3.90	3.59	3.81	3.97
Count	35	35	35	35

Static Pressure

The chamber pressures at the inlet and exhaust were monitored by electronic pressure transducers (BAPI Inc., model ZPS-EZ in WC, Gays Mills, WI). The pressure transducer output a 4-20 mA analog signal, which was recorded as inches of water column ("H_2O) in LabVIEW. Only the SAWB chamber readings are accurate readings of the pressures in the chamber relative to the pressure in the room. Due to the nature of the two converted activity box exposure chambers, they were not sealed well enough to show a real pressure difference inside the exposure chamber relative to room pressure. As such, the pressure readings for the activity box exposure chambers were an indication of the relative pressures in the inlet and exhaust tubing connections where they were connected and were used as a reference relative to each other to gauge system performance.

Table 10: Motor Activity Box Static Pressure

Supply Static Pressure ("H_2O)	Hood 1	Hood 2	Hood 3	Hood 4
Average	0.069	0.060	0.086	0.081
Min	0.055	0.033	0.080	0.050
Max	0.090	0.087	0.090	0.130
Std. Dev.	0.014	0.019	0.004	0.033
Count	25	25	25	25

Exhaust Static Pressure ("H_2O)	Hood 1	Hood 2	Hood 3	Hood 4
Average	-0.424	-0.435	-0.436	-0.434
Min	-0.480	-0.510	-0.490	-0.460
Max	-0.390	-0.387	-0.403	-0.360
Std. Dev.	0.031	0.051	0.034	0.024
Count	25	25	25	25

Table 11: SAWB Static Pressure

Supply Static Pressure ("H_2O)	Hood 1	Hood 2	Hood 3	Hood 4
Average	-0.327	-0.299	-0.250	-0.214
Min	-0.476	-0.565	-0.400	-0.470
Max	-0.195	-0.080	-0.012	-0.047
Std. Dev.	0.071	0.114	0.100	0.101
Count	20	20	20	20

Exhaust Static Pressure ("H_2O)	Hood 1	Hood 2	Hood 3	Hood 4
Average	-0.353	-0.330	-0.278	-0.248
Min	-0.503	-0.591	-0.428	-0.504
Max	-0.223	-0.112	-0.041	-0.081
Std. Dev.	0.071	0.112	0.099	0.101
Count	20	20	20	20

Table 12: Rotarod Static Pressure

Supply Static Pressure ("H_2O)	Hood 1	Hood 2	Hood 3	Hood 4
Average	0.432	0.440	0.423	0.380
Min	0.416	0.423	0.368	0.235
Max	0.459	0.451	0.441	0.401
Std. Dev.	0.010	0.008	0.011	0.030
Count	35	35	35	35

Exhaust Static Pressure ("H_2O)	Hood 1	Hood 2	Hood 3	Hood 4
Average	-0.125	-0.144	-0.133	-0.148
Min	-0.143	-0.156	-0.147	-0.181
Max	-0.112	-0.130	-0.110	-0.079
Std. Dev.	0.008	0.006	0.008	0.025
Count	35	35	35	35

Post-behavioral Blood Analysis

Blood collection

For pre exposure samples, blood was collected directly into 0.8mL MiniCollect tubes with Lithium Heparin using needles to avoid exposure to air and potential loss of target compounds. For post exposure sample collection, the blood was taken from the vena cava using a syringe, then placed in the MiniCollect tubes. Blood that was used for calibrations and blanks was taken from rats that were not exposed to the chemicals.

Sample preparation

Samples were prepped by weighing approximately 0.2g of sodium chloride (NaCl) (Fisher Scientific) into 10mL screw cap vials (Gerstel Inc., Linthicum Heights, MD). Deionized water and blood were added to the vials totaling 1mL, not to exceed more than 0.5mL 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.

Instrumentation

The analysis of TMS and/or TCM was done 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 micro extraction (SPME) on a Multi-Purpose Sampler (MPS) (Gerstel Inc., Linthicum Heights, MD). The analytical conditions were as follows: The MPS incubated the sample at 70ºC 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 30µm fiber thickness, Restek Corp., Bellefonte, PA) moved to the vial and with a 21mm 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 54mm. 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 2mL/min. The initial oven temperature was 35ºC with an initial hold time of 0 minutes. The oven temperature program was as follows: 1ºC/minute to 47ºC, 20ºC to 100ºC followed by 30ºC to 320ºC. 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.

Reference standards

TMS was made to a concentration of 81.4 µg/mL while TCM was 82.9 µg/mL in methanol.

Analysis

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 affects, 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 internal standard for TCM and TMS/TCM combo analysis was 2-Bromo-1-chloropropane, also form the 624 Internal Standard Mix, with the same surrogates as the TMS analysis. A different IS was used when chloroform was the target due to similar retention times and using SIM analysis. The calibration curve met 20% relative standard deviation or R^2 of >0.98 with five points minimum. The calibration curve for TMS ranged from 0.2442 µg/mL to 16.28 µg/mL. The calibration curve for TCM ranged from 0.2487 µg/mL to 16.58 µg/mL. 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 affects, where the CCV did not. The CCV, LCS

and surrogates were considered passing if they were 70-130% recovery. The IS was passing if it was 50-200% of the IS of the batch's CCV.

Post-mortem Measurement of Neurotransmitter Levels

Different sections of the rat brain from fourteen rats per group (same animals used in the rotarod test) were extracted at necropsy and the following sections were lysed and analyzed for the following neurotransmitters by enzyme-linked immunosorbent assay (ELISA): acetylcholine (pre-frontal cortex), dopamine (striatum), epinephrine (brainstem), γ-aminobutyric acid (GABA) (hippocampus), glutamate (cerebellum), norepinephrine (brainstem), serotonin (striatum), and Substance P (cortex).

Tissue preparation

The different brain sections were sectioned and weighed during necropsy, wrapped in aluminum foil, and stored at -80° C. For tissue homogenization, samples were stored/thawed on ice and homogenized with an electric tissue homogenizer for approximately 30 seconds with 1.0 mL phosphate buffered saline/0.5g of tissue. Samples underwent 2 freeze/thaw cycles at -20° C and room temperature. At the last thaw, samples were centrifuged for 15 minutes @1500xg (2-8° C) prior to analysis using ELISA according to the manufacturer's protocol.

Statistical Analysis

For Experiments 1 and 2, primary outcome measures were treated as continuous (such as time to fall from the rotarod or distance traveled in the motor activity box) or categorical (such as incidence of fall from rotarod) data and were analyzed using either analysis of variance (ANOVA) or Chi-Square test for each type of data, respectively. Additionally, for open field performance (motor activity), a two-way ANOVA was performed to assess habituation over six five minute blocks of time per exposure group. Both main effects (time block and exposure group) and any interactions were analyzed. Additional exposure group comparisons of total cumulative test session activity was performed. Furthermore, ANOVA was used to compare exposure groups for quantitative continuous data produced by biochemical assays (e.g. blood concentrations of each chemical and neurotransmitter levels using ELISA).

For the ANOVA, Levene's test was used to assess homogeneity of variance and the Shapiro-Wilk test was used to determine if data were normally distributed. If data were homogenous and normally distributed, one-way ANOVA was used to test for statistically significant differences between the control group and the four treatment groups. If variances are non-homogenous, Welch's ANOVA was used, and if data were not normally distributed, a Kruskal-Wallis ANOVA was used to test for statistical significance. An appropriate post-hoc test such as Tukey or Holm-Sidak multiple comparison procedure was used to find if animals exposed to the individual chemicals was different from the group of animals exposed to the combination of chemicals. Statistical analyses were performed using either Sigma Plot or the Statistical Package for the Social Sciences (SPSS) statistical software programs, as deemed appropriate.

A minimum of ten animals per group has been used in behavioral tests historically (Mattie et al., 2011; Baldwin et al. 2001). The number of animals per exposure group (2 chemicals x 3 exposures + 1 chemical mixture at the highest non-effective concentrations + 1 control) for Experiment 1 was determined by the number of animals necessary for neurobehavioral testing, which was a total of 240 animals. For Experiment 2, the number of animals per exposure group was determined by the number of animals necessary for blood collection following exposure to each of the two chemicals, a combination of the two chemicals and control, which was a total of 80 animals. Specifically, the number of animals per group for each experiment is summarized below:

1) Experiment 1:

a) For the rotarod testing, there were 14 animals per group (control, low, middle, and high for TMS and TCM or control and combination exposure), thus yielding a total of 56 animals per chemical and 28 animals for the combination exposures. Because it was expected that there would be greater variability using the rotarod, a sample size of 14 animals were used. A sample size of 14 animals per group allows a minimum detectable difference in means of 26% with a standard deviation of 20% or a minimum detectable difference in means of 22% with a standard deviation of 20% with a statistical power of 0.8 and an unadjusted alpha of 0.05 for four or two groups, respectively.

b) For the motor activity testing, there were 10 animals per group (control, low, middle, and high for TMS and TCM or control and combination exposure), thus yielding a total of 40 animals per chemical and 20 animals for the combination exposures. A sample size of 10 animals per group allows a minimum detectable difference in means of 29% with a standard deviation of 18% or a minimum detectable difference in means of 25% with a standard deviation of 18% with a statistical power of 0.8 and an unadjusted alpha of 0.05 for four or two groups, respectively.

2) Experiment 2:

a) For the SAWB exposure chambers and subsequent blood draws, there were eight animals per group (control, low, middle, and high for TMS and TCM or control and combination exposure), thus yielding a total of 32 animals per chemical and 16 animals for the combination exposure. For the bioassays (e.g. blood concentrations of the chemicals), a sample size of eight animals per group allows a minimum detectable difference in means of 45% with a standard deviation of 24% or a minimum detectable difference in means of 39% with a standard deviation of 24% with a statistical power of 0.8 and an unadjusted alpha of 0.05 for four and two groups, respectively.

Results

Exposure Data

The average daily temperatures and relative humidity within each of the hoods stayed within the ranges set forth in the *Guide for the Care and Use of Laboratory Animals* (hereafter referred to as *The Guide*) for animal exposures. The ranges for temperature and RH were 68 – 79 °F and 30% - 70%, respectively. The location of the probes was at the top of the exposure hoods so a small temperature gradient was likely to have formed causing the probes to show a slightly higher temperature than what the animals experienced.

The FTIR concentration data was displayed as two averages. The first was an overall average which encompassed the entire time the animal was inside the exposure chamber. The second was a "peak" average that encompassed an approximate twenty minute period starting after the roughly ten minute T-90 concentration equilibration period and ended at the end of the exposure period when the exposure switch was switched off.

Behavioral Data

Experiment 1: Open Field Motor Activity Test

Exposure of rats to 0, 134, 1068, and 2137 ppm TMS did not cause a significant change in the total distance moved for each exposure group relative to the control group (0 ppm) (Figure 8A). However, there was a slight trend in the decrease in total distance with increasing concentrations of TMS. Exposure of rats to increasing concentrations of TMS caused a concentration-dependent decrease in movement time, showing significance at the high concentration (2137 ppm) relative to the control group (Figure 8B). The ambulatory activity results were similar to the total distance the animals moved in that there was a slight concentration-dependent decrease in ambulatory activity but there was no significant difference between any of the exposure groups compared to the control group (Figure 8C). There were concentration-dependent decreases in both stereotypic (Figure 8D) and vertical activities (Figure 8E), which were significant at the middle and high concentrations for the stereotypic activity but only significant at the high concentration for the vertical activity.

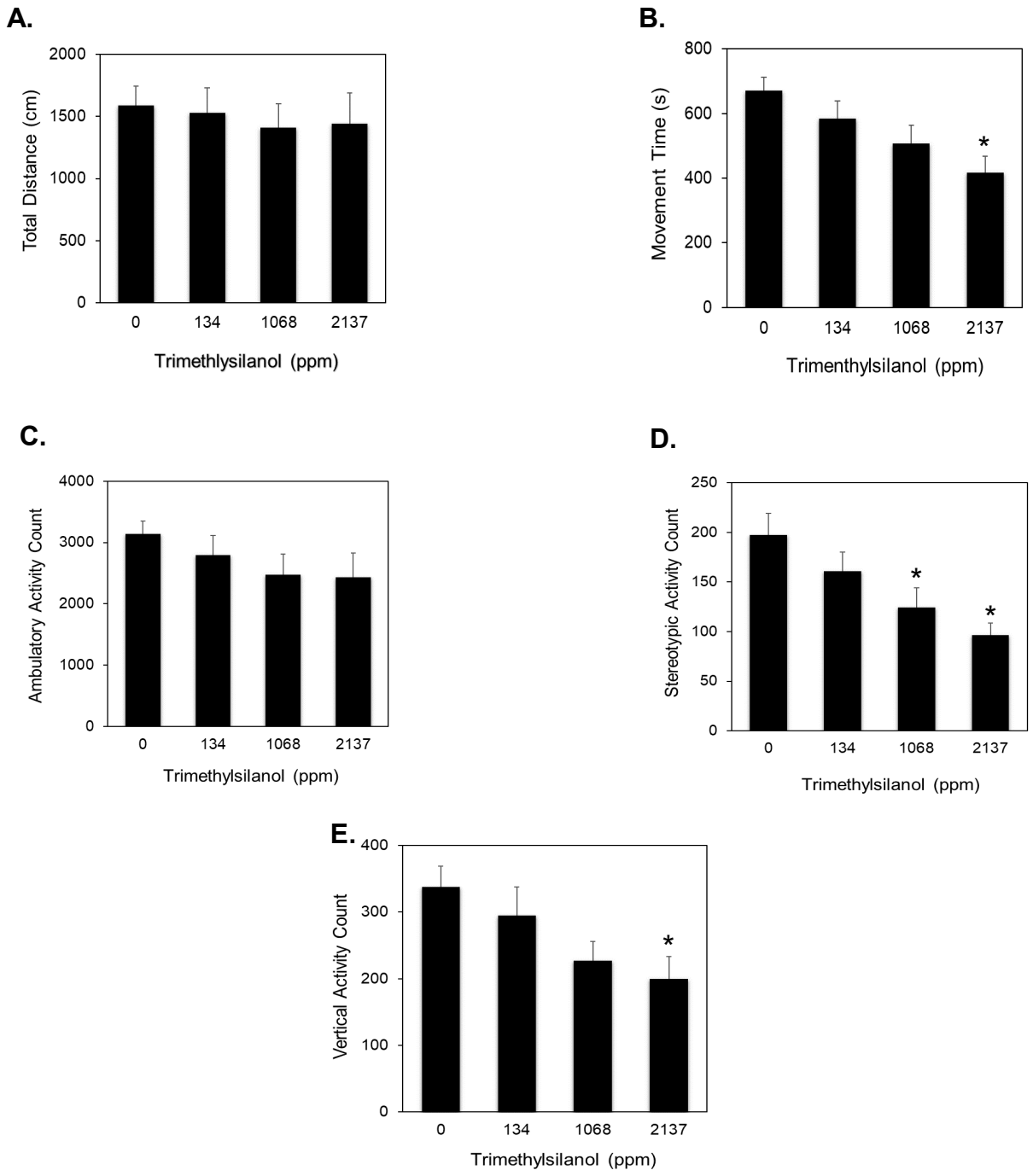

Figure 8. OPEN FIELD MOTOR ACTIVITY BOX TEST. Measurement of total distance (A), movement time (B), ambulatory activity count (C), stereotypic activity count (D), and vertical activity count (E) during exposure of rats to trimethylsilanol for 30 minutes (N=10 animals per group; *p<0.05 compared to the control group; Data are expressed as mean± standard error of the mean (SEM)).

Exposure of rats to 0, 401, 3206, and 6411 ppm TCM caused an initial slight but non-significant decrease in total distance at the lowest concentration (401 ppm); however, this was followed by a significant increase in total distance during exposure to the middle (3206 ppm) and high (6411 ppm) concentrations of TCM (Figure 9A). TCM caused a significant decrease in movement time during exposure to the low and high concentrations of TCM (Figure 9B). Although the middle concentration was not significant, it was trending downward compared to the control group (Figure 9B). The ambulatory activity mirrored the total distance in trend, but the low concentration of TCM caused a significant decrease compared to the control group and there was no significant change in the ambulatory activity for both the middle and high concentration groups compared to the control group (Figure 9C). Exposure of rats to all three concentrations of TCM caused a significant but threshold decrease in stereotypic activity (Figure 9D) but a concentration-dependent decrease in vertical activity (Figure 9E), showing significance at the middle and high concentrations only.

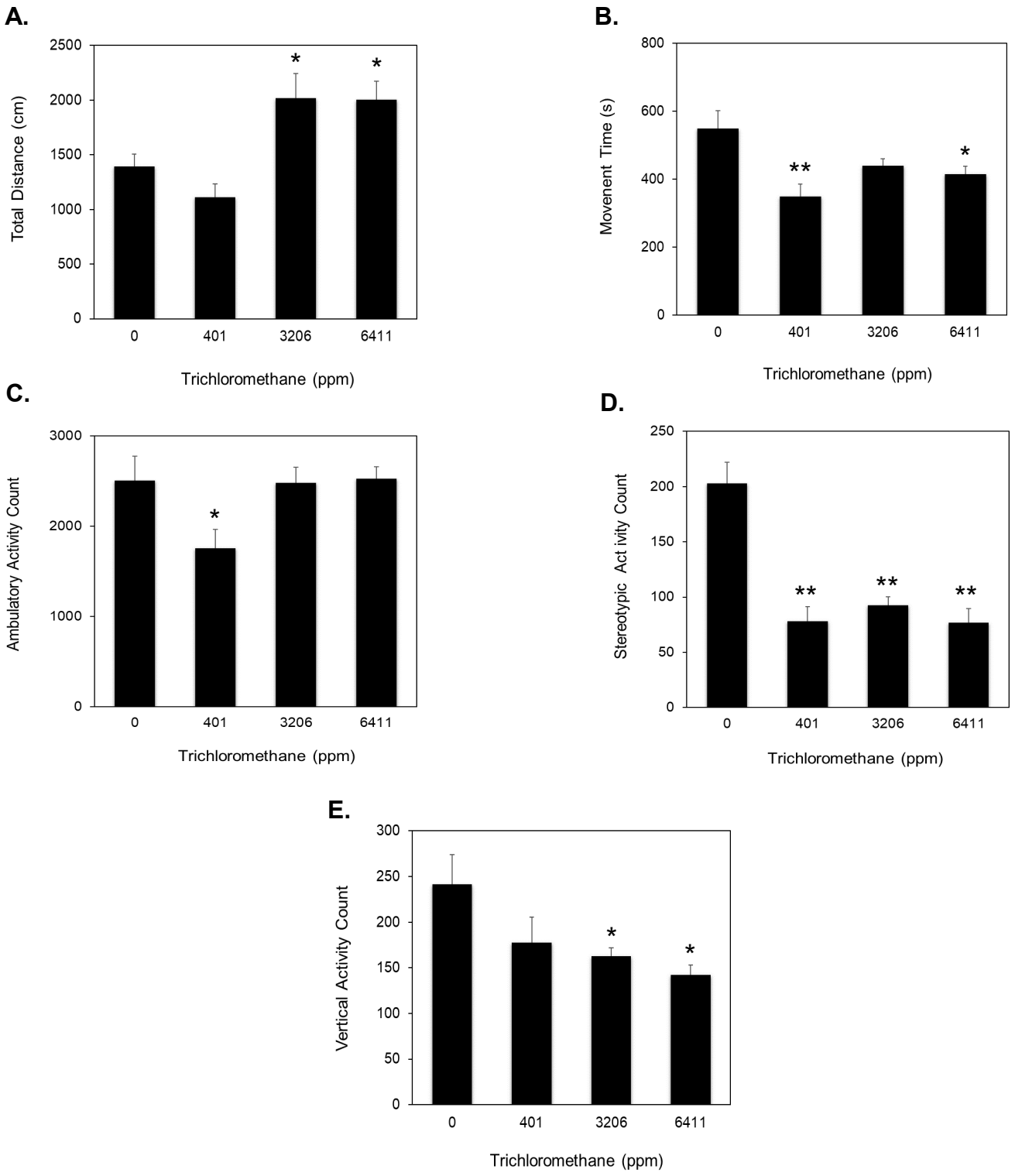

Figure 9. OPEN FIELD MOTOR ACTIVITY BOX TEST. Measurement of total distance (A), movement time (B), ambulatory activity count (C), stereotypic activity count (D), and vertical activity count (E) during exposure of rats to trichloromethane for 30 minutes (N=10 animals per group; *p<0.05 and **p<0.001 compared to the control group; Data are expressed as mean±SEM).

Based on the concentration curves for each chemical, it was determined that the overall NOAEL or LOAEL was at the lowest concentrations for each chemical: 134 ppm for trimethylsilanol and 401 ppm for trichloromethane. Therefore, the two chemicals were combined using their lowest concentrations and rats were exposed to either cleaned filtered air (control group) or to a combination of the two chemicals at their lowest concentrations for a period of 30 minutes while observing their behavior using the open field motor activity box in real-time. Exposure of rats to a combination of the two chemicals caused a 28% decrease in total distance (Figure 10A), a 42% decrease in movement time (Figure 10B), a 39% decrease in ambulatory activity (Figure 10C), a 64% decrease in stereotypic activity (Figure 10D), and a 56% decrease in vertical activity compared to the control group (Figure 10E).

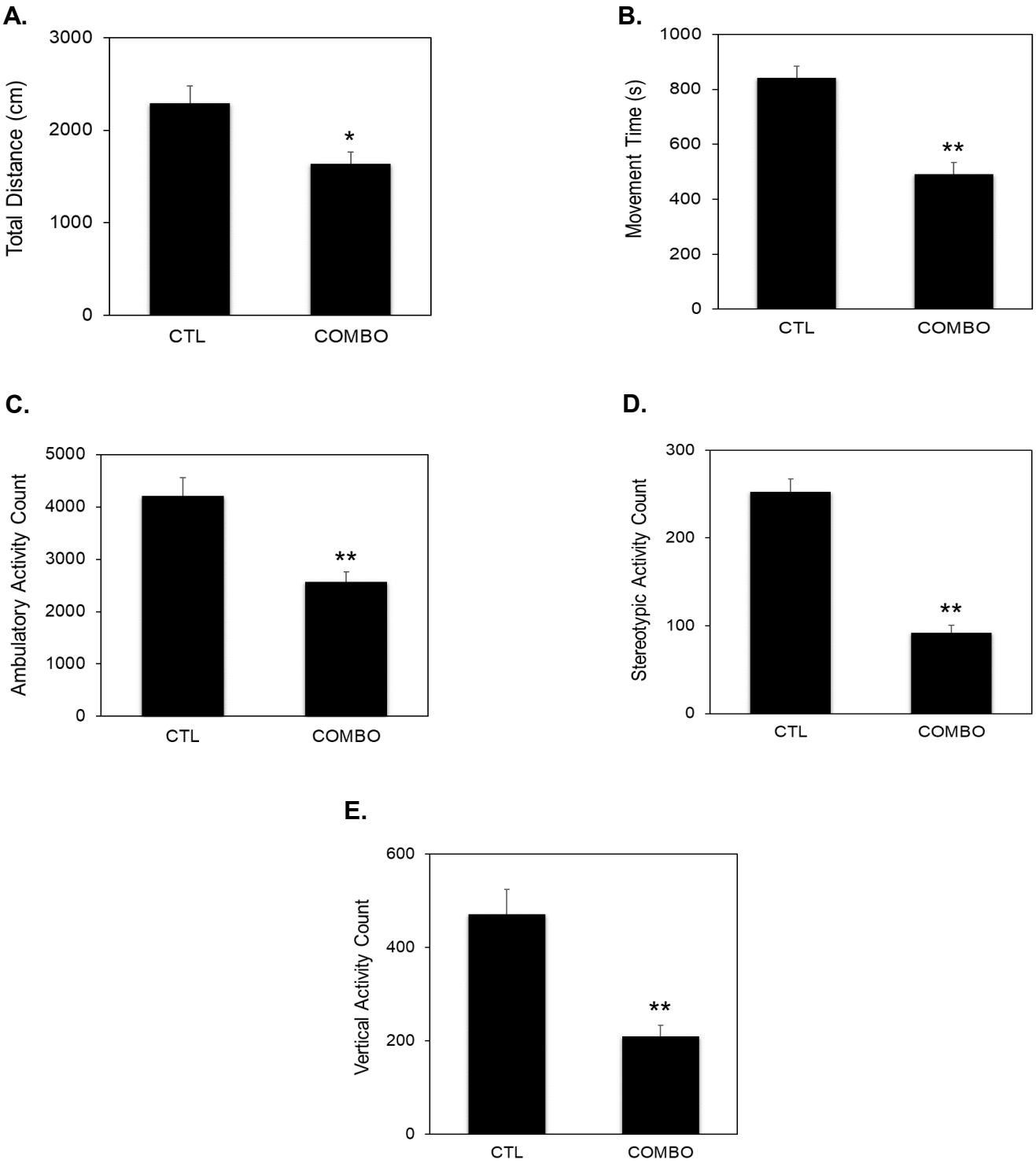

Figure 10. OPEN FIELD MOTOR ACTIVITY BOX TEST. Measurement of total distance (A), movement time (B), ambulatory activity count (C), stereotypic activity count (D), and vertical activity count (E) during exposure of rats to 134 ppm trimethylsilanol and 401 ppm trichloromethane for 30 minutes (N=10 animals per group; *p<0.05 and **p<0.001 compared to the control group; Data are expressed as mean±SEM).

Experiment 1: Rotarod Activity Test

Exposure of rats to 0, 134, 1068, and 2137 ppm TMS caused a slight but insignificant increase in the time spent on the rod (Figure 11A) and the distance traveled on the rod (Figure 11B) at 134 ppm; however, this was followed by a significant concentration dependent decrease by 43% that of the control group during exposure to the high concentration (2137 ppm) of TMS. Furthermore, exposure of rats to 0, 401, 3206, and 6411 ppm TCM caused a concentration dependent decrease by 71% and 75% at the middle and high concentrations of TCM, respectively, for duration (Figure 11C) and distance traveled (Figure 11D). Based on the individual exposures, the two chemicals were combined using their lowest concentrations and exposure of rats to a combination of the two chemicals did not affect the duration (Figure 11E) and rod distance (Figure 11F) compared to the control group.

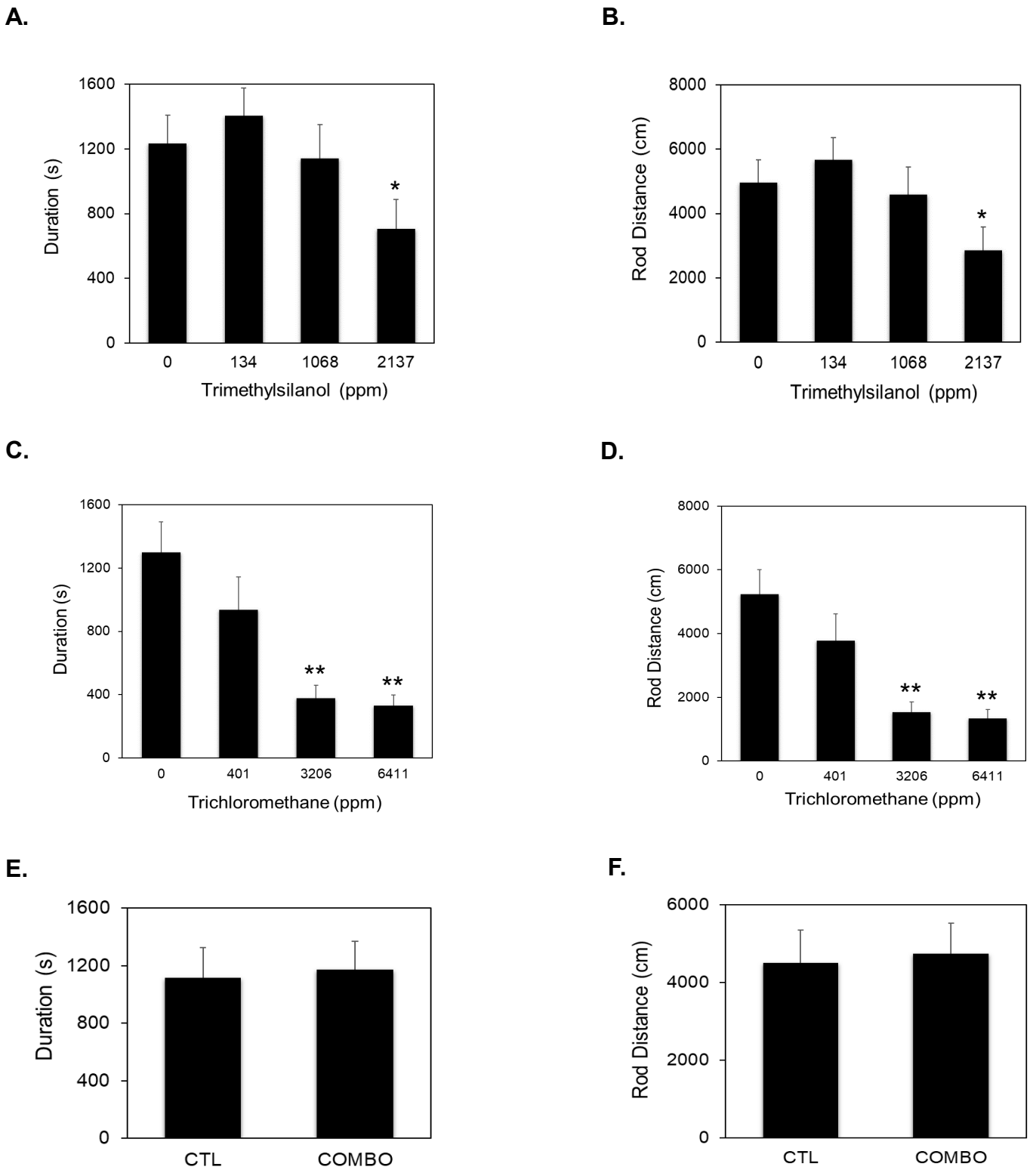

Figure 11. ROTAROD TEST. Duration (seconds) (A) and rod distance (centimeters) (B) during exposure to trimethylsilanol for 30 minutes. Duration (C) and rod distance (D) during exposure to trichloromethane for 30 minutes. Duration (E) and rod distance (F) during exposure to a combination of trimethylsilanol (134 ppm) and trichloromethane (401 ppm) for 30 minutes (N=14 animals per group; *$p<0.05$ and **$p<0.001$ compared to the control group; Data are expressed as mean±SEM).

Post-behavioral Blood Analysis Data

Experiment 2: Single Animal Whole Body Exposure

Rats were exposed to the same concentrations of TMS and TCM individually and in combination to measure the concentration of the chemicals in the blood in order to correlate those concentrations with observed neurobehavioral effects. The average corresponding blood concentrations to 0, 134, 1068, and 2137 ppm TMS were 0, 2.92, 22.7, and 51.4 µg/mL, respectively (Table 13). The average corresponding blood concentrations to 0, 401, 3206, and 6411 ppm TCM were 0, 4.36, 43.5, and 76.7 µg/mL, respectively (Table 14). When the two chemicals were combined at 134 ppm (TMS) and 401 (TCM), the corresponding concentrations in combination were 2.91 (Table 15) and 2.42 µg/mL (Table 16), respectively.

Table 13

Group	Average Blood Concentration (µg/mL)	S. E. M.
0	<0.4884	N/A
134	2.92	0.17 **
1068	22.7	1.36 **
2137 ppm TMS	51.4	2.92 **

Table 14

Group	Average Blood Concentration (µg/mL)	S. E. M.
0	<0.4884	N/A
401	4.36	1.55 **
3206	43.5	9.39 **
6411 ppm TCM	76.7	15.3 **

Table 15

Group	Average Blood Concentration (µg/mL)	S. E. M.
0	<0.4884	N/A
Combo (TMS)	2.91	0.172 **

Table 16

Group	Average Blood Concentration (µg/mL)	S. E. M.
0	<0.4884	N/A
Combo (TCM)	2.42	0.250 **

Tables 13-16. BLOOD CONCENTRATIONS. Blood concentrations post exposure to trimethylsilanol (Table 13), trichloromethane (Table 14), and a combination of 134 ppm trimethylsilanol (Table 15) and 401 ppm trichloromethane (Table 16) for 30 minutes (N=8 animals per group; **p<0.001 compared to the control group; Data are expressed as mean±SEM).

Substance P Levels In Post-mortem Rat Brian

Exposure of rats to TMS, TCM, and a combination of the two chemicals did not alter the levels of acetylcholine, dopamine, epinephrine, GABA, glutamate, and serotonin compared to the control group (data not shown). However, exposure to 134 ppm and 2137 ppm TMS caused a 20% decrease in the levels of Substance P compared to the control group (Table 17), but there was no change in Substance P levels during exposure to TCM (Table 18) or a combination of TMS and TCM (Table 19) compared to the control group.

Table 17

Group	Substance P Concentration (pg/mL)	S. E. M.
0	59.6	5.36
134	40.5	3.20 *
1068	48.8	3.92
2137 ppm TMS	39.9	3.07 *

Table 18

Group	Substance P Concentration (pg/mL)	S. E. M.
0	43.7	3.20
401	40.7	3.18
3206	42.6	4.04
6411 ppm TCM	42.8	3.22

Table 19

Group	Substance P Concentration (pg/mL)	S. E. M.
0	43.1	4.80
Combo (TMS and TCM)	42.6	2.72

Tables 17-19. SUBSTANCE P LEVELS. Exposure of rats to trimethylsilanol (Table 17), trichloromethane (Table 18), and a combination of 134 ppm trimethylsilanol and 401 ppm trichloromethane (Table 19) for 30 minutes (N=14 animals per group; *$p<0.05$ compared to the control group; Data are expressed as mean±SEM).

Discussion

As recent as 2017 and 2018, Navy T-45 and F-18 fighter pilots and weapons officers complained about experiencing PEs. A PE consists of a variety of symptoms including tingling and/or weaknesses in the limbs and extremities, headaches, vertigo, and possible changes in mental status, cognition, or loss of consciousness. Environmental conditions including altitude fluctuations, which result in changes in cockpit air pressure, and chemical contamination could contribute to PEs. Indeed, a plethora of low level chemical contaminants were discovered in the breathing air generated by the OBOGS and in sorbent tubes in the aircraft cockpits, and it was recommended by AESAB that several of those chemicals including TMS and TCM be evaluated further (Mumy, 2018). Therefore, the purpose of this study was to determine whether exposure of male Sprague-Dawley rats to a combination of these two chemicals based on their NOAELs/LOAELs could cause neurocognitive effects using two behavioral tests: the open field motor activity and rotarod tests.

Rats are commonly used in inhalation exposure studies because their anatomy is suitable for comparison to the effects that would be expected in humans. Additionally, utilization of rats in neurobehavioral research has been well characterized using a variety of neurobehavioral tests, including the open field motor activity and rotarod tests, which are used to measure neurological decrements that may occur as a result of being exposed to various drugs or environmental toxicants. In this study, rats were exposed to 0, 134, 1068, and 2137 ppm TMS and 0, 401, 3206, and 6411 ppm TCM to establish a concentration-response curve to determine the effects of each chemical separately, and then rats were exposed to a combination of the two chemicals based on their NOAEL or LOAEL to determine if the chemicals caused additive or synergistic adverse effects. Furthermore, this study also investigated the real-time neurobehavioral effects of exposure, as opposed to the standard procedure, which typically entails testing once exposure has ended.

Exposure of rats to increasing concentrations of TMS caused concentration-dependent decreases in the movement time, ambulatory activity, stereotypic activity, and vertical activity. The ambulatory and stereotypic activities are major contributors to movement time, and the significant decrease in movement time at the high concentration of TMS was likely due to the significant decrease in stereotypic activity. The total distance traveled was trending downward in a concentration-dependent manner and would have possibly reached significance if more animals were included in each group. The ambulatory activity had a more robust concentration-dependent decrease compared to the total distance, albeit the decrease was insignificant; however, the addition of more animals to each group would have possibly resulted in significant decreases in ambulatory activity with increasing concentrations. Similar to the motor activity, increasing concentrations of TMS caused a concentration-dependent increase in motor coordination at the middle and high concentrations, showing significance at the high concentration. An interesting finding in this study is that the low concentration of TMS caused a slight increase in the rats' ability to stay on the rotarod compared to the control group, therefore, slightly enhancing performance at the low concentration but impairing motor coordination at the high concentration. However, increasing concentrations of TMS impaired motor activity and motor coordination.

Exposure of rats to increasing concentrations of TCM caused a non-significant decrease in total distance traveled during exposure to the low concentration of TCM, which was followed by significant increases in total distance traveled during exposure to higher concentrations of TCM. Based on observations during the exposure, these significant increases can likely be attributed to rats making

several short but rapid bursts of movement during exposure to the middle and high concentrations of TCM. Similar to TMS, exposure of rats to increasing concentrations of TCM caused an overall impairment in motor activity and motor coordination.

According to the concentration-response data for TMS and TCM, the low concentrations were chosen because they had little to no effect for most of the parameters tested. Therefore, rats were subsequently exposed to 134 ppm TMS and 401 ppm TCM simultaneously during the motor activity and rotarod tests. Exposure to a combination of the two chemicals at low levels caused a significant decrease in every parameter of the motor activity test, with the combined effects being greater for four of the five measures of motor activity, suggesting the potential for additive or synergistic CNS depressive effects. Interestingly, a combination of the two chemicals did not impair motor coordination. Additive or synergistic effects as a result of combinational exposure to two or more different chemicals at low levels may be elicited via binding sites on a variety of receptors in the CNS. For example, TCM is an agonist of the γ-aminobutyric acid type-A receptor ($GABA_A$), which mediates inhibition of neurotransmission or the action potential (Jenkins et al., 2001) and induces CNS depression. Although some alcohols (e.g. ethanol) can bind to $GABA_A$, the exact molecular target of TMS is not known. However, it is plausible that the CNS depressive effects induced by TMS exposure occurred as a result of TMS binding to a different subtype of the $GABA_A$ receptor family, although further investigation is necessary to elucidate the mechanisms of action for TMS.

The robust differential effects that were observed in the rats during the motor and rotarod activity tests are not uncommon. For example, in a rat model of migraine headache, exposure of rats to 10% mustard oil and the environmental irritant umbellulone, which are both transient receptor potential ankyrin-1 receptor agonists, significantly impaired motor activity but not motor coordination (Edelmayer et al., 2012). In a mouse model of Huntington's disease, mice overexpressing the mutant huntingtin protein showed significant decreases in motor activity at 6-12 months but only showed a significant impairment in motor coordination at 12 months compared to the wild type group, respectively (Peng et al., 2016). Similar effects were also shown in a mouse model of mild traumatic brain injury in that repetitive mild traumatic brain injury in mice impaired motor activity but not motor coordination (Semple et al., 2016). Per the results reported by Peng et al. (2016), it is possible that exposure to a combination of TMS and TCM at low concentrations beyond the 30 minute real-time exposure period could have impaired motor coordination and thus decreased the rats' latency to fall off the rotarod; however, due to experimental limitations, the rats were unable to stay on the rotarod longer than 30 minutes under normal conditions.

Previously, it was shown that exposure of adult Fisher rats to trichloroethylene for six weeks via oral gavage decreased the levels of dopamine metabolites significantly, which corresponded with a significant decrease in motor coordination at the same time point (Liu et al., 2010). Although the levels of dopamine metabolites likely were attributed to the death of dopaminergic neurons in the striatum as shown by immunohistochemistry, it was of interest to ascertain whether exposure of rats to TMS, TCM, or a combination of the two chemicals via inhalation could alter the levels of neurotransmitters and possibly influence the behavioral outcomes as a result of the drastic change in the levels of specific neurotransmitters. Therefore, the levels of acetylcholine, dopamine, epinephrine, GABA, glutamate, norepinephrine, serotonin, and Substance P were measured. Exposure of rats to TMS and TCM individually and in combination did not have a significant effect on altering the levels of any of the neurotransmitters that were measured, with the exception of TMS, which decreased the levels of Substance P. The significant decrease in Substance P during exposure to 134 and 2137 ppm of TMS is interesting. Since Substance P is an undecapeptide that is synthesized by ribosomes (Harmar et al., 1982), it is possible that TMS could directly or indirectly

inhibit the synthesis of peptide neurotransmitters; however, further studies are necessary to elucidate the mechanistic actions of TMS and its effects on the levels of Substance P.

PEs can manifest as a variety of neurological symptoms during flight and can potentially place the aircrew in grave danger when flying under austere conditions. For example, symptoms can range from headaches to loss of consciousness. After a series of incidents, which involved T-45 and F-18 pilots who experienced PEs, public awareness of this problem and the ensuing discovery of low level chemical contaminants onboard these aircraft, resulted in AESAB recommending further evaluation of 17 compounds that are known to induce CNS effects (Mumy, 2018). The sole purpose of this study was to determine whether a combination of two of the chemicals, TMS and TCM, which were found onboard these aircraft, could cause adverse CNS effects when combined at low levels using a rat model. The overall results of this study suggest that a combination of low level chemical contaminants can cause differential CNS effects as displayed by the significant decrease in the rats' spontaneous motor activity/ability to explore a novel environment and the lack of impaired motor coordination. According to findings released by the Physiological Episodes Action Team, which was commissioned by the Naval Safety Center, PEs were not caused by contaminated air, a lack of oxygen or systems not designed well enough to keep humans safe in harsh environments (Eckstein, 2020). Although this recent report suggests that the low level chemical contaminants discovered onboard these aircraft are not the root cause of the PEs experienced by pilots who flew them, the results from this study conclude that a combination of low level chemical contaminants could possibly cause PEs, at least in part, in a rat model. Therefore, based on the findings of this study, uncertainty factor calculations (Dankovic et al., 2015) can be used to set new exposure limits for TMS and TCM to ensure the safety of Navy pilots.

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Appendix. Concentration Data

Motoractivity Single Chemical								
TMS	Hood 1 – Control		Hood 2 – Low		Hood 3 – Mid		Hood 4 – High	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	-6.35	-7.63	105	138	755	1032	1490	2048
Min	-24.2	-23.8	62.9	89.5	490	728	963	1429
Max	8.55	7.21	168	199	1146	1353	2229	2834
Std. Dev.	10.7	10.5	31.3	33.8	197	211	395	460
Count	10	10	10	10	10	10	10	10
TCM	Hood 1 – Control		Hood 2 – Low		Hood 3 – Mid		Hood 4 – High	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	-24.0	-27.0	298	351	2128	3109	3932	6477
Min	-148	-149	179	221	1604	2264	3294	5053
Max	12.3	11.8	405	410	2398	3533	5239	7176
Std. Dev.	58.3	64.0	56.2	58.4	217	332	537	560
Count	10	10	10	10	10	10	10	10

Motoractivity Combo								
TMS	Hood 1 – Control		Hood 2 – Exposure		Hood 3 – Control		Hood 4 – Exposure	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	-16.3	-18.5	109	135	-14.0	-16.4	107	132
Min	-22.9	-25.9	83.2	108	-25.4	-27.3	94.5	122
Max	4.82	3.37	156	184	9.42	7.80	127	150
Std. Dev.	11.9	12.3	29.4	31.0	13.8	14.0	12.2	11.6
Count	5	5	5	5	5	5	5	5
TCM	Hood 1 – Control		Hood 2 – Exposure		Hood 3 – Control		Hood 4 – Exposure	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	11.3	11.9	280	366	-19.7	-21.1	299	382
Min	5.59	5.58	89.0	117	-25.2	-26.3	129	162
Max	18.1	19.5	406	522	-8.45	-10.7	398	505
Std. Dev.	5.35	5.66	120	155	6.63	6.12	104	135
Count	5	5	5	5	5	5	5	5

SAWB Single Chemical								
TMS	Hood 1 – Control		Hood 2 – Low		Hood 3 – Mid		Hood 4 – High	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	56.7	63.1	141	163	770	1041	1381	1965
Min	28.4	33.5	112	130	713	948	1310	1821
Max	89.0	98.0	190	221	864	1142	1468	2159
Std. Dev.	20.8	24.1	24.1	28.2	48.5	72.2	59.6	105
Count	8	8	8	8	8	8	8	8
TCM	Hood 1 – Control		Hood 2 – Low		Hood 3 – Mid		Hood 4 – High	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	44.7	48.3	321	407	2416	3150	4720	6350
Min	25.9	24.7	244	377	2261	2905	4517	5947
Max	58.8	65.5	360	439	2526	3329	4901	6527
Std. Dev.	11.2	12.8	38.8	25.3	90.3	129	134	182
Count	8	8	8	8	8	8	8	8

SAWB Combo								
TMS	Hood 1 – Control		Hood 2 – Exposure		Hood 3 – Control		Hood 4 – Exposure	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	63.1	66.1	154	182	147	169	172	198
Min	53.8	59.4	131	160	61.9	69.3	132	158
Max	70.1	72.2	174	197	242	275	219	252
Std. Dev.	8.21	6.40	17.4	15.9	83.3	101	39.5	44.8
Count	4	4	4	4	4	4	4	4
TCM	Hood 1 – Control		Hood 2 – Exposure		Hood 3 – Control		Hood 4 – Exposure	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	48.4	51.9	359	440	80.3	89.3	363	445
Min	33.7	35.6	342	407	55.5	58.7	336	395
Max	62.7	68.3	377	468	110	125	421	527
Std. Dev.	12.5	14.3	19.6	30.2	24.0	30.1	39.9	57.0
Count	4	4	4	4	4	4	4	4

Rotarod Single Chemical								
TMS	Hood 1 – Control		Hood 2 – Low		Hood 3 – Mid		Hood 4 – High	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	1.99	0.47	113	135	772	995	1496	2064
Min	-11.7	-12.7	79.5	96.0	657	833	1338	1836
Max	10.1	9.17	258	301	912	1177	1712	2284
Std. Dev.	7.00	7.38	44.5	51.5	63.3	83.0	111	126
Count	14	14	14	14	14	14	14	14
TCM	Hood 1 – Control		Hood 2 – Low		Hood 3 – Mid		Hood 4 – High	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	2.13	2.01	327	394	2557	3236	4764	6426
Min	-11.3	-10.9	300	365	2244	2907	3199	4832
Max	12.1	13.5	365	445	2811	3495	5314	7209
Std. Dev.	7.02	6.90	18.3	23.9	162	181	511	541
Count	14	14	14	14	14	14	14	14

Rotarod Combo								
TMS	Hood 1 – Control		Hood 2 – Exposure		Hood 3 – Control		Hood 4 – Exposure	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	9.07	7.94	135	157	8.55	3.23	108	131
Min	4.34	1.64	105	123	1.53	-1.17	95.9	119
Max	11.2	11.0	152	179	28.3	7.22	124	147
Std. Dev.	2.38	3.03	16.5	18.1	9.16	3.00	9.72	9.77
Count	7	7	7	7	7	7	7	7
TCM	Hood 1 – Control		Hood 2 – Exposure		Hood 3 – Control		Hood 4 – Exposure	
	Overall	Peak	Overall	Peak	Overall	Peak	Overall	Peak
Average	2.80	2.21	318	388	15.8	15.0	351	422
Min	-8.52	-9.28	283	350	8.85	7.58	329	396
Max	16.8	17.3	358	431	24.6	24.1	402	481
Std. Dev.	8.46	8.94	27.6	30.4	6.23	5.74	24.8	28.6
Count	7	7	7	7	7	7	7	7