Crude Oil Vapor Neurotoxicity Study Dataset

Materials and Methods

Animals

Male Sprague-Dawley [Hla:(SD) CVF] rats (200 – 250 g) were procured from Hilltop Lab Animals (Scottdale, PA). Upon arrival, animals were housed in pairs in ventilated micro-isolator units with autoclaved ALPHA-dri® virgin cellulose chips (Shepherd Specialty Papers; Watertown, TN) and hardwood Beta-chips (NEPCO; Warrensburg, NY) for bedding, with provision for HEPA-filtered laminar flow air (Thoren Caging Systems; Hazleton, PA). Tap water and Harlan 2918 irradiated Teklad Global 18% rodent chow (Harlan Teklad; Madison, WI) was provided *ad libitum*. Rats were housed under controlled light cycle (12 h light/12 h dark), temperature (22 – 25 °C) and humidity (40 – 65 % RH) conditions. The NIOSH animal facility is specific pathogen-free, environmentally controlled, and accredited by AAALAC International. All animals were acclimated for at least 6 d after arrival prior to experimentation. All animal use and procedures have been reviewed and approved by the Institutional Animal Care and Use Committee.

Whole-body inhalation exposure to COV

COV was generated using Macondo well surrogate crude oil obtained from the Gulf of Mexico. The Macondo well is in close proximity to the DWH oil spill site. The COV was well characterized prior to use in animal exposures (McKinney *et al.,* 2022). An automated whole-body inhalation exposure system was developed to expose animals to COV (McKinney *et al*., 2022). Briefly, COV was generated using a collision type atomizer. Chamber concentration and pressure were regulated with software feedback loops to ensure that constant concentrations of COV (300 ppm × 6 h/d) were maintained throughout the duration of the exposure. Additionally, BTEX gases, temperature, humidity, and carbon dioxide levels were monitored throughout the duration of the whole-body inhalation exposure. The COV concentration chosen for the study is comparable to that reported in the breathing zone of workers conducting upstream operations in the oil and gas industry.

Prior to exposures, rats were acclimated in the inhalation chamber for 4 - 5 d. During inhalation exposures, the animals were housed individually inside stainless-steel wire mesh cages. The dimensions of individual cells in the cages are adequate to provide the animal sufficient room without confining them to an abnormal environment during exposure and provide unobstructed airflow. The chamber air flow was maintained at 40 – 50 L/min with 8 – 12 air changes/h. The chamber temperature was maintained between 66 – 79°F (usually 72°F) and chamber relative humidity was maintained between 35 – 60 % (usually 45%).

Rats were exposed by whole-body inhalation to 300 ppm of COV aerosol for 6 h/d for one of two durations: a single exposure for one day (300 ppm; 6 h/d × 1 d; acute exposure), or a repeated exposure of 4 d/wk for 4 consecutive wks (300 ppm; 6 h/d × 4d/wk × 4 wks; for a total of 16 d; sub-chronic exposure). Control animals were simultaneously exposed to HEPA-filtered air in an identical exposure system.

The exposures were conducted in two experimental blocks to obtain a final $n = 8$ for each experimental group. During the daily 6 h whole-body inhalation exposures, the animals did not have access to food or water. Animals exposed under such conditions did not exhibit any observable pain or distress, body weight changes, behavioral abnormalities or adverse health effects.

Euthanasia and sample collection

In the acute COV exposure studies, animals were euthanized at 1 or 28 d after exposure. In the sub-chronic COV exposure studies, animals were euthanized at 1, 28 or 90 d after cessation of exposure. Euthanasia was performed by administration of sodium pentobarbital euthanasia solution (Fatal Plus; >100 – 300 mg/kg; Vortech Pharmaceutical Ltd., Dearborn, MI; intraperitoneal injection), and the animals were exsanguinated by transecting the abdominal aorta to ensure death. All euthanasia and tissue collection procedures were performed between 7:30 – 11:30 am on the days of the sample collection. After euthanasia and collection of lung lavage fluid for pulmonary toxicity assessment, the brains were excised and discrete brain areas, i.e., olfactory bulb (OB), striatum (STR) and midbrain (MB) from the left and right hemispheres, were dissected free-hand. Tissues from the left hemisphere were collected in 1% perchloric acid and stored at -75 °C until processing for biogenic amine neurotransmitter analysis. Tissues from right hemisphere were collected in Tissue Protein Extraction Reagent (T-PER, Pierce Biotechnologies, Inc.; Rockford, IL) containing protease inhibitor cocktail and EDTA for subsequent isolation of protein for immunoblot analysis. Due to the cellular heterogeneity of the nervous system, as well as the progressive nature of neural injury, the timeline of expression of various injury markers (neurotransmitters, neuronal and synaptic proteins, glial proteins) are distinct. Through a careful understanding of the time course of expression of various neural makers based on our previous experience, we monitored their changes at relevant time points in this study. As synaptic and neuronal damage are typically delayed events, changes in their protein markers were monitored at 28 d after the acute exposure and all post-exposure time points after the sub-chronic exposure. Changes in neurotransmitters were monitored at all post-exposure time points after acute and sub-chronic exposures in order to determine both early and long-term/persistent alterations in their levels.

High performance liquid chromatography with electrochemical detection (HPLC-EC)

The biogenic amines, norepinephrine (NE), epinephrine (EPI), dopamine (DA), and serotonin (5-hydroxytryptamine, 5-HT) were measured by HPLC-EC. Briefly, brain tissues (OB, STR, MB) were homogenized in 1 % perchloric acid containing isoproterenol (as internal standard) and centrifuged for 10 min at 12,000 × g. The supernatant was filtered through a 0.2 µm nylon filter and 10 µl aliquots were injected onto a C-18 reverse phase HPLC column (Agilent Technologies; Santa Clara, CA) using an UltraFast Liquid Chromatography (UFLC) system (Shimadzu Instruments; Columbia, MD) attached to an autosampler. A BAS-LC4B amperometric detector (BASi Inc.; West Lafayette, IN) with a glassy carbon oxidative flow cell was used for detection at an electrode potential of 0.8 V. The mobile phase (pH 3.2) consisted of 0.15 M monochloroacetic acid, 0.115 M sodium hydroxide, 0.1 mM EDTA, 0.015 % sodium octyl sulfate, 3 % acetonitrile, 1.5 % methanol and 1.2 % tetrahydrofuran. Under these conditions NE, EPI, DA, 5-HT, DOPAC, HVA and 5-HIAA were chromatographically separated and detected. Analytes were quantified by comparing peak area detector responses in the sample with those produced by a series of standards similarly prepared in 1 % perchloric acid. The pellet from each of the processed sample was solubilized with 3 % sodium hydroxide and the protein content was estimated. The neurotransmitter content in each sample was then normalized to the amount of protein. The values were calculated as ng/mg total protein and are graphically represented as percent of air-exposed controls.

Preparation of brain tissues for protein isolation

Brain tissues (OB, STR, MB) collected in 1.5 ml microfuge tubes were homogenized using 0.3 ml of T-PER tissue protein extraction reagent (Pierce Biotechnologies, Inc.; Rockford, IL) containing protease inhibitors and EDTA. The homogenates were centrifuged at 9,600 × g for 5 min to pellet the cell/tissue debris. The supernatants were carefully recovered, without disturbing the pellet, and transferred to a new 1.5 ml centrifuge tubes. The supernatants were stored at -20 °C until protein concentrations were determined.

Protein estimation

On the day of protein estimation (usually the following day after supernatants were prepared), the samples were allowed to thaw on ice and vortexed. Total protein in the supernatant was determined according to the micro-bicinchoninic acid method (Pierce Biotechnologies, Inc.) using bovine serum albumin as a standard. For long-term storage, the protein extracts were stored at -75 °C.

Western immunoblotting

Aliquots of brain homogenates (6 µg total protein) diluted 1:1 in 2× Laemmli sample buffer were boiled and loaded onto 10 % SDS-polyacrylamide gels. Proteins then were electrophoretically resolved and transferred to 0.45 µm Immobilon-FL PVDF Membranes (cat# IPFL00010, Millipore; Billerica, MA). Following transfer, immunoblot analysis was performed. Briefly, membranes were blocked using Odyssey Blocking Buffer (LI-COR Biosciences; Lincoln, NE) for 1 h at room temperature, washed (1 × 5 min; 2 × 10 min) with phosphate-buffered saline containing Triton X-100 (PBST), and incubated overnight at 4 °C with the primary antibodies (30 – 50 ng/ml primary antibody buffer) to synaptophysin 1 (SYP; mouse monoclonal, Santa Cruz Biotechnology, Inc.; Santa Cruz, CA; 35 kDa), synaptotagmin 1 (SYT; mouse monoclonal, Santa Cruz Biotechnology, Inc.; 60 kDa), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (YWHAE or 14-3-3E; mouse monoclonal, Santa Cruz Biotechnology, Inc.; 28 kDa), glial fibrillary acidic protein (GFAP; rabbit polyclonal, DAKO North America Inc.; Carpinteria, CA; 50 kDa), tyrosine hydroxylase (TH; rabbit polyclonal, Millipore, Temecula, CA or mouse monoclonal, AVES Labs, Davis, CA; 56 kDa), PARK5 (UCHL1/PGP 9.5; rabbit polyclonal, Sigma-Aldrich, St. Louis, MO; 26 Kuda), and PARK7 (DJ1; goat polyclonal, Novus Biologicals, Centennial, CO; 22 kDa). β-Actin (ACTB; mouse monoclonal, Santa Cruz Biotechnology, Inc.; 46 kDa) was used as endogenous control. Following incubation with primary antibodies, the blots were washed with PBST (1 × 5 min; 3 × 10 min) and then incubated for 1 h at room temperature with appropriate secondary antibodies conjugated with near-infrared fluorescent dyes, IRDye 680 or IRDye 800 (LI-COR Biosciences; Lincoln, NE). The membranes were protected from light to minimize any photo-bleaching of the fluorescent dyes. Membranes were washed (1 × 5 min; 4 × 10 min) in PBST, followed by washes (2 × 3 min) in PBS. Near-infrared fluorescence detection was performed using the Odyssey Imaging System (LI-COR Biosciences). The fluorescent signal intensities (*k* counts) of the individual bands were determined and normalized to ACTB. The data are graphically represented as percent of air-exposed controls to depict increases or decreases in protein expression.