Materials and Methods

Animals. Male (n=18) Sprague-Dawley rats (H1a: (SD) CVF, approximate body weight of 200 – 230 g at arrival), were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). All animals were free of viral pathogens, parasites, mycoplasma, *Heliobacter,* and cilia-associated respiratory bacillus. Upon arrival, animals were acclimated to AAALAC International accredited animal facilities at NIOSH for one week. 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) *ad libitum*. Rats were housed in pairs, and under a controlled light cycle (12 h light/12 h dark) and temperature (22 – 25 °C) conditions. One week following acclimation to the facilities, animals were randomly assigned to control or to an applied force condition of 2 or 4 newtons (N). The exposure and all other procedures performed were approved by the Institutional Animal Care and Use Committee and were 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, animals 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 animals 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 animals 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; it was 4.49 mm for 2.07 N and 5.09 mm for 4.3 N (Dong et al., 2023). 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. Once the tail and pressure apparatus were in place, the tail was marked so that the same region was exposed each day. The exposure was 4 h/day for 10 consecutive days. Control animals were placed in a restrainer but their tails were not exposed to pressure. Pre-exposure body weights were collected on days 1, 5 and 10 of the experiment. Blood flow (measured by laser doppler) and sensitivity to applied pressure (measured using the Randall-Selitto test) were measured immediately before and after the exposure on days 1, 5 and 10, and nerve function was measured using the current perception threshold method immediately before and after exposures before exposures began and on days 2 and 9.

On the morning following the last exposure, animals were anesthetized using 100-300 mg/kg sodium pentobarbital and exsanguinated by cardiac puncture. Tails were dissected from rats after exsanguination and placed in cold Dulbecco's modified Eagle's medium with glucose (Invitrogen/Gibco; Carlsbad, CA).

Laser Doppler measurement of blood flow: Laser Doppler measurements were made using a Periflux system 5000 and PF 450 thermostatic small angle probe (Perimed, Stockholm, Sweden). At the beginning of each day, the machine was calibrated by placing the probe into the calibration solution supplied by the manufacturer. Once calibrated, the probe was secured in the opening of a plastic holder with holes in the bottom. Animals were weighed, placed in a

restrainer and then put into a sound attenuating chambers. Each animals tail was put into the holder that held the doppler probe stable. The probe was placed under the C15-16 region on the ventral surface of the tail and the animals tail was then covered with a piece of foam to keep it in place during the measurement. If the animal moved its tail away from the probe, the tail was quickly repositioned so that blood flow could be measured. Laser doppler recordings of perfusion units were made for 5 min at 0.2 Hz immediately before and after exposures on days 1, 5 and 10 of the experiment.

Because animals do occasionally move during the recording period, and this results either in a rapid, acute increase in the doppler signal, or a loss of signal, data were sent to a statistician for smoothing. Regions were identified as motion artifact if the recorded number of perfusion units (PUs) was greater than 200, and a loss of signal was if there were less than 2 PU (Krajnak, Waugh, and Sarkisian 2019). These regions, which were out of range, were identified and running means were calculated to replace the regions with motion artifact or loss of signal. To calculate the running mean, the 10 measures prior to and 10 measures following motion artifact were used to calculate an average and these averages (i.e., running means) were used to replace data that was identified as motion artifact or loss of signal.

Randall-Selitto test: The Randall-Selitto pressure test was performed after blood flow was collected on days 1, 5 and 10 of the study. Prior to testing, the Randall-Selitto pressure gauge was set to "0". To test an animal, the flat end of the gauge was placed on the dorsal surface of the tail, in the middle of the region exposed to pressure (approximately C15). Pressure was applied using the probe side (1 mm wide probe) of the gauge. The pressure was gradually increased until the animal responded by flicking its tail or vocalizing. The pressure that elicited a response from the animal was recorded. If the pressure reached 200 g, the test was stopped

because previous studies have demonstrated that applying over approximately 200 – 250 g can result in injury (Sittiracha, McLachlan, and Bell 1987; Krajnak, Miller, et al. 2012; Krajnak et al. 2016). If the animal did not respond prior to or at 200 g of pressure, the response was recorded as 200 g. Only 3 animals had a response recorded as 200 g and this was not on every trial so changes in detectability of the stimulus did not go beyond the measurable range of the test. This test also was not done using repeated trials because the animals quickly exhibited a learned response to the tactile sensation of the probe and responded to the probe touching them instead of changes in pressure. Immediately following the pre-exposure test, animals were placed in the exposure chambers for their respective exposure. After the post test, animals were placed back into their home cages and returned to the colony room.

Current Perception Threshold (CPT). The rapid CPT test was performed using a Neurometer (Neurotron; Colorado). Three measurements are collected at each stimulation frequency (i.e., 2000, 250 and 5 Hz). CPTs were collected prior to the beginning of the study for the 4N group, and pre-and post-exposure on days 2 and 9 of the experiment in all groups. To perform the CPT, animals were placed in their restrainer and their tail was cleaned. The dispersion electrode was placed on the proximal end of the tail, near the region exposed to the front of the force plate. The stimulating electrode was filled with electrode gel and attached more distally on the tail using Soft-Tape (Neurotron), near the far end of the location at which the force plate contacted the tail. To begin testing, the Neurometer was set to deliver a 2000 Hz stimulus. The stimulus began at 5 mAmp and increased 5 mAmp approximately every 5 sec. When the animal responded (tail flick or vocalization), the test was stopped and the CPT (or amplitude of the stimulus was recorded. The test was run 2 more times and the average of the measures was used as the CPT for that frequency. After running the 2000 Hz stimulus, the

stimulating frequency was changed 250 Hz and the tail was tested for sensitivity as described above except that the test started at 1 mA and increased by 1mA every 5 seconds until the animal responded. After 3 trials, the frequency was changed to 5 Hz and the test was run in a manner identical to that described for 250 Hz. The tail was tested at all three frequencies because each frequency stimulates a different population of nerves; A-beta fibers (or large myelinated fibers) are stimulated at 2000 Hz and are sensitive to vibration and light touch, A-delta fibers (or small myelinated fibers) are stimulated at 250 Hz and are sensitive to pressure, and C-fibers are unmyelinated fibers and are sensitive to painful stimuli (mechanical and temperature). Immediately following the pre-exposure test, animals were placed in the exposure chambers for their respective exposure. After the post-exposure test, animals were placed back into their home cages and returned to the colony room.

Microvessel physiology: On the day of euthanasia, ventral tail arteries from the C18-20 region of the tail were dissected shortly, mounted on glass pipettes in a microvessel chamber (Living System; Burlington, VT), and perfused with sodium bi-carbonated HEPES buffer (130 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 4mM $NaHCO_3$,.8 mM $CaCl_2$, 10 mM HEPES, 1.80 mM KH_2PO_4, 0.03 mM EDTA) plus 10 % glucose added just prior to use and warmed to 37°C. Arteries were pressurized to 60 mm Hg and allowed to equilibrate for approximately 1 h. After an hour acclimation period, the chamber buffer was replaced with fresh HEPES biocarbonate buffer and vascular responsiveness to phenylephrine (PE)-induced vasoconstriction and acetylcholine (ACh)-induced re-dilation was measured. All chemicals for microvessel exposures were purchased from Sigma (Indianapolis, IN) unless otherwise, noted. To assess the effects of treatment on sensitivity to α_1-adrenoreceptor-mediated vasoconstriction, PE was applied to the chamber so that changes in the concentration occurred in half-log increments (-9.0 to -5.5 M) and

the internal diameter of the artery was recorded after vessels stabilized (approximately 5 min between concentrations). After assessing vasoconstriction, the chamber buffer containing PE, was removed and replaced with fresh, oxygenated HEPES buffer. After rinsing, arterial diameter returned to levels that were near baseline. Because ventral tail arteries usually display little basal tone, endothelial-mediated re-dilation was assessed after arteries were pre-constricted to approximately 50% of their baseline diameters with PE. We have demonstrated that re-constricting arteries with PE does not affect subsequent responses to ACh. To assess the dilatory effects of ACh, the agonist was added cumulatively in half-log increments (-10.0 to -5.0) and changes in the internal diameter of the vessel were measured as described for PE.