

## Materials and Methods.

Animals. Male (n=18) Sprague-Dawley rats (Hla<sup>®</sup>(SD)CVF<sup>®</sup>, approximate body weight of 200 – 230 g at arrival), were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). All rats were free of viral pathogens, parasites, mycoplasma, *Helicobacter*, and cilia-associated respiratory bacillus. Upon arrival, rats were acclimated to the AAALAC International accredited animal facilities at NIOSH for one week. The NIOSH animal facility is a specific, pathogen-free, environmentally controlled facility. They were housed in ventilated micro-isolator units supplied with HEPA-filtered laminar flow air (Lab Products OneCage; Seaford, DE), Teklad Sanichip and Shepherd Specialty Paper's Alpha-Dri cellulose, tap water, and autoclaved Teklad rodent diet (Harlan Teklad; Madison, WI) available *ad libitum*. Rats were housed in pairs, and under controlled light cycle (12 h light/12 h dark) and temperature (22 – 25 °C) conditions. One week following acclimation to the facilities, rats were randomly assigned to restraint control conditions or to an applied force condition of 2 or 4 newtons (N). The use of animals, housing, exposures, and all other procedures performed were reviewed and approved by the Institutional Animal Care and Use Committee and are in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals.

Exposure. After acclimation to the facilities, rats were acclimated to restraint for 5 d. Acclimation to restraint was performed by putting animals into Broome Style restrainers for gradually longer lengths of time until the total time in the restrainer was 4 h. The restrainers were large enough so that animals could move but they could not turn around or rear up onto their hind legs. Acclimation to restraint was performed by starting with 1h of exposure in the restrainer, and then increasing the length of the exposure by 1h/day until the rats were acclimated

to 4h of continuous restraint. After 5d of exposure to restraint, the experiment began, and animals were exposed to applied force or control conditions. The tails of rats exposed to applied force were gently placed on the holding platform, and the pressure platform was gently lowered onto the middle of their tail (approximately at C12-20), as shown in Figure 1. The length of the loading plate acting on the tail was 53 mm. The tail contact width was measured in a test with cadaver tails was 4.49 mm for 2.07 N and 5.09 mm for 4.3 N [41]. Hence, it is estimated that the average contact pressure on the living tail was approximately 8 kPa for 2 N force and 14 kPa for 4 N force. Additional details regarding the characterization of the system can be found in (Dong et al. 2023). Once the tail and pressure apparatus were in place, the tail was marked so that the same region was exposed each day. Each animal was exposed to control or applied pressure conditions for 4 h/day for 10 consecutive days.

On the morning following the last exposure, rats were anesthetized using 100-300 mg/kg sodium pentobarbital euthanasia solution and exsanguinated by cardiac puncture. Tails were dissected and sections of skin, nerve, ventral tail artery, DRG and spinal cord were collected and stored in cryovials for measurement of another set of sections were put into Tissue Tek ( ) in cryomolds. All tissues were stored at -80C other for morphological analyses and localization of proteins using immunohistochemistry.

*Serum Hormone Assays:* Blood was collected via cardiac puncture during euthanasia was centrifuged at 1500 rpm. Serum was collected and stored in 100  $\mu$ l aliquots at -80°C. Estradiol, testosterone, progesterone, follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) were measured in duplicate serum samples (10-50  $\mu$ l, depending on the assay). All assays were from Calbiotech (El Cajon, CA), and were performed using the manufacturer's protocol.

*Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR):* qRT-PCR was performed to determine if exposure to applied force resulted in changes in transcript levels in the artery, nerve, skin, DRG and spinal cord using the methods described in (Hughes et al. 2009). We examined transcripts for cytokines (interleukin (*Il*)-1 $\beta$  and *Il6*) and tumor necrosis factor (*Tnf*)- $\alpha$ ), hypoxia-induced factor 1, (*Hif1*), vasomodulating factors (neuronal, inducible and endothelial nitric oxide synthase (*nNos*, *iNos* and *eNOS*) respectively), factors involved in vascular remodeling [vascular endothelial growth factor (*Vegf*) and endothelin (*Et1*)], and antioxidant enzymes [catalase (*Cat*) and superoxide dismutase-2 (*Sod-2*), metallothioneine (*Mt1*)]. These transcripts were chosen based on their responses to vibration exposures in tail tissues in previous studies.

*Processing of tissue for morphology and immunohistochemistry.* Skin from the exposed area of the tail, a segment of the ventral tail artery and ventral tail nerve, and the DRG from the L1-5 region of the spinal cord were processed as follows. Tissue was placed on a mounting platform, frozen in Tissue Tek and 20  $\mu$ m cross-sections were cut on a cryostat. A total of 5 slides/animal was collected. The first section was placed on the first slide, the second on the second slide etc. Once there was a section on each of the 5 slides, the next section was placed back onto slide 1. Sections (5-10 depending on the tissue type) were collected on slides, with each section on a slide being 100  $\mu$ m in distance from the adjacent section.

*Immunohistochemistry.* Immunohistochemistry was performed as described in (Krajnak et al. 2006; Kiedrowski et al. 2015). Briefly, one slide was chosen from each tissue type. Slides were thawed and tissue circled with a pen that produced a liquid proof barrier. Sections were fixed for 5 min in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), rinsed in PBS and incubated overnight in primary antibody diluted 1:1000 in PBS + 0.3% TX-100 (buffer).

Table 2 has a list of the specific antibodies used, and the tissue(s) they were used in. Slides were rinsed, incubated in the appropriate secondary antibody diluted 1:800 in buffer at room temperature for 1h, rinsed again in PBS, and the procedure was repeated using another primary and secondary antibody. After completing the immunohistochemistry, slides were air dried in the dark overnight, cover slipped using Fluoromont G with DAPI (Fisher Scientific, Pittsburgh PA) and stored at 4°C until imaged and quantified. All primary antibodies were from Santa Cruz Biotechnology (Dallas, Tx) and fluorescent secondary antibodies (Cy2 and Cy3) were from Invitrogen Life Sciences Technologies (Eugene Oregon).

All sections were imaged on a DP73 Olympus microscope at 20 x magnification. Photomicrographs were taken of each tissue type (3-5). To ensure all pictures were taken using the same settings, several sections were imaged to determine the best threshold for identifying immunostaining. That threshold was then used for photographing all sections of a particular tissue type.

Quantification of immunostaining was done using ImageJ (National Institutes of Health). Photos were imported into ImageJ, set to grayscale, and a threshold was set for identifying staining. This threshold was maintained for all sections of a specific tissue type and stained for a particular protein. The area of interest was outlined and the percent of the outlined area that was immune-stained was measured along with the intensity of the stained area. Immuno-labelled neurons were also counted in the DRG for antigens that labelled neurons. The average cell number or area that was immuno-stained in a particular region was then used for data analysis.