Development of attenuated baroreflexes in obese Zucker rats coincides
with impaired activation of nucleus tractus solitarius

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Adult obese Zucker rats (OZR; >12 wks) develop elevated sympathetic nerve activity (SNA) and mean arterial pressure (MAP) with impaired baroreflexes compared to adult lean Zucker rats (LZR) and juvenile OZR (6-7 wks). In adult OZR, baroreceptor afferent nerves respond normally to changes in MAP, whereas electrical stimulation of baroreceptor afferent fibers produces smaller reductions in SNA and MAP compared to LZR. We hypothesized that impaired baroreflexes in OZR are linked to reduced activation of brainstem sites that mediate baroreflexes. In conscious adult rats a hydralazine (HDZ)-induced reduction in MAP evoked tachycardia that was initially blunted in OZR, but equivalent to LZR within 5 minutes. In agreement, HDZ-induced expression of c-Fos in the rostral ventrolateral medulla (RVLM) was comparable between groups. In contrast, phenylephrine (PE)-induced rise in MAP evoked markedly attenuated bradycardia with dramatically reduced c-Fos expression in nucleus tractus solitarius (NTS) of adult OZR compared to LZR. However, in juvenile rats PE-induced hypertension evoked comparable bradycardia in OZR and LZR with similar or augmented c-Fos expression in NTS of the OZR. In urethane-anesthetized rats, microinjections of glutamate into NTS evoked equivalent decreases in SNA, HR, and MAP in juvenile OZR and LZR, but attenuated decreases in SNA and MAP in adult OZR. In contrast, microinjections of glutamate into the caudal ventrolateral medulla, a target of barosensitive NTS neurons, evoked comparable decreases in SNA, HR, and MAP in adult OZR and LZR. These data suggest OZR develop impaired glutamatergic activation of the NTS that likely contributes to attenuated baroreflexes in adult OZR.
INTRODUCTION

Accumulation of excess body fat is an independent risk factor for increased basal sympathetic nerve activity (SNA) and elevated mean arterial pressure (MAP) in humans and animals (8, 39, 49, 55). In addition, obesity is associated with impaired short-term control of MAP by arterial baroreflexes (4, 5, 19, 47, 59). In obese subjects evoked changes in MAP yield significantly blunted compensatory baroreflex-mediated changes in SNA and heart rate (HR). In humans and animal models of obesity, impaired baroreflex-mediated regulation of HR is associated with reduced variability in HR, a hallmark for increased risk of poor outcomes in patients with cardiovascular disease (29, 36, 59). These baroreflexes provide powerful moment-to-moment buffering against changes in MAP, and increased variability of MAP is a significant independent risk factor for end organ damage and detrimental cardiovascular incidents (34, 41). Therefore, understanding the basis for obesity-related impairment of baroreflexes is essential for reducing morbidity and mortality.

The use of obese animal models greatly facilitates the invasive study of mechanisms underlying altered autonomic regulation of cardiovascular function. In one such model, obese Zucker rats (OZR), excess weight gain occurs due to the mutation of the leptin receptor, which promotes hyperphagia compared to lean Zucker rats (LZR) with functional leptin receptors (26, 36). Adult OZR display many of the abnormal physiological attributes observed in obese humans, including hyperlipidemia and insulin resistance with eventual hyperglycemia (10, 14). Although OZR begin to weigh more than LZR shortly after weaning at 4 weeks of age (10), autonomic and cardiovascular deficits emerge later in life, suggesting these deficiencies are not due to the mutation of the leptin receptor itself but rather are a consequence of the progressing metabolic syndrome. Juvenile OZR (7-8 wks old) have SNA, MAP, and baroreflex control of
HR and SNA that are comparable to age-matched LZR (35, 47). However, by 12 wks of age
adult OZR have elevated renal and splanchnic SNA and MAP with significantly impaired
baroreflex control of HR and SNA compared to age-matched LZR and juvenile OZR (9, 33, 35,
47).

The mechanisms underlying impaired baroreflex-mediated control of SNA and HR in
adult OZR are not known. We recently reported that baroreceptor afferent nerves, as represented
by whole aortic depressor nerve activity (ADN), appear to respond normally to acute, evoked
changes in MAP (22). In addition, the threshold MAP for the onset of afferent activity and gain
of ADN activity in relation to MAP is comparable in adult OZR and LZR. In contrast, direct
electrical stimulation of baroreceptor afferent fibers evokes blunted decreases in SNA and MAP
in adult OZR compared to LZR (22). These data suggest that OZR have impaired baroreflexes
due to changes in processing of baroreceptor inputs by the brain.

Three brain stem nuclei are essential for baroreflex-induced changes in SNA to
cardiovascular targets (e.g. 16). Increased MAP stimulates baroreceptor afferent nerves to excite
second order neurons in the NTS by activation of glutamatergic receptors (1, 62). Second order
neurons in the NTS provide a glutamatergic activation of GABAergic inhibitory neurons in the
caudal ventrolateral medulla (CVLM). These inhibitory neurons in the CVLM in turn project to
the rostral ventrolateral medulla (RVLM) to inhibit the activity of presympathetic RVLM
neurons and decrease SNA, HR, and MAP (16). Conversely, lowering MAP decreases
baroreceptor afferent nerve activity to reduce tonic activation of critical neurons in the NTS and
CVLM, thereby allowing activation of presympathetic RVLM neurons to increase SNA and raise
HR and MAP. Altered central processing of baroreceptor inputs could potentially arise from
changes in any of these brain stem regions.
The present study examines whether activation of the NTS, CVLM, or RVLM by acute changes in MAP is altered in adult OZR. Specifically, we determined whether hypotension-induced activation of the RVLM, as evidenced by c-Fos expression, is blunted in adult OZR compared to LZR. Furthermore, we sought to determine whether acutely raising MAP results in reduced expression of c-Fos in the NTS of adult OZR compared to LZR. In addition we sought to determine whether the ability of exogenous glutamate microinjected into the NTS or CVLM to decrease SNA, HR, and MAP is attenuated in adult OZR compared to LZR.

MATERIALS AND METHODS

Animals. Male OZR (fa/fa), LZR (fa/+ and +/+), and Sprague-Dawley rats were purchased from Harlan (Indianapolis). Rats were housed in centralized animal care facilities kept at consistent humidity (60 ± 5%), temperature (24 ± 1 °C), and light cycle (0600-1800). Rats were given free access to tap water and standard rat chow (Teklad 8640 or Purina 5GL3). Lean and obese rats were housed separately, with rats housed 2-4 in a cage. Experiments were performed on age-matched juvenile (6-7 weeks old) Zucker rats or adult (13-17 weeks old) Zucker rats and Sprague-Dawley rats. All experiments were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the American Physiological Society’s Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training. The animal protocols were reviewed and approved by the Institutional Animal Care and Use Committees at University of North Texas Health Science Center and Medical College of Georgia.
Evoked changes in MAP to induce c-Fos expression in conscious rats. Under isoflurane anesthesia (initially with 5% in 100% oxygen in a secured box and then maintenance with 2.5% through a nose cone), catheters were inserted into a femoral artery and into a femoral vein for recording arterial pressure and infusing drugs, respectively. The catheters were tunneled beneath the skin to exit between the scapulae. To allow free movement of the rat in the conscious state, catheters were run through a tether attached to a swivel (Instech Solomon). The rats regained consciousness in individual cylindrical Plexiglas cages (MTANK, Instech Solomon) and recovered one day prior to experiments. The baseline arterial pressure, MAP, and HR were monitored in quiet conditions for 60 minutes. Baseline values were recorded for 4 minutes prior to the onset of a treatment. Rats were subjected to one of two treatments for 90 minutes; a phenylephrine (PE)-induced increase in MAP or a hydralazine (HDZ)-induced decrease in MAP. Control rats received volume- and rate-matched infusions of saline. Juvenile and adult rats were examined in age-matched pairs of LZR and OZR separated by 30 minutes to accommodate perfusions and alternating whether a treated or control rat started first. The PE (0.5mg/ml) was infused through the venous line to increase and maintain MAP approximately 40 mmHg from baseline (4–30µl/minute; Razel pump). During the last 30 minutes of infusion, the PE-filled syringe was exchanged with a saline-filled syringe to flush the PE from the line connecting the syringe to the rat. The HDZ (7-15 mg/kg/2 ml) was infused through the venous line over 1 minute. A single infusion of HDZ produces a significant decrease in MAP that is sustained over the 90-minute protocol period (17). After 90 minutes, rats were anesthetized with urethane (1.5 g/kg/5 ml, iv) and perfused transcardially with phosphate-buffered saline (250 ml, pH 7.4) followed by 4% phosphate-buffered formaldehyde (500 ml; Electron Microscopy Sciences). The
brain was removed and fixed for 48 hours in the same formaldehyde solution for later histological analysis.

Histology for c-Fos and tyrosine hydroxylase. Brain stems were sectioned in the coronal plane (30 µm) with a Vibratome and stored in a cryoprotectant solution at -20°C. (52). Histological protocols were performed using a subset of 1 in every 6 sections free floating at room temperature on an orbital shaker in solutions prepared in Tris-buffered saline (TBS, pH 7.4) unless otherwise noted. Sections were incubated with 1% hydrogen peroxide (30 minutes) to block endogenous peroxidases, rinsed in TBS, and then incubated in 10% horse serum (45 minutes) to block nonspecific staining. For detection of c-Fos, sections were incubated with a goat-anti Fos primary antibody (1:2000; 48 hours; 4°C; Santa Cruz, sc-52G), followed by a biotinylated donkey anti-goat secondary antibody (1:400; 1 hour; Jackson 705-066-147 or Invitrogen D-20698), and then an avidin-biotin solution (1 hour; Vector, PK-6100). Immunoreactivity for c-Fos was revealed by incubation with a nickel-intensified 3-3’diaminobenzadine (DAB) solution for approximately 8-10 minutes. The reaction was terminated by rinsing with TBS.

Presympathetic RVLM neurons are comprised of C1 adrenergic neurons and noncatecholaminergic neurons (43), so the catecholaminergic phenotype of the Fos+ neurons in the RVLM was also examined by staining for tyrosine hydroxylase (TH). At this rostro-caudal level of the ventrolateral medulla all neurons that express TH are C1 neurons (50). Tissue was incubated simultaneously with primary antibodies for c-Fos and TH (mouse anti-TH, 1:2000; Chemicon MAB5280). The protocol for detection of c-Fos in the nucleus was performed to completion as previously described, followed by detection of TH to avoid false positive staining
for TH in the soma and dendrites. After rinsing sections from the nickel-DAB solution, sections were incubated with a biotinylated donkey anti-mouse secondary antibody (1:400, Jackson), followed by an avidin-biotin solution (1 hour; Vector, PK-6100). Immunoreactivity for TH was revealed by incubation with a DAB solution for approximately (8-10 minutes). This double staining protocol produced black Fos+ nuclei in brown TH+ neurons for activated C1 neurons.

After completion of immunohistochemical protocols the sections were mounted onto gelatin-coated slides and serially dehydrated and delipidated in alcohols and xylenes. Coverslips were affixed with DPX mounting media (Sigma-Aldrich). Sections were examined in brightfield using an Olympus BX40 microscope. Fos immunoreactive (Fos+) neurons were mapped and counted in the NTS of rats treated with phenylephrine and in the RVLM of rats treated with hydralazine using the Neurolucida system (MicroBrightfield, Inc.) as previously described (52). In addition, TH+ neurons with or without c-Fos immunoreactivity were mapped and counted in the RVLM. To account for activation unrelated to evoked changes in MAP, Fos+ neurons were also counted in the NTS and RVLM of saline-treated rats. Several rostro-caudal levels of the NTS and the RVLM were analyzed separately, and bilateral counts were averaged for each section. Typical examples were photographed (Magnafire SP camera and Magnafire software, Optronics) and imported into Adobe Photoshop. Images were converted to gray scale for sections containing only Fos+ neurons. The output levels on all images were adjusted to the range of levels containing pixels. Contrast, brightness, and sharpness were also individually adjusted to best reflect the original material.

Microinjections into brainstem of anesthetized LZR and OZR. We sought to determine whether reduced PE-induced c-Fos expression in the NTS of adult OZR was due to an attenuated ability of glutamate to activate the NTS, as indicated by evoked changes in SNA, HR, and MAP.
To determine whether responses observed with activation of the NTS were due glutamatergic activation of the CVLM; a target of barosensitive NTS neurons for mediating baroreflexes (16). We also examined glutamatergic activation of the NTS in juvenile rats, because at this age baroreflexes are comparable in OZR and LZR (47), and preliminary data showed that PE-induced c-Fos expression was not blunted in juvenile OZR versus LZR. Anesthetized rats were used in these experiments to facilitate direct measurement of SNA and acute microinjections into the exposed brain stem. We have previously shown that intravenously administered urethane does not prevent the observations of elevated baseline SNA and MAP or reduced baroreflexes in adult OZR versus LZR (47).

Each rat was initially anesthetized with 5% isoflurane in 100% O$_2$ in a secured box. Then the rat was moved to a heated pad and received 2.5-3.5% isoflurane through a nose cone. Adequate anesthesia was confirmed by absence of leg flexion in response to a firm toe pinch. Catheters were implanted into the femoral artery and vein to record MAP and inject drugs, respectively. The trachea was cannulated toward the lungs and rats were ventilated at approximately 1ml/100g of body weight with 2.0-2.5% isoflurane in 100% O$_2$ (Model 683, Harvard Apparatus). Respiratory frequency was adjusted to maintain end-tidal CO$_2$ at 3.8-4.2% (CapStar-100, Charles Ward Electronics). The rat was placed in a stereotaxic apparatus (David Kopf Instruments) with the bite bar at -11mm. The left greater splanchnic sympathetic nerve was isolated, placed on two silver wires (teflon-coated and bared 250µm at tips, A-M Systems) and covered with kwik-sil (World Precision Instruments). The wound was closed to maintain core temperature and prevent desiccation. A partial occipital craniotomy was performed to expose the dorsal surface of the brain stem caudal to the cerebellum.
After surgical procedures were completed, isoflurane anesthesia was replaced by urethane (1.5 g/kg LZR body weight in 1.5 g/5ml at 50 µl/minute, iv), as previously described (23, 47). Once anesthetized with urethane, rats were allowed to recover for 30–45 minutes. After confirmation of an adequate level of anesthesia (<10 mmHg change in MAP to firm toe pinch, lack of corneal reflex, and stable MAP and HR), the neuromuscular blocker, pancuronium, was administered (1 mg/kg, iv; supplemented hourly at one third the initial dose).

Rectal temperature was maintained at 37°C (TC-1000, Charles Ward Electronics) throughout the experimental protocols.

Microinjections were performed using single barrel glass pipettes pulled and cut to a 50 µm diameter tip. Glutamate was dissolved in artificial CSF to deliver 1 nmol in 50 nl into previously established stereotaxic coordinates for the NTS and CVLM (46). Coordinates for the NTS were 0.5 mm lateral to the midline, 0.5 mm rostral to calamus scriptorius (caudal tip of area postrema), and 0.5 mm ventral to the dorsal surface of the brain stem. Coordinates for the CVLM were 1.3 mm rostral to calamus scriptorius, 1.9 mm lateral to the midline, and 2.4-2.8 mm ventral to the dorsal surface of the brain stem. In this case, three depths were explored (2.4 mm, 2.6 mm, and 2.8 mm), and the site producing the largest depressor response was used for analysis. Drugs were microinjected over 4-6 seconds by pressure (Pressure system Ille, Toohey), and the volume was estimated by observing the movement of the meniscus in the calibrated pipette. The drug solutions contained 5% green latex microspheres (Lumiphore) for histological confirmation of the injection sites as previously shown (32). The drugs were injected on each side of the medulla, and the values of the two responses were averaged for each rat. After the completion of the experimental protocol, the rats were treated with a ganglionic antagonist (mecamylamine, 3 mg/kg, iv) to estimate the minimum SNA. Then the rats were deeply
anesthetized with addition of isoflurane and were perfused transcardially with phosphate-buffered saline (250 ml, pH 7.4) followed by 4% paraformaldehyde (500 ml). The brains were removed and stored the fixative for 48 hours and then sectioned using a Vibratome (50 µm sections, coronal plane). The sections were mounted onto glass slides, and coverslips were applied with Krystalon. The microinjection sites were visualized via epifluorescence (Olympus BX40) and were verified to be in the region of the NTS or CVLM.

Data Collection and Statistical analysis. To measure arterial pressure the catheter line from the femoral artery was connected to a pressure transducer and amplifier (Neurolog System, Digitimer). The MAP and HR were derived from the arterial pressure pulse using an integrator and a spike trigger respectively (Neurolog System, Digitimer). The SNA was amplified 25,000X and filtered at 10-3 kHz with a 60 Hz notch filter (Differential AC amplifier 1700, A-M Systems). For integrated SNA the raw signal was full-wave rectified and averaged into 1-second bins (Digitimer). The baseline integrated SNA (100%) was defined as the activity 2 minutes preceding each stimulus. The minimum (0%) SNA was measured after ganglionic blockade with mecamylamine. Changes in integrated SNA were measured as % change from baseline.

Differences in baseline raw SNA were measured from the full-wave rectified voltage with the voltage due to noise subtracted (23). All analog signals were converted to digital (Micro 1401, Cambridge Electronic Design) and viewed on-line using Spike2 software (Cambridge).

All group data are expressed as mean ± SEM. Significant statistical difference was set at $P < 0.05$. Comparisons of baseline parameters or changes in SNA, MAP, and HR with microinjections between age-matched OZR and LZR were performed using unpaired $t$-tests. Comparisons of changes in MAP or HR along the 90-minute protocol and the expression of Fos
neurons at multiple rostro-caudal levels were performed using two-way ANOVA repeated
measures followed by Bonferroni post hoc tests. Statistical analyses were performed with
SigmaStat software version 3.5.

RESULTS

Baseline values for conscious and anesthetized rats are shown in Tables 1 and 2
respectively. Juvenile and adult OZR had higher body weights in comparison to age-matched
LZR in all experiments. Conscious juvenile OZR had a higher MAP compared with age-
matched LZR, but under anesthesia baseline MAP was not different in juvenile OZR and LZR as
previously reported (47). In the adults, OZR had a significantly higher MAP than age-matched
LZR in the conscious state and under anesthesia. There were no differences in HR between age-
matched OZR and LZR at either age range or anesthetic condition as previously reported (47).

Hydralazine-induced c-Fos expression in the RVLM of conscious Zucker rats.

Hydralazine (HDZ) produced a significant decrease in MAP that was comparable in the adult
OZR and LZR (Fig. 1A). The initial reduction of ~20mmHg was reached within 15 minutes and
the hypotension was sustained for the 90-minute protocol. The HDZ-induced hypotension
evoked a considerable baroreflex-mediated tachycardia that was significantly blunted by 16% in
the OZR ($n=7$) compared to LZR ($n=8$) in the initial phase of the response (Fig. 1B).
However, the HR values became comparable after 5 minutes and were not different through the
remainder of the 90-minute protocol. Infusion of an equivalent volume of saline evoked minimal
and comparable changes in MAP and HR in OZR ($n=5$) and LZR ($n=5$; Fig. 1). Because this
dose of HDZ did not evoke the expected 40 mmHg reduction in MAP previously reported in
conscious Sprague-Dawley rats (17), we examined a set of this strain under the same conditions 
\( (n = 8) \). Interestingly, in Sprague-Dawley rats HDZ (10 mg/kg) reduced MAP 39 ± 4 mmHg 
within 10 minutes, but evoked a weaker baroreflex-mediated tachycardia (peak rise was 116 ± 7 
beats/minute in 3-5 minutes; compare to Fig. 1B).

Hydralazine-induced hypotension produced robust c-Fos expression in the RVLM of 
OZR and LZR (representative maps, Fig. 2; representative photomicrographs, Figs. 3A and B; 
group data, Fig. 4). In contrast, rats treated with saline had few c-Fos+ neurons in the RVLM, 
and some sections contained no c-Fos+ neurons (Figs. 3C and 4A). In the HDZ-treated rats the 
majority of Fos+ neurons in the RVLM were not catecholaminergic (>80%), and number of 
Fos+/non-TH neurons was comparable in OZR and LZR at all levels of the RVLM examined 
(Fig. 4B). Of the ~20% of TH+ neurons that were Fos+, there was a trend for a reduced number 
of Fos+ neurons in the adult OZR compared to LZR that did not reach statistical significance 
(Fig. 4C; \( P = 0.053 \) at -12.2 mm caudal to bregma). However, the total number of HDZ-induced 
Fos+ catecholaminergic neurons in the RVLM was low (2 – 4 on each side of the section; Figs. 
2, 3A and B, and 4C).

Because the percentage of activated C1 neurons was substantially lower than previously 
reported for Sprague-Dawley rats (80%; 53), we also examined HDZ-induced c-Fos expression 
in the RVLM in this strain. Although the total number of c-Fos+ neurons was similar to the 
Zucker rats (12 ± 1 at -12.0 mm, 17 ± 2 at -12.2 mm, and 24 ± 3 at -12.4 mm, compare to Fig. 
4A), the percentage of c-Fos+ neurons that were catecholaminergic at the most rostral RVLM 
was ~60% (example in Fig. 3D). Thus, the activation of the RVLM in terms of c-Fos expression 
was comparable, but the proportion of C1 versus noncatecholaminergic RVLM neurons was 
different between the strains.
Phenylephrine-induced c-Fos expression in the NTS of conscious Zucker rats. The maximal rise in MAP (40 mmHg) by intravenous injection of PE was reached within 5 minutes of the onset of the infusion and sustained for at least 60 minutes in the PE-treated groups (Fig 5A). The rise in MAP induced a robust baroreflex-mediated bradycardia in both OZR ($n=9$) and LZR ($n=7$), but the magnitude of the decrease in HR was markedly blunted in OZR for the first 20 minutes of infusion in comparison to LZR (47% of LZR response; Fig 5B). Saline infusion in OZR ($n=7$) and LZR ($n=5$) evoked minimal and comparable changes in MAP and HR (Fig 5).

The PE-induced hypertension produced significant expression of c-Fos in the NTS of OZR and LZR (representative maps, Fig. 6; representative photomicrographs, Fig. 7; group data, Fig. 8). In contrast, rate- and volume-matched saline expression yielded little c-Fos expression in the NTS at all levels examined (Fig. 8). In contrast to the comparable HDZ-induced c-Fos expression in the RVLM of OZR and LZR (Fig. 4), the PE-induced c-Fos expression was substantially less in the adult OZR compared to age-matched LZR at all levels of the NTS examined (44% of LZR expression; Figs. 6-8).

Because juvenile OZR appear to have normal baroreflex-mediated responses to evoked increases in MAP (47), we examined whether PE-induced c-Fos expression would be comparable in juvenile OZR and LZR. As seen in the adult rats, PE increased MAP by 40 mmHg within 5 minutes of the onset of the infusion, and the rise in MAP was sustained for at least 60 minutes (Fig. 9A). As seen in the adult rats, PE produced a substantial baroreflex-mediated bradycardia in the juvenile OZR and LZR ($n=5$ in each group). However, unlike the adult rats, the magnitudes of the PE-induced decreases in HR were comparable in OZR and LZR for the duration of the 90-minute protocol (Fig. 9B).
In stark contrast to the adult rats and despite the similar changes in MAP and HR, juvenile OZR treated with PE had significantly more Fos+ neurons in the NTS in comparison to PE-treated juvenile LZR at 3 of the 4 levels examined (Fig. 10). Because the average volume of infusion needed to reach the 40 mmHg increase in MAP was significantly higher in juvenile OZR (1.2 ± 0.13 ml) than in LZR (0.7 ± 0.05 ml), we evaluated c-Fos expression in the NTS in two OZR and LZR that received 0.9% saline solution in the higher volume. In these animals, saline infusion evoked minimal and comparable changes in MAP and HR, and similar Fos expression in the NTS (total Fos+ cells for all 4 levels of the NTS combined was approximately 30, data not shown).

Microinjections of glutamate into the brainstem of anesthetized Zucker rats. The attenuated PE-induced c-Fos expression in adult OZR suggested that the NTS may be less responsive to excitatory inputs. To examine this possibility we stimulated the NTS directly by microinjections of glutamate and measured the evoked sympathoinhibition, bradycardia, and hypotension. In juvenile rats, microinjections of glutamate produced comparable physiological responses in OZR and LZR (n = 10 in each group; Figs. 11A1 – A3), in agreement with the equivalent PE-induced bradycardia observed in OZR and LZR of this age (Fig. 9B). In contrast, microinjections of glutamate into the NTS of adult OZR (n = 12) evoked a blunted sympathoinhibition compared to adult LZR (n = 10; Fig. 11B1). This observation is consistent with reduced PE-induced c-Fos expression and markedly attenuated PE-induced bradycardia observed in adult OZR. However, glutamatergic stimulation of the NTS evoked an exaggerated decrease in HR in adult OZR versus LZR (Fig.11B2). These two responses together coincided with a comparable decrease in MAP in OZR and LZR (Fig. 11B3).
To determine whether the apparent reduced sympathoinhibition in adult OZR had a functional significance for regulation of MAP, we prevented the changes in HR by treating another set of rats with methylatropine (2 mg/kg/0.1 ml, iv) and propranolol (5 mg/kg/0.1 ml, iv) to block parasympathetic and sympathetic inputs to the heart. In this preparation, microinjections of glutamate into the NTS evoked the same attenuated sympathoinhibition observed in the untreated rat (Fig. 11C1 versus 11B1) and was accompanied by a blunted hypotension in adult OZR (n = 7) compared to LZR (n = 8; Fig. 11C3). These data suggest an impaired ability to inhibit sympathetic vasomotor tone in adult OZR compared to LZR. In these rats the methylatropine was given before the propranolol, so the rats were also tested with selective antagonism of parasympathetic inputs to the heart. In this condition, microinjections of glutamate evoked comparable reductions in HR in adult OZR and LZR (-8 ± 1 versus -7 ± 1 beats/minute). These data suggest the exaggerated bradycardia evoked by microinjection of glutamate into the NTS was due to enhanced parasympathetically-mediated bradycardia in the OZR.

Baroreflex-mediated responses require glutamatergic activation of the CVLM (15), a target of barosensitive glutamatergic NTS neurons (61). To determine whether blunted sympathoinhibition after microinjections of glutamate into the NTS could be due to targets downstream of the NTS, we examined the physiological effects of microinjections of glutamate into the CVLM of adult OZR and LZR. In contrast to the differences observed after stimulation of the NTS (Fig. 11B and C), activation of the CVLM evoked comparable decreases in SNA, HR, and MAP in adult OZR (n = 14) and LZR (n = 13; Fig. 11D1-3). These data suggest that the differences observed after microinjections of glutamate into the NTS between adult OZR and LZR were due to changes at the NTS.
DISCUSSION

Previous studies have shown impaired baroreflex-mediated control of SNA and HR in adult OZR compared to age-matched LZR (3, 9, 47). The present study highlights the disproportionate degree of impairment to evoked increases versus decreases in MAP in conscious adult OZR and provides insights into potential mechanisms for the compromised responses. Although HDZ-induced tachycardia was significantly attenuated for 5 minutes in adult OZR, the evoked rise in HR was equivalent to the LZR for the next 85 minutes of sustained hypotension. This response was reflected in a comparable HDZ-induced c-Fos expression in the RVLM of OZR and LZR. In contrast, the attenuation of PE-evoked bradycardia in adult OZR was much more pronounced and continued for 20 minutes. In agreement, PE-induced c-Fos expression in the NTS was significantly diminished in adult OZR compared to LZR. Furthermore, the ability of glutamate in the NTS to decrease SNA and MAP was also reduced in adult OZR, suggesting the attenuated c-Fos expression reflected changes in NTS neurons that are relevant to the autonomic control of MAP. In contrast, glutamatergic activation of the CVLM, a target of the NTS in the baroreflex pathway, evoked comparable changes in SNA, HR, and MAP in adult OZR and LZR. Unlike adult rats, PE-induced bradycardia was not impaired in juvenile OZR, as previously reported (47). In agreement, in juvenile OZR and LZR glutamatergic activation of the NTS evoked equivalent decreases in SNA, HR, and MAP. Unexpectedly, the PE-induced c-Fos expression in NTS of the juvenile OZR was exaggerated compared to LZR. Together, these data suggest that changes are occurring in the NTS of OZR prior to the detection of overt autonomic and cardiovascular deficits, and that the NTS is a critical brain stem site for the production of attenuated baroreflexes in adult OZR.
The baroreflex is often depicted as a continuum of changes in HR to induced increases and decreases in MAP, but responses to these opposing changes in MAP involve distinct underlying mechanisms that may be differentially affected by metabolic syndrome. Lowering MAP reduces the ongoing activity of baroreceptor afferent nerves to eventual inactivity (e.g. 22). The observed reflex responses are a reflection of elimination or significant diminution of existing tonic activity in neurons of the baroreflex pathway that are baroreceptor-driven (the NTS and CVLM). Furthermore, hypotension-induced tachycardia is primarily mediated by sympathetic stimulation of the heart, with a smaller contribution from vagal withdrawal (3, 51). The SNA rises due to reduced CVLM-mediated tonic inhibition of presympathetic neurons in the RVLM. The adult OZR appears to have reduced basal tonic CVLM-mediated inhibition of the RVLM, which is also reflected in a reduced drive from the NTS. The diminished tonic influences of the NTS and CVLM may explain the initially blunted HDZ-induced tachycardia observed in adult OZR in the present study, which is in agreement with previous reports of impaired rises in HR that focus on the initial responses observed with brief infusions of nitroprusside (3, 37).

Highlighting the relatively small magnitude of the deficit, several reports in obese rats fail to see significant differences in hypotension-induced tachycardia, even when hypertension-induced bradycardia is clearly attenuated (5, 6, 11, 47). Regardless of the differences observed at the onset of hypotension, within minutes HDZ-induced tachycardia was comparable in OZR and LZR, suggesting other mechanisms activated by hypotension provided a delayed compensation that allowed the OZR to effectively respond to a sustained decrease in MAP.

Infusion of HDZ to decrease MAP produced robust c-Fos expression in the RVLM that was not observed in rats treated with saline, suggesting the hypotension and not the protocol itself was the effective stimulus. In agreement with the largely comparable HDZ-induced
tachycardia, the HDZ-induced c-Fos expression in the RVLM was not different between adult OZR and LZR. Because the full expression of sympathoexcitatory responses mediated by the RVLM requires activation of both C1 catecholaminergic and non-catecholaminergic neurons (7, 30, 44), both populations were examined. The majority of Fos+ RVLM neurons were not catecholaminergic (80%), and there was no significant difference in the c-Fos expression of either cell group between adult OZR and LZR. Thus, the two populations of presympathetic RVLM neurons each appear to respond comparably to hypotension in adult OZR versus LZR. With the substantial and sustained hypotension used for activating c-Fos expression in the RVLM, the observed c-Fos expression is likely to be more representative of the upper plateau of the baroreflex rather than the gain, which is more readily observed with the onset of changes in MAP.

The more striking deficit in the adult OZR lies in their reduced ability to combat a hypertensive stimulus. In contrast to hypotension-evoked responses, which rely upon the reduction of ongoing baroreceptor afferent nerve activity to the brainstem, raising MAP stimulates baroreceptor afferent nerves to produce a glutamatergic excitation of the NTS (1, 2, 54, 62). The sympathetic response is highly affected by the ability to acutely activate the NTS and its target in the baroreflex pathway, GABAergic neurons of the CVLM. Although the ability of GABA to inhibit the RVLM can also alter the efficacy of baroreflex-mediated reductions in SNA, we have previously shown that GABAergic inhibition of the RVLM produces comparable decreases in SNA, HR, and MAP in adult OZR and LZR (23). In contrast, the NTS appears to become less responsive to glutamatergic activation in the OZR at the same age when PE-induced bradycardia and c-Fos expression in the NTS become reduced (Figs. 5-10; 47). The attenuated baroreceptor-mediated activation of the NTS in OZR does not appear to be due to impaired
baroreceptor afferent function (22) or diminished release of glutamate in the NTS, because direct application of exogenous glutamate into the NTS also produced a smaller reduction in sympathetic vasomotor tone in the adult OZR. Furthermore, the impaired regulation of SNA appears to be localized to the NTS, because activation of target neurons in the CVLM by microinjections of glutamate evoked comparable decreases in SNA, HR, and MAP in adult OZR and LZR. Further study will be needed to determine the cellular mechanisms that underlie the reduced physiological responses to glutamate in the NTS of adult OZR.

Baroreflex-mediated reductions in HR are predominantly produced by activation of vagal parasympathetic efferents to the heart with a smaller contribution from withdrawal of cardiac sympathetic tone (3, 51). Activation of parasympathetic control to the heart is initiated by baroreceptor-driven glutamatergic stimulation of NTS neurons, which in turn provide glutamatergic activation of vagal motor neurons in the nucleus ambiguus (e.g. 60). In agreement, selective antagonism of vagal inputs to the heart greatly reduces the bradycardia evoked by raising MAP with PE (3) or by microinjecting glutamate into the NTS (present study). After blockade of sympathetic inputs to the heart with propranolol, PE-induced bradycardia is still smaller in adult OZR compared to LZR, suggesting a reduced vagally-mediated inhibition of HR contributes to the blunted baroreflex in OZR. In addition, impaired PE-mediated sympathoinhibition in the adult OZR also appears to contribute, because after selective antagonism of parasympathetic inputs to the heart with atropine, adult OZR still have smaller reductions in HR to evoked rises in MAP compared to LZR (3). Impaired sympathoinhibitory responses extend beyond the heart because PE-induced rises in MAP also produce blunted inhibition of splanchnic SNA in adult OZR (47). Although the diminished PE-evoked sympathoinhibition in adult OZR appears to be mediated by impaired glutamatergic activation of
the NTS, whether the same mechanism attenuates vagal responses to increased MAP in adult OZR is not known.

Unexpectedly, although PE-induced bradycardia was significantly reduced in adult OZR versus LZR, glutamatergic activation of the NTS produced an exaggerated bradycardia in adult OZR. The enhanced glutamate-induced bradycardia appeared to be vagally-mediated because blockade of parasympathetic inputs to the heart with methylatropine equalized the responses in OZR and LZR. It is most likely that microinjections of glutamate into the NTS stimulated neurons in addition to those that mediate arterial baroreflexes. Interestingly, not all vagal inputs to the NTS evoke significantly blunted bradycardia in adult OZR. Activation of the Bezold-Jarisch reflex produces a similar pattern to that observed with glutamatergic activation of the NTS with blunted sympathoinhibition, a trend for larger bradycardia, and comparable depressor response in adult OZR versus LZR (22). The Bezold-Jarisch reflex, which is initiated by stimulation of cardiopulmonary vagal afferents to the NTS, utilizes a brainstem pathway that is parallel to and overlapping with the arterial baroreflex. Within the NTS, distinct neurons process the two types of inputs, whereas neurons in the CVLM and RVLM respond to activation of both reflexes (38, 45, 48, 57, 58). Electrical stimulation of whole vagal afferent nerves in adult OZR and LZR elicit comparable, blunted, or exaggerated changes in HR in OZR, depending on the frequency of the stimulation (22), highlighting the heterogeneity of vagal inputs and their processing in the NTS. In obese rats, the arterial baroreflex is not the only impaired sympathoinhibitory reflex that occurs by vagal activation of the NTS. Rats made obese by a high fat diet with impaired arterial baroreflexes also show blunted sympathoinhibitory responses to gastric cholecystokinin, a reflex that is initiated by gastric vagal afferents to the NTS (21).

Thus, it appears that metabolic syndrome differentially affects the processing of functionally
diverse vagal inputs to distinct NTS neurons that regulate autonomic function to cardiovascular targets. To our knowledge, it is not known whether arterial baroreceptor-driven control of sympathetic and parasympathetic outflows is directed by common or distinct NTS neurons. Clearly, a more cellular analysis of NTS neurons with identified inputs and projections would elucidate synaptic mechanisms underlying impaired baroreceptor-mediated activation of the NTS in adult OZR.

The underlying causative attributes of metabolic syndrome that lead to the development of impaired NTS function and baroreflexes are also not known. Hypertension and blunted baroreflexes often occur together, but in OZR the development of impaired baroreflexes and changes in NTS function precede measurable differences in MAP between OZR and LZR. At ~7 weeks of age the gain of the sympathetic baroreflex is reduced in OZR compared to LZR (47), and PE-induced c-Fos expression in the NTS is different between the two groups (Fig. 10). However, significant differences in MAP cannot be reliably detected in conscious undisturbed OZR and LZR until 10 weeks of age (28) with a clear separation of MAP levels by 12 weeks of age (35). In agreement, rats made obese by a high fat diet develop impaired baroreflexes within 4 weeks on the diet (63), prior to the onset of elevated MAP that is present after 13 weeks (21, 49). Just as animal models of obesity show a separation of baroreflexes and elevated MAP, obese patients can have significantly impaired baroreflexes in the absence of hypertension (18). Conversely, impaired baroreflexes in OZR are coincident with diminished basal tonic inhibition of SNA by the NTS and CVLM and reduced tonic GABAergic inhibition of the RVLM neurons that drive SNA to maintain MAP (23). These changes in the tonic regulation of the baroreflex pathway are likely to contribute to the elevated basal levels of SNA and MAP in adult OZR.
In contrast to the onset of cardiovascular deficits in OZR, metabolic parameters are altered at an earlier age, and many of these attributes have been shown to affect basal SNA and MAP as well as baroreflex efficacy. At 7 weeks of age, fasted OZR have elevated plasma levels of triglycerides and insulin (12, 35, 42). Although reports of differences in fasted levels of blood glucose between OZR and LZR at 7 weeks are inconsistent, insulin resistance is unmistakable as evidenced by the hyperinsulinemia and impaired glucose tolerance, (12, 35, 42). By adulthood OZR have fasting hyperglycemia with elevated blood cholesterol and nonesterified fatty acids compared to juvenile OZR and age-matched LZR (12, 37). In addition, compared to LZR, adult OZR have elevated fasting plasma levels of proinflammatory markers such as interleukin-6 and tumor necrosis factor-α (56). Although OZR do not respond to their elevated leptin levels, the excess white adipose tissue in OZR shows increased expression of inducible nitric oxide synthase and reduced expression of adiponectin (but elevated plasma levels) to further promote insulin resistance (24). In addition, whereas young LZR show rising testosterone that remains stable through adulthood, juvenile OZR show a decline in testosterone with levels that are significantly lower than LZR in adulthood (20). These factors, and others not mentioned here, each have the potential to impact baroreflexes in the adult OZR, making unraveling the underlying deleterious mechanisms and restoration of normal function in the obese state a challenge.

Perspectives and Significance

The occurrence of metabolic syndrome or cardiometabolic disease has reached epidemic proportions in the United States and now across the world. From the cardiovascular perspective elevated arterial pressure is used as a key indicator of the syndrome. However, cardiometabolic
disease is also highly and independently associated with impaired short-term control by baroreflexes, which are vital for stabilization of MAP. When baroreflex sensitivity is diminished, spontaneous variability of MAP is increased and HR variability is reduced (e.g., 25, 27). In humans, these attributes are highly predictive of increased risk for poor cardiovascular outcomes (31, 40). Patients treated for hypertension with residual increased variability of MAP are at higher risk for deleterious cardiovascular events (41). Like obese humans, OZR develop metabolic syndrome complete with elevated MAP and significantly impaired baroreflexes, making the OZR an excellent model to investigate mechanisms and effective treatments for the cardiovascular attributes of cardiometabolic disease. This study illustrates the development of an impaired baroreflex in OZR and pinpoints a region of the brainstem, the NTS, which appears to be a key mediator of the observed deficits in short-term, and perhaps long-term control of MAP. Future studies will be necessary to determine the causes and cellular mechanisms of impaired glutamatergic activation of the NTS in the adult OZR.

GRANTS
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DISCLOSURES
The authors have no competing interests to disclose.
AUTHOR CONTRIBUTIONS

P.S. Guimaraes performed the studies in conscious rats with immunohistochemical analyses and D.A. Huber performed the microinjection studies in anesthetized rats. M.J. Campagnole-Santos and A.M. Schreihofer supervised the design, implementation, and interpretation of experiments within the study. All authors contributed to the analysis of the data and drafts of the manuscript and take full responsibility for the content of the study.

REFERENCES


FIGURE LEGENDS

Fig. 1. Changes in mean arterial pressure (A) and heart rate (B) produced by intravenous injection of HDZ or saline in adult LZR and OZR. Data are means ± SE. *Significant difference between LZR and OZR at 3 and 4 minutes after injection of HDZ, P < 0.05.

Fig. 2. Representative maps of c-Fos expression in the RVLM of an adult lean rat (left) and an adult obese rat (right) after 90 minutes of HDZ-induced hypotension. Tyrosine hydroxylase immunoreactivity depicts coincidence of c-Fos expression with the C1 cell group. Open squares are catecholaminergic (tyrosine hydroxylase- immunoreactive; TH+) neurons, filled circles are Fos+/non-TH neurons, and asterisks are Fos+/TH+ neurons. Amb, nucleus ambiguus; FN, facial nucleus; ION, inferior olivary nucleus; sp5, spinal trigeminal nucleus. Location relative to bregma is depicted in mm in the middle of each pair of maps.

Fig. 3. Representative photomicrographs of c-Fos expression (black nuclei) and catecholaminergic neurons (TH+, brown somas and dendrites) in the RVLM of after 90 minutes of HDZ-induced hypotension or infusion of saline. A: Section from one HDZ-treated LZR. B: Section from one HDZ-treated OZR. C: Section from one saline-treated LZR. D: Section from one HDZ-treated Sprague-Dawley rat. Arrows: Fos+/TH+ neurons; Arrowheads: TH+ neurons without Fos; asterisks: Fos+/non-TH neurons. Scale bar is 50 µm.
Fig. 4. Expression of c-Fos in C1 catecholaminergic and non-catecholaminergic neurons in the RVLM after infusion of HDZ or saline in OZR and LZR. 
A: Fos$^+$ neurons in the RVLM and 3 rostro-caudal levels. 
B: Fos$^+$/non-TH cells/level of RVLM. 
C: c-Fos in TH$^+$ cells. These are the same rats that are depicted in Fig. 1. Data are means ± SE. *Significant difference from respective saline-treated group, $P < 0.05$. 

Fig. 5. Changes in mean arterial pressure (A) and heart rate (B) produced by intravenous infusion of PE or saline for 90 minutes in LZR and OZR. Data are mean ± SE. *Significant difference between LZR-PE and OZR-PE at 5, 10, 15 and 20 minutes, $P < 0.05$. 

Fig. 6. Representative maps of c-Fos expression in the NTS of an adult lean rat (left) and an adult obese rat (right) after 90 minutes of a PE-induced increase in MAP. Filled circles represent c-Fos+ nuclei. Location of section relative to bregma is depicted in mm on the left side of the sections. AP, area postrema; NTS, nucleus tractus solitarius; 10, dorsal vagal motor nucleus; 12, hypoglossal nucleus. 

Fig. 7. Representative photomicrographs of c-Fos expression in the NTS of an adult lean rat (A-C) and an adult obese rat (D-F) after 90 minutes of a PE-induced increase in MAP. Photos are from 3 rostro-caudal levels in relation to bregma: -14.2 mm (A and C), -13.8 mm (B and E), and -13.4 mm (C and F). AP, area postrema; CC, central canal; TS, tractus solitarius; 4V, 4th
ventricle; 10, dorsal motor nucleus of the vagus; 12, hypoglossal motor nucleus. Scale bars are 250 mm.

Fig. 8. Expression of c-Fos in the NTS of LZR and OZR after infusion of PE or saline. These are the same rats that are depicted in Fig. 5. Data are means ± SE. *Significant difference from respective saline-treated rats, P <0.05. #Significant difference from LZR-PE at that bregma level, P <0.05.

Fig. 9. Changes in mean arterial pressure (A) and heart rate (B) produced by intravenous infusion of PE for 90 minutes in juvenile LZR and OZR. Data are mean ± SE.

Fig. 10. Expression c-Fos in the NTS of juvenile LZR and OZR after intravenous infusion of PE. These are the same rats that are depicted in Fig. 9. Data are means ± SE. *Significant difference from LZR-PE at that bregma level, P <0.05.

Fig. 11. Changes in MAP, HR, and SNA after microinjections of glutamate into the NTS (A-C) or caudal ventrolateral medulla (CVLM, D) in juvenile (A) and adult (B-D) LZR and OZR. In C, rats were treated with atropine and propranolol before microinjections of glutamate into the NTS to prevent changes in HR. Data are mean ± SE. *Significant difference from respective LZR, P <0.05.
Table 1. Baselines of conscious juvenile and adult OZR and LZR

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>weight (g)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/minute)</th>
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</thead>
<tbody>
<tr>
<td>Juvenile</td>
<td></td>
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<tr>
<td>LZR</td>
<td>7</td>
<td>175 ± 4</td>
<td>108 ± 1</td>
<td>408 ± 5</td>
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<tr>
<td>OZR</td>
<td>7</td>
<td>233 ± 1*</td>
<td>121 ± 2*</td>
<td>422 ± 2</td>
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<tr>
<td>Adult</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LZR</td>
<td>25</td>
<td>372 ± 7</td>
<td>116 ± 1</td>
<td>363 ± 4</td>
</tr>
<tr>
<td>OZR</td>
<td>28</td>
<td>540 ± 7*</td>
<td>128 ± 1*</td>
<td>360 ± 5</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. $n$ = number of rats. *$P < 0.05$ compared to LZR in that age group.

MAP, mean arterial pressure; HR, heart rate.
### Table 2. Baselines of anesthetized juvenile and adult OZR and LZR

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>weight (g)</th>
<th>SNA (µV)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/minute)</th>
</tr>
</thead>
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<td>Juveniles</td>
<td></td>
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<tr>
<td>LZR</td>
<td>10</td>
<td>211 ± 6</td>
<td>0.9 ± 0.2</td>
<td>118 ± 2</td>
<td>457 ± 6</td>
</tr>
<tr>
<td>OZR</td>
<td>10</td>
<td>309 ± 1*</td>
<td>1.4 ± 0.4</td>
<td>120 ± 3</td>
<td>431 ± 8</td>
</tr>
<tr>
<td>Adults</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LZR</td>
<td>30</td>
<td>375 ± 5</td>
<td>1.5 ± 0.2</td>
<td>116 ± 3</td>
<td>427 ± 3</td>
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<tr>
<td>OZR</td>
<td>34</td>
<td>567 ± 7*</td>
<td>2.3 ± 0.2*</td>
<td>127 ± 2*</td>
<td>418 ± 4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *n* = number of rats. *P* < 0.05 compared to LZR in that age group.

SNA, sympathetic nerve activity.
Figure 1. Guimaraes et al.
Figure 2. Guimaraes et al.
Figure 4. Guimaraes et al.
Figure 5. Guimaraes et al.
Figure 6. Guimaraes et al.
Figure 7. Guimaraes et al.
**Figure 8.** Guimaraes et al.
Figure 9. Guimaraes et al.
Figure 10. Guimaraes et al.
**Figure 11.** Guimaraes et al.