Elevated corticosterone in the dorsal hindbrain increases plasma norepinephrine and neuropeptide Y, and recruits a vasopressin response to stress
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Abstract: Repeated stress and chronically elevated glucocorticoids cause exaggerated cardiovascular responses to novel stress, elevations in baseline blood pressure and increased risk for cardiovascular disease. We hypothesized that elevated corticosterone (Cort) within the dorsal hindbrain (DHB): (1) enhances arterial pressure and neuroendocrine responses to novel and repeated restraint stress, (2) increases c-Fos expression in regions of the brain involved in sympathetic stimulation during stress and, (3) recruits a vasopressin-mediated blood pressure response to acute stress. Small pellets made of 10% Cort were implanted on the surface of the DHB in male Sprague-Dawley rats. Blood pressure was measured by radiotelemetry. Cort concentration was increased in the DHB in Cort-treated compared with Sham-treated rats (60±15 vs. 14±2 ng Cort/g of tissue, P<0.05). DHB Cort significantly increased the integrated arterial pressure response to 60 min of restraint stress on days 6, 13 and 14 following pellet implantation (e.g. 731±170 vs. 1204±68 mmHg/60 min in Sham- vs. Cort-treated rats, day 6, P<0.05). Cort also increased baseline blood pressure by day 15 (99±2 vs. 108±3 mmHg for Sham- vs. Cort-treated rats, P<0.05) and elevated baseline plasma norepinephrine and neuropeptide Y concentrations. Cort significantly enhanced stress-induced c-Fos expression in vasopressin-expressing neurons in the paraventricular nucleus of the hypothalamus, and blockade of peripheral vasopressin V1 receptors attenuated the effect of DHB Cort to enhance the blood pressure response to restraint. These data indicate that glucocorticoids act within the DHB to produce some of the adverse cardiovascular consequences of chronic stress in part by a peripheral vasopressin-dependent mechanism.

Abbreviations: AVPX, vasopressin V1 receptor antagonist; Cort, corticosterone; CVLM, caudal ventral lateral medulla; DβH, dopamine-β-hydroxylase; DHB, dorsal hindbrain; HR, 11βHSD1, 11-beta-hydroxysteroid dehydrogenase1, 11βHSD2, 11-beta-hydroxysteroid dehydrogenase2; heart rate; MAP, mean arterial pressure; NPY, neuropeptide Y; NTS, Nucleus of the Solitary Tract, PBS, phosphate buffered saline; PVN, paraventricular nucleus; RVLM, rostral ventral lateral medulla; SC, subcutaneous; SON, supraoptic nucleus
Introduction

Chronic or repeated stress enhances cardiovascular and neuroendocrine responses to acute novel stress and can increase baseline blood pressure and sympathetic nervous system activity, all of which increase cardiovascular disease risk (1, 4, 8, 12, 21, 25, 27, 28, 54, 59, 64). However, the central nervous system mechanisms that link chronic stress to these adverse cardiovascular outcomes are not well understood. Baseline glucocorticoids (cortisol in humans and corticosterone (Cort) in rats) are elevated by repeated or chronic stress, and elevated glucocorticoids also increase cardiovascular disease risk (9, 41, 58, 60). Furthermore, inhibition of the enzyme 11-beta-hydroxysteroid dehydrogenase1 (11ßHSD1), which converts inactive metabolites back into corticosterone, reduces blood pressure in both lean and obese Spontaneously Hypertensive/NDmcr-cp rats, indicating that endogenous corticosterone can contribute to the development of hypertension in this model of metabolic syndrome (46). Taken together, these previous studies suggest that glucocorticoids could mediate some of the adverse cardiovascular consequences of stress.

Numerous studies indicate that glucocorticoids act in the periphery to increase vascular tone and reactivity (16). Fewer studies have investigated the central nervous system actions of glucocorticoids on blood pressure regulation, and the results have been equivocal (15, 52, 53, 56, 57). These previous studies have mostly delivered glucocorticoid receptor agonists or antagonists by intracerebroventricular administration, which could be problematic since central administration of glucocorticoids would inhibit peripheral glucocorticoid secretion via feedback inhibition of the hypothalamic-pituitary-adrenal axis (24), and glucocorticoids could exert differential effects that depend on the brain region and receptor subtype being influenced. We developed a novel method to chronically deliver Cort to the region of the dorsal hindbrain (DHB) that includes the Nucleus of the Solitary Tract (NTS), which is a key region for cardiovascular regulation within the brain (42, 43). We have previously reported that high levels of Cort act in the DHB to enhance the arterial pressure response to acute novel restraint stress, supporting our overall hypothesis that elevated Cort levels within the DHB can mediate, at least in part, cardiovascular effects of chronic stress. The present study used a lower more physiological dose of Cort (44) and investigated potential mechanistic explanations for the effects of DHB Cort on blood pressure regulation in the context of both acute and chronic stress. Psychological stress-induced elevations in blood pressure are mediated at least partially by increased sympathetic activity (29), so we tested the specific
hypothesis that elevated DHB Cort enhances the arterial pressure and neuroendocrine responses to novel and repeated restraint stress. Measurements of plasma norepinephrine, neuropeptide Y (NPY) and Cort were used as indices of sympathetic and neuroendocrine activity. NPY is released from peripheral sympathetic nerves and is considered to be a marker of prolonged increases in sympathetic nerve activity (66). In conjunction with this, we tested the hypothesis that elevated DHB Cort increases neuronal activation (as determined by c-Fos expression) in regions of the brain involved in sympathetic stimulation during restraint stress. c-Fos is an early immediate gene product whose expression is linked to neuronal depolarization (19, 32, 47). Plasma vasopressin is also increased with some, but not all, stressors in rats and humans, and could potentially mediate stress-induced increases in blood pressure (22, 23). Based on this fact and the results from the first two series of experiments, we also tested the hypothesis that DHB Cort-induced augmentation of the cardiovascular response to acute novel restraint stress is mediated, at least in part, by peripherally-acting vasopressin.

These hypotheses were tested in conscious male Sprague-Dawley rats treated for 6 to 18 days with pellets implanted on the DHB to chronically deliver Cort to this brain region. Control rats received Sham pellets. Rats were stressed by placing them for 60 min in clear plexiglass restraining tubes, which is an established method for producing psychological stress in rats (10). Blood pressure and heart rate were measured by radiotelemetry and blood samples for measuring plasma Cort, catecholamines and NPY levels were obtained from indwelling arterial catheters. Peripheral actions of vasopressin were acutely blocked using the vasopressin V1 receptor antagonist d(CH(2))(5)(1)-O-Me-Tyr(2)-Arg(8)]-vasopressin (Manning compound). Neuronal activation in response to restraint stress was estimated by quantifying the number of neurons expressing the c-Fos protein visualized by immunohistochemistry (19, 32).

Methods

Ethical Approval. All animal housing, handling, surgical and experimental procedures were conducted within an Association for the Assessment and Accreditation of Laboratory Care (AALAC) International-accredited animal care facility at the University of Florida, in accordance with the U.S.A. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of
the University of Florida approved all animal housing, handling, surgical and experimental procedures.

**Animals.** The data presented in this paper were obtained from male Sprague-Dawley rats weighing 275-300g at the time of procurement from Charles River Laboratories (Raleigh, North Carolina). All rats were housed in a Specific Pathogen Free temperature- and humidity-controlled animal care facility with a 12:12 hour light-dark cycle and had free access to standard rat chow and water. The rats were allowed a minimum of 5 days following arrival to acclimate to the new environment before any surgery or procedure was performed. For euthanasia, the rats were deeply anesthetized with isoflurane (5% in oxygen), then killed either by rapid decapitation or perfused transcardially with phosphate buffered saline followed by 4% paraformaldehyde in 100mM phosphate buffered saline (pH=7.4).

**Dorsal hindbrain pellet preparation.** Small pellets containing either silastic (Sham pellets) or 10% Cort in cholesterol were manufactured in the lab, sterilized using ethylene oxide then implanted on the dorsal surface of the hindbrain, as previously validated using 100% Cort pellets and both silastic and cholesterol-containing sham pellets (42, 43). Previous experiments showed no differences in the effects of cholesterol compared with silastic pellets. To make 10% Cort pellets, Cort and cholesterol powders (Sigma-Aldrich, St. Louis, MO) were gently melted over a Bunsen burner and pipetted into a mold. The mixture cooled to form pellets with the approximate dimensions of 1.5 mm (l) x 1.75 mm (w) x 1.0 mm (h). The pellets were removed from the mold and trimmed under a surgical microscope. The final weight of the pellets was between 3 and 4 mg. The silastic sham pellets were made from hardened Kwik-Sil silastic gel (World Precision Instruments, Sarasota, FL) then carved to the same dimensions as the Cort pellets.

**Surgical Procedures.** For all surgical procedures, anesthesia was induced in a chamber filled with 5% isoflurane in oxygen. Anesthesia was maintained in the surgical plane using isoflurane (2-3.0% in oxygen) throughout all surgical procedures. Adequate anesthesia was evidenced by lack of reflex response to pinch of the hind paw or to any surgical manipulation. Penicillin (Extended Action Penicillin, 30,000 U s.c., Hanford’s US Vet) was administered following induction of anesthesia. The analgesic carprofen (5mg/kg/day, Webster Veterinary Supply, Inc., Alachua, FL) was administered just before the surgery and for the following 72 hr. All surgical procedures were performed using aseptic technique and all incisions were closed in layers (muscle, subcutaneous
and cutaneous). Animals were carefully monitored following surgery for signs of pain or distress and any concerns were addressed in consultation with the veterinary staff.

*Pellet implantation.* Pellets were implanted using procedures similar to those described previously (43). Rats were placed in a stereotaxic frame with the neck slightly ventroflexed. An incision was made in the skin along the midline between the occipital crest and the atlas. The dorsal surface of the hindbrain was surgically exposed without removing any bone by blunt dissection of the overlying muscle followed by removal of the dura and pia mater that lay directly over the site of pellet implantation. The bottom surface of the pellet was coated with a thin layer of mineral oil to aid diffusion of the Cort into the brain. The pellet was then placed on the surface of the hindbrain at the level of calamus scriptorius and secured in place with Vetbond surgical glue. The surface of the pellet was protected with silastic gel (Kwik-Sil, World Precision Instruments). Pellets (3-4 mg) made of silastic or 10% Cort were implanted subcutaneously in two additional groups of control rats used for the measurement of brain tissue Cort concentrations.

*Telemetry transmitter implantation.* Telemetry transducers for the measurement of arterial pressure, heart rate and activity (model PA-C40, Data Sciences International, St. Paul, MN) were implanted into the descending aorta via a midline abdominal incision. The aorta was isolated and the tip of the catheter was inserted using a 21-gauge needle. Surgical glue (3 M Vetbond Tissue Adhesive) and a nitrocellulose patch were applied to secure the catheter in place. The transducer was sutured to the abdominal muscle.

*Arterial Catheters.* Indwelling arterial catheters were implanted to obtain blood samples for the measurement of plasma Cort, catecholamine and NPY concentrations. Catheters were made in the lab and implanted into the femoral artery as previously described (3).

**Experimental Protocols**

**Experiment 1** tested the hypothesis that a physiological dose of DHB Cort enhances the arterial pressure (protocol 1A) and plasma catecholamine, NPY and Cort responses (protocol 1B) to novel and repeated restraint stress. Some of these animals were also used to measure tissue Cort concentrations within the brain.

**Protocol 1A.** Telemetry transducers were implanted and the rats were allowed a minimum of 3 weeks to recover, then a DHB Sham or DHB 10% Cort pellet was implanted (n=8 per group). Rats were subjected to 60 min of restraint stress on days 6, 13, 14 and 15 following pellet implantation. Rats were always stressed in the morning commencing between 8 and 10:45 AM. Separating the initial novel stress from the start
of the repeated stress by 1 week allowed us to collect blood samples during a novel stress and final episode of repeated stress in the same rats without compromising their blood volume. Restraint stress involved placing the rats in a clear plastic restrainer with the length adjusted such that the rat could not turn around but was not in pain and could breathe easily. Arterial pressure and heart rate data were collected continuously from 30 min prior to restraint until 30 min after restraint. At other times, 20 seconds of data were collected every 10 min. The investigator did not enter the rat housing room until just before the onset of restraint, so rats were undisturbed during collection of the baseline data.

**Protocol 1B.** Plasma hormones. A single surgery was performed to implant a DHB Sham (n=14) or DHB 10% Cort (n=15) pellet followed by implantation of a femoral arterial catheter. Catheters were flushed daily with sterile saline. Rats were subjected to 60 min of restraint stress on days 6, 13, 14 and 15 following pellet implantation. On days 6 and 15, while the rat remained in his home cage, the arterial catheter was connected to a piece of extension tubing so that the blood could be obtained from outside the cage without disturbing the animal. Initially a baseline blood sample was obtained 1 hour after handling the animal and connecting the extension tubing, however some of these rats had high baseline plasma Cort levels, indicating they had not returned to a basal state. In subsequent animals the baseline sample was obtained 2 hours after connecting the extension tubing. Additional samples in all rats were taken at 10 and 60 min of restraint. For each sample the catheter dead space was withdrawn, then 600 µl of blood (representing approximately 2% of the rats’ blood volume) was drawn and aliquoted into separate tubes containing either 0.5 µl of heparin (1000 U/mL, Webster Veterinary Supply Inc., Alachua, Florida) for measurement of Cort, 6 µL of glutathione-EGTA (Fisher Bioreagents, Fair Lawn New Jersey) for measurement of catecholamines or 31.8 µl of aprotonin (1.4 mg/ml, Sigma Aldrich, St. Louis, MO) and 2 µl of heparin for the measurement of NPY. The catheter was then flushed with sterile saline to replace the volume of blood that had been removed. Due to issues with the catheters, samples were not collected on both days 6 and 15 in all animals. Data from experiments in which the rats had very high baseline Cort (>20 µg/dl) prior to stress were excluded from the analysis. The samples were kept on ice then centrifuged at 4°C, and the plasma was stored at -80°C until being assayed.

**Experiments 2a** and 2b tested the hypothesis that DHB Cort would increase neuronal activation, as estimated by c-Fos expression, in the NTS and in two regions that are

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involved in the neuroendocrine and sympathetic nervous system responses to stress, the paraventricular nucleus of the hypothalamus (PVN) and the rostral ventral lateral medulla (RVLM) (17, 31, 55). We also determined the effect of DHB Cort on c-Fos expression in the caudal ventral lateral medulla (CVLM), which is important for arterial baroreflex-mediated control of blood pressure (17). Other areas were initially analyzed but no effects of DHB Cort on neuronal activation in response to restraint stress were observed, and the data are not reported. Expression of c-Fos in the supraoptic nucleus of the hypothalamus (SON) was subsequently quantified based on the results from the PVN, and additional immunohistochemistry experiments were performed to determine if DHB Cort treatment altered the expression of c-Fos within vasopressinergic neurons in the PVN and SON.

Rats used in experiment 2a underwent surgery to implant either a DHB Sham or DHB 10% Cort pellet (n=14 per group). Six days later the rats were brought to the procedure room and either restraint-stressed for one hour and then returned to their home cage for one hour (n=9 per DHB treatment group) or allowed to rest quietly in their home cage for one hour (n=5 per DHB treatment group).

NTS catecholaminergic neurons are known to express c-Fos in response to restraint stress and robustly express the glucocorticoid receptor (10, 18). To determine if the DHB Cort treatment had any selective effects on c-Fos expression within these catecholaminergic neurons, the hindbrains were immunochemically labeled for the catecholamine synthesizing enzyme dopamine-beta-hydroxylase (DBH) in addition to c-Fos. Forebrain sections were immunochemically stained only for c-Fos.

**Experiment 2b.** Experiments was performed to determine if the increased c-Fos labeling in the PVN and SON neurons in the DHB Cort-treated rats co-localized with vasopressin. The protocol outlined in experiment 2a was performed on DHB Sham and DHB 10% Cort rats (n=6 per group). In this experiment all rats were stressed as described above. The tissue from 1 Sham-treated rat was lost during processing. **Experiment 3** was based on the results from experiments 1 and 2, and was designed to test the hypothesis that peripheral blockade of vasopressin V1 receptors would attenuate the Cort-induced augmentation of the arterial pressure response to stress. First, radiotelemetry transmitters were implanted. The animals recovered for at least two weeks, and then a second surgery was performed to implant DHB Sham (n=8) or 10% Cort pellets (n=7) along with an indwelling femoral venous catheter. Results from Experiment 1 demonstrated that there was no difference in the integrated changes in the
mean arterial pressure response to restraint stress on days 6 and 13 (see results). Therefore, we used a randomized crossover study design in which rats were subjected to 1 hour of restraint stress 6 and 13 days following pellet implantation. Rats were pre-treated 2 hours prior to restraint either on day 6 or day 13 with d(CH(2))(5)(1)-O-Me-Tyr(2)-Arg(8)-vasopressin (AVPX, Manning compound, 10µg/kg i.v., ). On the other day they were pretreated with 1 ml/kg of 0.9% saline i.v. (vehicle). The drugs were given as single injections via the indwelling venous catheter and the process involved handling the animals. The blockade of vasopressin V1 receptors was confirmed by recording the blood pressure response to an intravenous bolus of vasopressin (50 ng in 200 µl of 0.9% saline, data not shown). At the conclusion of the experiment the animals were deeply anesthetized with isoflurane (5% in oxygen) and killed by rapid decapitation. Some rats could be used on only one day due to technical difficulties related to the catheters and transmitters, so data from Sham- and Cort-treated rats from experiment 1 were combined with the saline treatment data from experiment 3 for analysis. To incorporate the data from experiment 1, data from days 6 and 13 were averaged for each rat in Experiment 1 and the averaged data used for analysis in Experiment 3.

**Measurement of Tissue Cort Concentration.** Thirteen animals from protocols 1A and 1B were used to measure tissue Cort concentrations in selected brain regions. Brain tissue Cort was also measured in the following control treatment groups: subcutaneous sham (n=6), subcutaneous 10% Cort (n=6) and following 60 min of restraint stress (n=8). Tissue from the dorsal hindbrain (average weight 29.6±1.0 mg), ventral hindbrain (average weight 21.9±1.0 mg) and forebrain (averages weight 32±1 mg) were dissected out and processed as previously described (42). In short, each sample was homogenized in 300 µl of ethanol-saline (1:1). The homogenates were transferred into 3 ml of methylene chloride, vortexed for 30 min then centrifuged at 4°C. The organic layer was set aside and the remaining aqueous layer was extracted a second time as above, this time using 2 ml of methylene chloride. The organic layers from the first and second extractions were combined and vacuum-dried, then reconstituted in 1 ml of extraction buffer, allowed to sit overnight then assayed in duplicate or triplicate using a commercially available enzyme immunoassay (Oxford Biomedical Research, Oxford MI, USA, product no. EA66). The extraction recovery was previously measured as 93±3% (42). Any contamination of the methylene chloride layer with contents from the pellet causes the assay to report very high levels of Cort, so outliers were identified by calculating the interquartile range, multiplying this value by 1.5 and subtracting and
adding the resulting number from/to the lower and upper quartiles, respectively, then the outliers were removed from the data set.

**Plasma Hormone assays.** Plasma Cort was measured using a commercially available double antibody I^{125}\textsuperscript{+} radioimmunoassay kit (minimum detectable dose of 0.77 µg/dl determined by the manufacturer, MP Biomedicals, Solon, OH). The kit was used at half volume and a 1.25 ng/dl standard was added to the standard curve, as in previous experiments (45). Plasma norepinephrine and epinephrine were measured by HPLC by the Vanderbilt University Hormone Assay & Analytical Services Core (Nashville, TN). However, in one set of samples the values for epinephrine were almost all undetectable, so the data for epinephrine are not reported. Plasma NPY was measured in duplicate using Neuropeptide Y EIA kits purchased from Peninsula Laboratories, Inc. (#S-1145.001). Samples were first extracted using a procedure modified from the kit instructions and provided by Dr. Zofia Zukowska (26). Briefly, equal amounts of 1% trifluoroacetic acid (TFA; buffer A) and plasma were mixed and centrifuged at 1700 g at 4°C for 20 min. The supernatant was transferred to a new tube and the pellet discarded. C18 Sep-columns were equilibrated with 60% acetonitrile and 1% TFA (buffer B) followed by buffer A. The plasma solution was then loaded onto the column, the column was washed with buffer A (discarded) followed by buffer B (collected). Samples were dried using a speed vacuum, and then the sample was reconstituted in EIA buffer.

**Immunohistochemistry for c-Fos, DBH and Arginine Vasopressin.** Brains were post-fixed overnight in 4% paraformaldahyde, placed in 30% sucrose for two days at 4°C, then cut on a freezing stage microtome into 40 µm sections and collected serially into 4 wells for the hindbrain and 6 wells for the forebrain. The sections were stored in freezing solution (39 mM dibasic sodium phosphate, 12 mM monobasic sodium phosphate, 30% sucrose (w/v), and 30% ethylene glycol (v/v)) at -20°C until use. Slides were cover-slipped using Vectamount (Vector Laboratories) mounting medium following enzymatic processing or ProLong Gold (Invitrogen.com) following processing with fluorescent secondary antibodies.

**c-Fos labeling for Experiment 2a.** Immunohistochemistry analysis was performed on every fourth section for the hindbrains and every 8\textsuperscript{th} section for the forebrains. Sliced brain sections were rinsed in 100 mM phosphate buffered saline (PBS) before all steps other than prior to incubation with the primary antibody. The sections were incubated for thirty minutes in 0.3% hydrogen peroxide, incubated for two hours with blocking solution (0.25% Triton X-100, 3% normal goat serum in PBS), then placed in a 1:22,000 dilution
of rabbit anti-c-Fos IgG (Calbiochem) in blocking solution for 40-48 hours at 4°C. The sections were then incubated for two hours in biotinylated goat anti-rabbit IgG (1:800 in blocking solution; Jackson ImmunoResearch) and processed using a Vectastain ABC kit (Vector Laboratories) followed by incubation for ten minutes in 3,3'-diaminobenzidine with nickel and hydrogen peroxide (0.05% 3,3'-diaminobenzidine with nickel, 35% hydrogen peroxide in 50mM tris-HCl, pH=7.2, Vector Laboratories). Forebrain sections were mounted and cover slipped.

**DβH labeling.** Hindbrain sections that had been processed for c-Fos labeling were then immunochemically stained for DβH to determine the number of catecholaminergic neurons activated by stress in sham- and Cort- treated rats. Due to the localization of c-Fos to the nucleus and DβH to the cytoplasm, enzymatic immunohistochemistry was sufficient for the visualization of these two proteins in the same neuron. The procedure was the same as for c-Fos immunohistochemistry detailed above with the following exceptions: 1) sections were incubated for one hour in blocking solution; 2) the primary antibody was mouse anti-DβH, clone 4F10.2 (1:30,000; Millipore) 3) the secondary antibody was biotinylated goat anti-mouse IgG (1:4000; Jackson ImmunoResearch) 4) sections were incubated for 30 minutes in an avidin/biotin-peroxidase solution (0.3% Triton X-100, 1.2 µg/ml avidin, 0.3 µg/ml biotin-peroxidase in 50mM tris-HCl pH=7.6) instead of the Vectastain ABC kit; and 5) sections were incubated for ten minutes in a 3,3'-diaminobenzidine / hydrogen peroxide mixture that did not contain nickel.

**Fluorescent labeling for Arginine Vasopressin and c-Fos.** Forebrain sections were incubated for two hours with blocking solution (0.25% Triton X-100, 3% normal donkey serum in PBS), then placed in rabbit anti-c-Fos (1:1000, Calbiochem) and guinea pig anti-arginine vasopressin (1:250, Peninsula Laboratories) for 40-48 hours at 4°C. The sections were then incubated for 2 hours in Alexa Fluor 488 donkey anti-rabbit (1:200, Invitrogen) and DyLight 594 donkey anti-guinea pig (1:200, Jackson ImmunoResearch).

**Imaging and analysis.** Brain sections were imaged using an Olympus BX41 microscope fitted with an Olympus DP71 microscope digital camera. The NTS, RVLM, CVLM, PVN and SON were identified using a standard stereotaxic atlas for the rat brain (35). The subnuclei of the PVN (anterior, lateral magnocellular, medial parvocellular, dorsal parvocellular and ventral parvocellular) were identified using the atlas and counted separately because they are associated with different anatomical projections sites (38, 49-51, 62). The anterior PVN (Bregma -1.3 to Bregma -1.6) provides innervation to the median eminence and some innervation to the spinal cord, but spinal
cord projecting neurons are more numerous further caudally in the PVN (38, 51). The dorsal and ventral parvocellular regions (Bregma -1.80 to Bregma -1.88) send projections to the RVLM, dorsal hindbrain region and the spinal cord (38, 49-51). The lateral magnocellular division of the PVN (Bregma -1.60 to -1.88) projects to the posterior pituitary and the medial parvocellular PVN (Bregma -1.80 to Bregma -2.12) projects to the median eminence (50, 51, 62). In preliminary experiments double labeling for c-Fos and corticotropin releasing factor was used to help confirm the landmarks used for identification of the subregions (data not shown). The number of sections representing each PVN subdivision varied between brains. PVN subregions were identified and counted for each section, and the results were analyzed using the average number of c-Fos-positive neurons per section. The number of c-Fos-positive neurons in the SON was determined in 3-9 sections per brain. The average number of sections per brain that was analyzed for each forebrain region was not different between experimental groups. The NTS was further divided into the more caudal cardiovascular-related NTS (at the level of and caudal to the area postrema, Bregma -13.68, referred to as the caudal NTS in this study) and the rostral NTS (rostral to the area postrema). Neuronal counts were used from 8 sections per animal for the caudal NTS and 4 sections per animal for rostral NTS, RVLM and CVLM, and the results were analyzed and reported as total number of neurons per region (total c-Fos-positive neurons, neurons double-labeled for c-Fos and DßH and c-Fos-positive only neurons). Outliers were identified by calculating the interquartile range, multiplying this value by 1.5 and subtracting and adding the resulting number from/to the lower and upper quartiles, respectively, then the outliers were removed from the data set. The number of neurons double-labeled for c-Fos and arginine vasopressin was determined in sections with the most abundant expression of arginine vasopressin (2 sections of the PVN and 3 sections of the SON per rat); all vasopressin- and c-Fos expressing neurons were counted. The data were quantified as the average number of double-labeled neurons per section. The images in Figure 5 were adjusted for brightness and contrast.

Data Analyses

Absolute mean arterial pressure and heart rate were averaged into 5 min periods. The average baseline value was calculated during the 30 min prior to stress, excluding the final 4 minutes. For analysis of the baseline period, time points during which the telemetry system recorded animal movement were removed. The average baseline was then subtracted from the values measured during the 60 min of stress and
the 30 min recovery period following stress. Average results are expressed as means ± SE. Statistical significance was evaluated with the use of one-, two- or three-way ANOVA as appropriate. Fos data from experiment 2a were analyzed using a 2x2 factorial between subjects ANOVA. When significant interactions between main effects were detected, additional ANOVA was performed as appropriate. Post-hoc analyses were performed using Duncan’s or least squares means tests. Differences were considered significant at \( P < 0.05 \).

**Results**

**Tissue Cort Concentrations**

Eighteen days of DHB Cort treatment significantly increased tissue Cort concentration within the DHB (\( P<0.05 \) relative to DHB sham-treated rats), but did not alter tissue Cort concentrations within the ventral hindbrain or forebrain (Figure 1, n=4-8 per group). Acute stress significantly increased tissue Cort concentrations in all three regions relative to DHB Sham-treated rats, while subcutaneous Sham and subcutaneous Cort pellets did not alter tissue Cort concentrations within the brain. The increases in DHB tissue Cort concentration with stress and DHB Cort treatment were not significantly different. Therefore, the DHB 10% Cort pellets produced a chronic physiologically relevant increase in tissue Cort concentration within the DHB without altering tissue Cort concentrations elsewhere in the brain.

**Effects of DHB Cort on arterial pressure and heart rate**

Figure 2 shows mean arterial pressure from 30 min prior to restraint through 30 min following the end of restraint. DHB Cort treatment had no effect on average baseline mean arterial pressure in the 30 min pressure prior to restraint stress until day 15 when it was significantly greater in the DHB Cort-treated rats (108±3 mmHg) compared with the DHB Sham-treated rats (99±2 mmHg). This increase in baseline pressure was only evident just prior to restraint stress and not at other times during the day or night (data not shown). There were no effects of DHB Cort treatment or repeated restraint stress on baseline heart rate. Average values were, 328±13 (Day 6), 330±5 (Day 13), 329±8 (Day 14) and 319±7 (Day 15) beats per minute for Sham-treated rats, and 321±7, 324±8, 330±10 and 320±6 beats per minute respectively for Cort-treated rats. During restraint stress mean arterial pressure was significantly greater in Cort-treated compared with Sham-treated rats on all days (Figure 2). There was no significant interaction between the effects of pellet treatment and time over the 60 min of restraint, indicating this effect of DHB Cort was maintained throughout the period of stress. During the 30 min post-
stress period there were significant interactions between the effects of the day of stress, time and pellet treatment on blood pressure. Arterial pressure was greater in the Cort-treated rats at several time points following stress on days 6 and 13, at one time point on day 15 and at no time points on day 14. The response to restraint was also analyzed as the integrated change from baseline over the 60 min period of stress and the 30 min period of recovery following stress (Figure 3). DHB Cort significantly augmented the integrated change in mean arterial pressure during stress on days 6, 13 and 14, but not on day 15 (Figure 3). DHB Cort treatment did not significantly affect the integrated increase in arterial pressure during the post-stress period (Figure 3). There were no significant effects of DHB Cort on the integrated changes in heart rate during the stress or post-stress periods (Figure 3).

Effects of DHB Cort on plasma norepinephrine, NPY and Cort concentrations

Rats were subjected to 1 hour of restraint stress on days 6, 13, 14 and 15 following implantation of either a 10% Cort or Sham pellet on the DHB. Blood samples to measure plasma hormone concentrations were obtained on days 6 and 15. DHB Cort treatment significantly increased baseline plasma norepinephrine on day 6 prior to any episode of restraint stress (Figure 4, top, n=9-12 per group for Day 6 and 6-10 per group for Day 15). On day 15, following repeated episodes of stress, baseline plasma norepinephrine was similar in Sham- and Cort-treated rats. During the period of restraint stress plasma norepinephrine rose to similar levels in all rats.

The effects of DHB Cort treatment and stress on plasma NPY were similar to the effects on plasma norepinephrine. Compared with DHB Sham treatment, DHB Cort treatment significantly increased baseline plasma NPY prior to the first episode of stress (Figure 4, bottom). Restraint stress produced an overall increase in plasma NPY that was not significantly different amongst the treatment groups. Plasma NPY in Sham-treated rats was significantly increased on day 15 relative to day 6, but this was not the case for DHB Cort-treated rats. Thus on day 15, following repeated episodes of stress, plasma NPY was similar in Sham- and Cort-treated rats.

The effects of DHB Cort treatment and stress on plasma Cort concentration were complex. DHB Cort significantly reduced baseline plasma Cort on day 6, prior to any stress (Table 1). Comparing baseline plasma Cort concentration on days 6 and 15 indicated that repeated stress significantly increased baseline plasma Cort in the DHB Cort-treated but not in the Sham-treated rats, so by day 15 baseline plasma Cort in the DHB Cort-treated rats was significantly higher than in all other groups. During restraint
stress plasma Cort was elevated in all groups of rats, with plasma Cort concentration after 10 min of restraint being similar in all groups. On day 6 DHB Cort significantly attenuated the plasma Cort response to the restraint stress at 60 min. Repeated restraint reduced the plasma Cort response to stress in DHB Sham-treated but not in DHB Cort-treated rats.

Effects of DHB Cort on Fos expression

Figure 5A-F illustrates subdivision of the PVN used for counting c-Fos, as determined using Paxinos and Watson (35). Figure 5G provides an example of double-labeling for DβH and c-Fos. There was a main effect of restraint stress to significantly increase the number of c-Fos-expressing neurons in in all hindbrain and forebrain regions analyzed with the exception of the rostral NTS (Figures 6 and 7, n=4-9 per group). This result was observed in both non-DβH- and DβH-positive neurons in the hindbrain. In the lateral magnocellular PVN there was a borderline significant (P=0.087) main effect of Cort treatment to increase c-Fos expression. There were no significant effects of Cort treatment on c-Fos expression in any other brain regions examined.

Double-labeling for c-Fos and arginine vasopressin in experiment 2b showed that, compared with Sham-treated rats (7±2 cells/section), DHB Cort-treated rats had significantly more (19±2 cells/section) neurons double-labeled for c-Fos and arginine vasopressin in the PVN but not in the SON (74±18 vs. 73±9 cells/section for Sham- vs Cort-treated rats).

Effect of peripheral vasopressin V1 receptor blockade on the stress response

Based on the above results, the effect of peripheral blockade of vasopressin V1 receptors on the arterial pressure response to restraint stress was determined in Sham- and Cort-treated rats. Administration of saline or AVPX had no effect on baseline blood pressure, and mean arterial pressure during the 30 min prior to restraint stress averaged 100±2, 101±2, 100±3 and 100±3 mmHg for Sham-, Cort-, Sham + AVPX- and Cort + AVPX-treated rats, respectively. Figure 8 shows the change in mean arterial pressure over time (left) and the integrated change in mean arterial pressure from baseline for the 60 min of restraint and the 30 min period of recovery following restraint (right). DHB Cort significantly increased the mean arterial pressure responses to restraint and recovery from restraint. Peripheral blockade of vasopressin V1 receptors significantly reduced the overall integrated mean arterial pressure response to restraint and stress recovery in DHB Cort-treated but not Sham-treated rats. Post-hoc analysis showed significant differences between Cort- and AVPX + Cort-treated rats at 15, 20, 35, 40 and 60 min of
stress and during the last 10 min of the recovery period. The difference in mean arterial pressure between these two groups of rats also tended to be significantly different during stress at 25 min (P=0.08) and 55 min (P=0.07), and during the recovery period at 65 min (P=0.07), and 80 min (P=0.06) following the initiation of the 60 min restraint period.

Discussion

There are multiple similarities between the adverse effects of chronic stress, which increases glucocorticoid secretion, and the adverse effects of chronic elevations in systemic glucocorticoids due to endogenous overproduction or exogenous administration (5, 9, 11, 25, 27, 28, 34, 36, 41, 48, 58-61, 63). These effects include exaggerated cardiovascular responses to novel psychological stress, elevations in baseline blood pressure and increased risk for cardiovascular disease. The present study investigated the cardiovascular-related effects of chronic elevations in glucocorticoids within a specific region of the brain, the DHB, and several novel findings are reported. First, a physiologically relevant increase in tissue Cort concentration within the DHB enhanced the blood pressure response to novel restraint stress and increased baseline blood pressure in the period just prior to the stress caused by repeated restraint. Second, these chronic elevations in DHB Cort concentration increased baseline plasma norepinephrine and NPY concentrations, suggesting that activity of the sympathetic nervous system was elevated even the absence of stress. Third, novel restraint stress induced a significantly greater increase in c-Fos expression in vasopressin-positive PVN neurons in DHB Cort- compared with Sham-treated rats, suggesting the possibility that vasopressin might be secreted in response to stress in the DHB Cort-treated rats, but not the Sham-treated rats. Fourth, peripheral blockade of vasopressin V1 receptors attenuated the arterial pressure response to stress in the DHB Cort-treated rats but had no effect in the Sham-treated rats. Taken together these results support the overall idea that a physiologically relevant elevation in DHB Cort can reproduce some adverse effects of repeated stress including enhanced blood pressure responses to novel stress and increased basal sympathetic nervous system activity. The data also indicate that increased peripheral effects of vasopressin contribute to the Cort-induced enhancement of the cardiovascular response to acute novel restraint stress.

We previously reported that a higher dose of DHB Cort, using pellets made of 100% Cort, enhanced the arterial pressure response to novel restraint (44). However, we subsequently determined that 6 days of treatment with the 100% pellets produced
supra-physiologic levels of Cort within the DHB, while a pellet made of 10% Cort produced physiological levels of Cort within the DHB (42). The present data indicate that the 10% pellets continued to produce physiological concentrations of Cort within the DHB for at least 18 days without increasing Cort levels in other brain regions, and this lower dose of Cort also enhanced the arterial pressure response to novel restraint stress. The results also extend these findings to show that the effect of DHB Cort to augment the arterial pressure response to restraint was diminished with repeated exposure to the stress. Blockade of DHB glucocorticoid receptors can partially prevent the adaptation of the arterial pressure response to a repeated stressor (2), which could account for the reduced effect of Cort with repeated restraint.

The present data do not support a portion of the original hypothesis, which predicted that DHB Cort treatment would heighten the stress-induced increases in plasma NPY and norepinephrine, two indices of sympathetic nervous system activity. The hypothesis was based on the premise that the sympathetic nervous system is the primary mediator of stress-induced increases in blood pressure (55). Nonetheless, the data do not rule out a role for increased stress-induced activation of the sympathetic nervous system in the DHB Cort-treated rats for several reasons. First, plasma norepinephrine and NPY provide only rough estimates of sympathetic nerve activity and they were measured at only two time points during the 60 min of stress. Second, the plasma NE response to stress might represent a maximum NE response in the Sham rats, so that Cort treatment could not augment this response. Third, peripheral vasopressin blockade did not completely reverse the enhanced blood pressure response to restraint that was induced by DHB Cort, suggesting that some factor in conjunction with vasopressin contributes to the exaggerated stress response in these rats. Fourth, although DHB Cort had no significant effects on c-Fos expression in regions of the brain known to control sympathetic nerve activity (17), c-Fos expression is only a surrogate marker for neuronal activity, so all c-Fos data should be interpreted with caution (19, 32, 47). Future work will more thoroughly investigate the effect of DHB Cort on sympathetic nervous system activity.

The tendency for increased stress-induced c-Fos expression in the magnocellular PVN in the DHB Cort-treated rats was unexpected, but suggested that DHB Cort recruited stress-induced secretion of either vasopressin or oxytocin (37). Previous work has shown that vasopressin synthesized in the medial parvocellular PVN as a neurotransmitter is recruited to promote activation of the hypothalamic-pituitary-
adrenal axis under conditions of chronic stress, while under basal conditions the hypothalamic-pituitary-adrenal axis is driven primarily by corticotropin releasing factor (37). The combination of the increased number of c-Fos and vasopressin double-labeled neurons in the PVN with the effect of vasopressin V1 receptor blockade to attenuate the stress response only in DHB Cort-treated rats suggests that DHB Cort caused recruitment of a systemic vasopressin response to psychological stress.

We have previously observed a variable effect of DHB Cort on baseline arterial pressure. When we measured blood pressure daily following DHB surgery in rats with arterial catheters, baseline arterial pressure was increased by about 7 mmHg by 4 days after surgery (3, 43). When rats were allowed to recover for 4-6 days following surgery without being subjected to experimental procedures, there was no increase in baseline blood pressure in DHB Cort-treated compared with Sham-treated rats (44). We suggested that the rats studied for four sequential days following surgery may have been stressed relative to the other rats, and that an interaction between repeated stress and DHB Cort levels could lead to elevated baseline blood pressure (44). The results from the present study support this hypothesis, since baseline blood pressure just prior to stress was elevated in the chronically stressed DHB Cort rats. The rats were undisturbed by any human activity during this period, so one can speculate that the rats were anticipating the restraint stress based on the memory of the previous episodes of stress. Interestingly, DHB Cort had a clear effect to increase two indices of sympathetic nerve activity, plasma norepinephrine and NPY, under baseline conditions, even prior to the first stress. Repeated stress in Sham-treated rats produced similar elevations in both neurohormones, raising the possibility that stress-induced increases in plasma Cort, acting within the DHB contribute to elevations in baseline sympathetic nerve activity with chronic stress. The increase in baseline NPY is of particular physiological significance, given that NPY is released from sympathetic postganglionic neurons during more intense and prolonged increases in sympathetic nerve activity and elevated NPY can promote stress-induced development of peripheral vascular disease, metabolic syndrome and obesity (20).

DHB Cort treatment had opposing effects on plasma Cort in response to novel compared with repeated restraint stress. DHB Cort treatment decreased baseline plasma Cort concentrations and inhibited the plasma Cort response after 60 min of novel restraint stress. With repeated stress, DHB Cort increased plasma Cort both prior to stress, but had no significant effect on the response to restraint. These results are in
agreement with some, but not all of our previous work. Chronic treatment of the DHB with the glucocorticoid receptor antagonist Mifepristone elevated baseline plasma Cort concentrations but inhibited restraint stress-induced increases in plasma Cort (2). However in our previous studies using 100% DHB Cort pellets, plasma Cort has been unaltered by DHB Cort treatment (3, 43, 44). Since there was no increase in tissue Cort within the forebrain, and DHB Cort did not alter stress-induced c-Fos expression in the medial parvocellular PVN, it seems very unlikely that Cort from the DHB pellet diffused to forebrain regions to cause feedback inhibition of the hypothalamic-pituitary-adrenal axis (9). There is also no evidence that Cort from the DHB pellet is diffusing into the periphery in physiologically significant amounts in adrenal-intact rats, and we have previously reported no effects of systemic 100% Cort pellets on blood pressure, baroreflex function or stress responsiveness(3, 43, 44). Thus, although we sometimes do see some physiological effects of even small doses of systemic Cort, these peripheral actions of Cort cannot account for the effect of DHB Cort reported in this study. Our results are in keeping with other reports that glucocorticoids can act within the NTS to activate or inhibit the hypothalamic-pituitary-adrenal axis (6, 14, 30, 39), however further research is required to determine the mechanisms accounting for these state-dependent effects of glucocorticoids on hypothalamic-pituitary-adrenal axis regulation.

The data presented here do not address the cellular mechanisms of Cort actions within the DHB. Glucocorticoids can bind to glucocorticoid and mineralocorticoid receptors, both of which are present in the NTS (13, 18). Some of the mineralocorticoid receptors in the NTS function selectively as aldosterone receptors due to the co-expression of the enzyme 11-beta-hydroxysteroid dehydrogenase 2 (11ßHSD2) within the same neurons; 11ßHSD2 metabolizes active endogenous glucocorticoids to inactive compounds (13). Mineralocorticoid receptors are also present in some NTS neurons that do not express 11ßHSD2, and higher levels of Cort could exceed the capacity of 11ßHSD2 to protect the mineralocorticoid receptors from Cort, so it is likely that both mineralocorticoid receptors and glucocorticoid receptors mediate actions of elevated Cort within the NTS (13). Glucocorticoid receptors are highly expressed in NTS catecholaminergic neurons, and Cort can act in the NTS to modulate stress-associated memory by a catecholamine-dependent mechanism (40). Stress induces an increase in tyrosine hydroxylase mRNA in the NTS, which can be glucocorticoid-dependent or independent, depending on the stress paradigm and other experimental factors (33, 65), so it is possible that some effects of DHB Cort reported here are mediated by NTS.
catecholaminergic neurons. Other NTS neuronal phenotypes such as glucagon like peptide-1 neurons mediate some effects of psychological stress and are modulated by glucocorticoids, and thus may mediate effects of elevated DHB Cort on stress responses (65).

**Perspectives and Significance**

Growing evidence implicates chronic stress as an important risk factor for cardiovascular disease, but the mechanistic explanation for the association is poorly understood. The data reported here support the idea that glucocorticoids, which are elevated with chronic stress, can act within the DHB to produce some of the adverse cardiovascular consequences of chronic stress. The data also suggest that glucocorticoids can recruit the involvement of vasopressin to the stress response, which is especially interesting given recent findings that centrally acting vasopressin influences stress-associated behaviors such as aggression, anxiety and depression (7). Improved understanding of the mechanistic links between stress and cardiovascular disease can lead to identification of susceptible individuals and improved treatment options.
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Figure Legends

Figure 1. Tissue corticosterone (Cort) concentrations within the dorsal hindbrain (DHB; top), ventral hindbrain (middle) and forebrain (bottom) after 18 days of treatment with DHB Sham pellets (open bars), DHB Cort pellets (cross-hatched bars), subcutaneous (SC) Sham pellets (horizontal striped bars), SC Cort pellets (vertical striped bars), acutely stressed rats with no pellet treatment (solid black bars). *=P<0.05 relative to DHB Sham treatment group.

Figure 2. Mean arterial pressure over time from 30 min prior to through 30 min following 60 min of restraint stress in DHB Sham- (solid squares and lines) and DHB Cort-treated (open triangles and dashed lines) rats. Rats (n=8 per group) were stressed on days 6, 13, 14 and 15 following the pellet implantation. Compared with Sham treatment, DHB Cort treatment significantly increased mean arterial pressure during the period of stress on all days and increased baseline pressure prior to stress on day 15. There were also some time points during the 30 min following stress at which blood pressure was higher in DHB Cort-treated rats compared with Sham-treated rats on days 6, 13 and 15. *=P<0.05 for Cort-treated relative to Sham-treated rats.

Figure 3. Average values for integrated change from baseline for mean arterial pressure (top row) and heart rate (bottom row) during the 60 min stress period (left graphs) and post-stress period (right graphs). Rats were stressed on days 6, 13, 14 and 15 following implantation of either a DHB Cort (solid bars) or Sham (open bars) pellet. DHB Cort treatment significantly increased the change in mean arterial pressure during stress on days 6, 13 and 14 relative to Sham-treated rats. The average integrated change in MAP during the post-stress period was not significantly different between Sham- and Cort-treated rats. There were no significant effects of DHB Cort on the average integrated change in heart rate during the stress and post-stress periods. *=P<0.05 for Cort-treated relative to Sham-treated rats.

Figure 4. Plasma hormone values at baseline and at 10 and 60 min of restraint stress on Day 6 (solid symbols and lines) and Day 15 (open symbols and dashed lines) in dorsal hindbrain (DHB) Sham-treated (squares) and DHB Cort-treated (triangles) rats. Top: Plasma norepinephrine, Bottom: plasma neuropeptide Y (NPY). P≤0.05 as follows for all
graphs: * for DHB Sham Day 6 compared with DHB Cort Day 6; § for DHB Sham Day 6 compared with Day 15; **for DHB Sham Day 6 compared with DHB Cort Day 15.

Figure 5. Example photomicrograph illustrating double-labeling for c-Fos (green) and vasopressin (red) in the PVN (approximately -1.88 mm from Bregma) in a Sham-treated rat (A-C) and Cort-treated rat (D-F). A and D are c-Fos only, column and E are vasopressin only and column and F show superimposed images. White arrows in D-F indicate sample neurons labeled for both c-Fos and vasopressin. The regions of the PVN for counting c-Fos expression are diagramed in the top left photomicrograph, and were determined using the atlas by Paxinos and Watson (35). DP=dorsal parvocellular, MP=medial parvocellular, VP, ventral parvocellular and LM, lateral magnocellular. G is an example photomicrograph of the NTS illustrating double labeling for dopamine-ß-hydroxylase (DßH; light brown) and c-Fos (dark brown/black). Black arrows indicate example neurons labeled for both DßH and c-Fos. The brown arrow indicates an example of labeling for c-Fos only.

Figure 6. Number of neurons expressing c-Fos in the caudal and rostral nucleus of the solitary tract (NTS, top first and second rows), the rostral ventral lateral medulla (RVLM, third row) and caudal ventral lateral medulla (CVLM, fourth row). Rats were treated with DHB Sham or DHB Cort pellets for 6 days then either subjected to restraint stress or left undisturbed (not stressed). Counts are provided for neurons expressing only c-Fos (left column), both c-Fos and dopamine-beta-hydroxylase (DßH; middle column) and for the total number of neurons expressing c-Fos in each region (right column). Values were =0 in unstressed rats where bars appear to be missing. *P<0.05 for main effect of stress.

Figure 7. Number of neurons expressing c-Fos in five subregions of the paraventricular nucleus of the hypothalamus (PVN): lateral magnocellular, dorsal parvocellular, anterior, ventral parvocellular and medial parvocellular, and in the supraoptic nucleus of the hypothalamus (SON). Rats were treated with DHB Sham or DHB Cort pellets for 6 days then either subjected to restraint stress or left undisturbed (not stressed). *P<0.05 for main effect of stress.

Figure 8. Left: Average change in mean arterial pressure from baseline over the 60 min of restraint and 30 min of post-restraint recovery period for DHB Sham (solid squares),
DHB Cort (solid circles), DHB Sham + intravenous infusion of a V1 vasopressin receptor antagonist (AVPX; open squares) and DHB Cort + intravenous infusion of a V1 vasopressin receptor antagonist (AVPX; open circles). **Right**: integrated change in mean arterial pressure from baseline for the 60 min of restraint and 30 min of post-restraint recovery period for DHB Sham (open bars), DHB Cort (solid bars), DHB Sham + intravenous infusion of a V1 vasopressin receptor antagonist (AVPX; slashed bars) and DHB Cort + intravenous infusion of a V1 vasopressin receptor antagonist (AVPX; cross-hatched bars). *=P<0.05 compared to Sham group; +=P<0.05 compared to Cort +AVPX group.
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§ =P<0.05 for Sham Day 15 compared with Sham Day 6; # for Cort Day 15 compared with Cort Day 6; † for Cort Day 15 compared with Sham Day 15; * for Cort Day 6 compared with Sham Day 6; ** for Cort Day 15 compared with Sham Day 6.