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ORIGINAL RESEARCH

MEMRI reveals altered activity in brain regions associated with anxiety, locomotion, and cardiovascular reactivity on the elevated plus maze in the WKY vs SHR rats

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Abstract

Individuals with anxiety/depression often have exaggerated cardiovascular responses to stressful stimuli and a comorbidity with hypertension. Alternatively, individuals with hypertension can be more anxious. In the present study cardiovascular changes were evaluated during behavioral testing of anxious behavior on the elevated plus maze (EPM) in the spontaneously hypertensive rat (SHR), a rodent model of neurogenic hypertension, and compared to the response of the more anxious, but normotensive, Wistar-Kyoto rat (WKY). Manganese-enhanced magnetic resonance imaging (MEMRI) was used to identify regional differences in baseline brain activity. Parallel to indicators of elevated behavioral anxiety on the EPM, WKYs had a greater increase in blood pressure but not heart rate when compared to the SHR while on the EPM. Associated with differences in anxiety-related behavior and autonomic responses, we observed increased baseline activity in the amygdala, central gray, habenula and interpeduncular nucleus with MEMRI of the WKY compared to the SHR. Conversely, elevated baseline brain activity was found in regions associated with blood pressure control and system arousal, including the hypothalamus, locus coeruleus and pedunculopontine tegmental nucleus, in the SHR vs WKY, in-line with increased resting blood pressure and increased mobility in this strain. Lastly, reduced activity in hippocampal regions was identified in the SHR compared to the WKY and may be associated with cognitive impairment previously reported in the SHR. Thus, autonomic reactivity may be a true measure of stress in rodent models of anxiety and MEMRI presents a powerful technique to uncover novel brain mechanisms of blood pressure control.

Keywords Elevated plus maze · Anxiety · Blood pressure · Heart rate · MEMRI · SHR · WKY

Introduction

When individuals with cardiovascular disease are screened for mental disorders, or tested for stress responsiveness, they often have high depression and anxiety scores (Davies et al. 1997; Sunbul et al. 2014) as well as heightened cardiovascular responses to stress (Garafova et al. 2014). Conversely, individuals with certain forms of chronic mental stress, like post-traumatic stress disorder, anxiety and/or depression have an increased incidence of cardiovascular

Highlights
- Increased behavioral anxiety in WKY rats is linked to heightened autonomic activity.
- High trait anxiety in the WKY is associated in increased resting activity in the amygdala.
- MEMRI identifies elevated neuronal activity in the pons of the SHR linked to hypertension/increased mobility.

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disease (Bacon et al. 2014; Burg and Soufer 2016; Debert et al. 2010; Ginty et al. 2013; Meng et al. 2012) and typically demonstrate exaggerated cardiovascular responses to acute exposure to stressful stimuli (Beckham et al. 2002). These outcomes suggest there is a strong interconnection between stress pathways in the brain and some element of cardiovascular disease (Bali and Jaggi 2013).

Animal studies have identified a strong link between angiotensin II (AngII), stress reactivity and anxiety. For example, physical restraint stress in rodents has been shown to trigger a significant increase in circulating AngII, while blockade of AngII receptors, either peripherally (Pavel et al. 2008) or centrally (Erdos et al. 2010; Yamazato et al. 2006), significantly attenuates the cardiovascular response to restraint stress. Alternatively, when healthy animals are chronically infused with a sub-pressor dose of AngII, the cardiovascular response to restraint stress is exaggerated (Pelaez et al. 2003). Chronic infusion of AngII has also been shown to increase indicators of anxiety in rats and mice (Duchemin et al. 2013), measured by less time spent in the open arms on the elevated plus maze (EPM). Conversely, administration of an Ang II type 1 receptor (AT1R) antagonist significantly increases time spent the open arms of the EPM and increases the number of entries into the open arms in normotensive rats, suggesting reduced anxiety (Pavel et al. 2008). Furthermore, knock-out mice lacking AT1Rs in the hypothalamus were recently reported to spend significantly more time in the open arms on the EPM when compared to wild-type controls (Wang et al. 2016), suggesting that AngII acting specifically in the hypothalamus plays a significant role in behavioral anxiety.

Interestingly, the proposed interconnection between elevated AngII levels, acute stress responsiveness, and indicators of anxiety does not hold up in all animal models of chronic cardiovascular disease. For example, the spontaneously hypertensive rat (SHR) has been shown to have elevated levels of circulating Ang II and exaggerated autonomic responses to acute physical stress (Martin et al. 2016; Palmer and Printz 1999, 2002; Sudo et al. 2004). However, when behavioral anxiety is measured, SHRs typically spend significantly more time in the open arms of the EPM when compared to normotensive controls (Nam et al. 2014). This suggests that in this model of cardiovascular disease, elevated circulating AngII and exaggerated cardiovascular responses to physical stress are coupled to a *low anxiety* profile. Similarly, in a rodent model of congestive heart failure, circulating levels of AngII are chronically elevated and the cardiovascular response to acute physical stressors is exaggerated when compared to sham operated controls (Cudnoch-Jedrzejewska et al. 2014). However, when placed on the EPM, the heart failure animals spend either significantly more time (Henze et al. 2008) or a similar amount of time (Gouweleeuw et al. 2016) in the open arms of EPM, compared to controls, suggesting reduced or unchanged anxiety. These observations contrast with a wealth of data from human subjects, which have identified that anxiety is a serious mood disorder in heart failure patients (Herr et al. 2014; Hopper et al. 2016).

To further evaluate the interplay between anxiety and cardiovascular function, the present study was undertaken. We hypothesized that the cardiovascular response during EPM testing would dissociate from behavioral anxiety and would be higher in the SHR, an animal model of low anxiety with underlying cardiovascular disease when compared to the more anxious normotensive Wistar Kyoto (WKY) strain (Nam et al. 2014; Pollier et al. 2000; Ramos et al. 1997). Telemetry technology that allowed continuous recording of cardiovascular parameters before and during EPM testing was used to test this hypothesis. In addition, we utilized manganese-enhanced magnetic resonance imaging (MEMRI) in WKY and SHR, to identify baseline activity differences in brain regions associated with low vs high anxiety. The results of this study have previously been presented in abstract form.

Methods

Animals

All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee and followed the National Institutes of Health guidelines for animal use in research. Experiments were performed on 12–14 weeks old male SHR (n = 23) and WKY rats (n = 21) that weighed between 300 and 400 gm (Charles River Laboratories, Wilmington, MA). Animals were housed in a temperature controlled room (24 °C) that was maintained on a 12 h lights on/off cycle (lights on at 6AM) with food and water available ad libitum. Following experimentation, all animals were euthanized with an overdose of sodium pentobarbital (200 mg/kg) or 4% isoflurane.

Telemetry surgical preparation

One group of animals (n = 7 WKY and n = 7 SHR) underwent survival surgery for placement of a Stellar telemetry probe (TSE Systems, Inc., Chesterfield, MO) in the femoral artery for measurement of arterial pressure (AP). These probes are unique because the data collected are stored on the probe until downloading, allowing continuous recording in an unrestricted environment and eliminating the need for an underlying trans-receiver. Briefly, animals were given a subcutaneous injection of carprofen (0.1 mg/kg) and buprenorphine (0.05 mg/kg) and then anesthetized to a surgical level (2–4% isoflurane in 100% O_2). While

deeply anesthetized, the abdominal and hindlimb regions were shaved and incisions were made along midline on the ventral surface of the abdomen and the left hindlimb. The body of the probe was inserted into the abdominal cavity and sutured to the abdominal musculature. The pressure probe lead was then tunneled subcutaneously to the hindlimb. The femoral artery was identified, isolated, tied distally and the probe was inserted into the artery and directed toward the abdominal aorta. The probe was secured to the vessel with suture and all incisions were closed, treated with triple antibiotic ointment, and the animals received a subcutaneous injection of saline (2–3 ml) for fluid replacement. Animals recovered on a heating pad and received an additional injection of buprenorphine (0.02–0.05 mg/kg) before returning to their home cages. All animals received additional doses of carprofen and buprenorphine hydrochloride for the next 2–3 days as needed and were allowed a minimum of 10 days to recover from surgery before tested on the elevated plus maze.

Elevated plus maze (EPM) test

The EPM (Stoelting, Wood Dale, IL) consists of a plus-shaped apparatus with two open and two enclosed arms, elevated off the floor by ~2 feet. The arms are ~4 inches wide and ~18 inches in length. For all animals (n = 10/strain, including 7 per strain with telemetry probes), the EPM test started with the animal being placed at the intersection of the four arms of the maze, facing an open arm. Movement on the EPM was monitored and quantified by Any-Maze Video tracking software (Stoelting). Following 5 min of EPM monitoring, animals were returned to their home cages. For those animals instrumented with a telemetry probe, prior to being placed on the maze the Stellar telemetry probe, was programed to record continuously for 12 min at a sampling frequency of 500 Hz (Biopac Software v 4.42,Goleta, CA), including 2 min of baseline AP while the animals were in their home cages, 5 min while they were on the EPM and 5 min following the return of the animal to its home cage.

Cardiovascular data analysis

To quantify the cardiovascular responses during the EPM test, the AP data from the Stellar/Biopac system was transferred to Spike 2 (Cambridge Electronics Design, Cambridge, UK). Spike 2 software was then used to identify both mean arterial pressure (MAP) and the interval between successive systolic AP (SAP) peaks. SAP interval values were converted to heart rate (HR) by calculating the 1/inter-systolic-peak interval *60. Next, three time periods were selected for analysis, including baseline (2 min in home cage), 5 min during the EPM test and the first 3 min of recovery in the home cage. Data were averaged into 1 min intervals and the change from baseline (Δ) in MAP and HR was calculated by subtracting the average value during the 2-min baseline from the 1 min averages taken during and after EPM testing for each animal. The Δ MAP and Δ HR during the 5 min of EPM testing was also calculated as the area under the curve (AUC) for each animal.

Manganese-enhanced magnetic resonance imaging (MEMRI) in WKY and SHR

MEMRI was performed on the remaining rats (n = 11 WKY and n = 13 SHR), as recently described (Perez et al. 2017). Briefly, manganese (II) chloride tetrahydrate ($MnCl_2$; Sigma–Aldrich, St. Louis, MO) was dissolved in sterile filtered ddH_2O prior to use. Twenty-two hours prior to imaging, all rats were briefly restrained and given an i.p. injection of $MnCl_2$ (70 mg/kg), and then returned to their home cages. The dose of MnCl2 used has been previously optimized for use in tracing brain activity in rodents by other labs (Brunnquell et al. 2016; Lee et al. 2005) and was used in our recent publications (Perez et al. 2017; Zubcevic et al. 2014b). On the day of testing, anesthesia was initially induced under 3–4% isoflurane delivered in 100% medical grade oxygen for 30–60 s (flow rate 1 L/min), and the levels of isoflurane were then maintained between 1.5–2.0% throughout the entire setup and imaging. Rats were placed prone on a plastic cradle with a respiratory pillow connected to a force transducer placed underneath the abdomen. Body temperatures were maintained using a warm air recirculation system that received feedback from a fiber optic thermocouple probe (SA Instruments, Inc., New York). Respiratory rates were monitored continuously and averaged between 50 and 70 breaths per minute across subjects. Images were collected on a 4.7 T Magnex Scientific MR scanner controlled by Agilent Technologies VnmrJ 3.1 console software. A 38-mm quadrature transmit/receive radiofrequency coil tuned to 200.6 MHz was used (airmri, LLC, Holden, MA). Images were acquired using a T_1-weighted multi-slice multi-echo sequence with the following parameters: repetition time (TR) = 460 ms, echo time (TE) = 16 ms, data matrix 256 × 256, field of view 25.6 × 25.6 mm, 20 slices at 0.8 mm thickness. Slices were in axial view (coronal in the rat) with the first slice starting at the rostral-most extension of the prefrontal/motor cortex and excluding the olfactory bulb. Scan time was 60 min per rat for 30 averages. All rats were euthanized with overdose of isoflurane (4%) following scanning.

MEMRI data processing

MEMRI data were processed and analyzed using previously published methods (Perez et al. 2017). Briefly, brain masks were manually drawn over T_1 anatomical scans using the

drawing tool in itkSNAP (http://www.itksnap.org). The masks were then used to remove non-brain signal from each scan (including the pituitary, which showed highest signal intensity that could skew the distribution of voxel signal intensities on histograms). The resulting cropped images were aligned with a rat brain template (Ekamsolutions, LLC, Holden, MA) using the FMRIB Software Library's automated linear registration tool flirt as previously described (Perez et al. 2017). Cropped T_1 scans were converted to z score maps through a voxel-wise signal-to-noise processing step. Both the global mean signal intensity (representing intensity of signal, SI) and volume (i.e. #voxels) of activation (representing the area of activation of each region) were evaluated for each region of interest (ROI). Forty-four brain ROIs were evaluated, including the following: supraoptic nucleus (SON), pontine nuclei, pontine reticular nucleus caudal, pontine reticular nucleus oral, locus coeruleus, reticular nucleus midbrain, central gray, ventral tegmental area, lateral amygdala, habenula nucleus, hippocampus, substantia nigra reticularis, lateral hypothalamus, dorsal raphe, parabrachial nucleus, substantia nigra compacta, and interpeduncular nucleus, among others.

Statistical analysis

All data were analyzed using Prism 6.0 software (GraphPad Software Inc.). Group means are expressed as mean ± SEM. A Student's t-test was used to identify differences between strains for behavioral measures on the EPM, cardiovascular AUC data, and ROIs from MEMRI. Cardiovascular changes over time were analyzed with a repeated measures ANOVA. When a significant interaction was indicated, differences between treatment groups were identified by post hoc analysis using a Holm-Sidak's multiple comparisons test. $P < 0.05$ was considered statistically significant.

Results

EPM testing and cardiovascular/autonomic responses

As shown in Fig. 1, analysis of behavior during the 5 min of EPM testing identified several significant differences between the SHRs and WKYs (n = 10/group). In general, SHRs were more active, and had a greater number of mobile episodes, (SHR: 24 ± 2 vs WKY:15 ± 2, $P < 0.004$), had more line crossings (SHR: 42 ± 4 vs WKY:28 ± 4, $P < 0.02$ and generally traveled a greater distance (SHR: 5 ± 0.7 vs WKY:4 ± 07, $P < 0.18$), although this did not reach significance. Additionally, SHRs spent less time in the closed arm (SHR:31 ± 5% vs WKY:54 ± 8%, percent total time; $P = 0.02$) and more total time in the open arm and center of the EPM (SHR:69 ± 5% vs WKY:46 ± 8%;

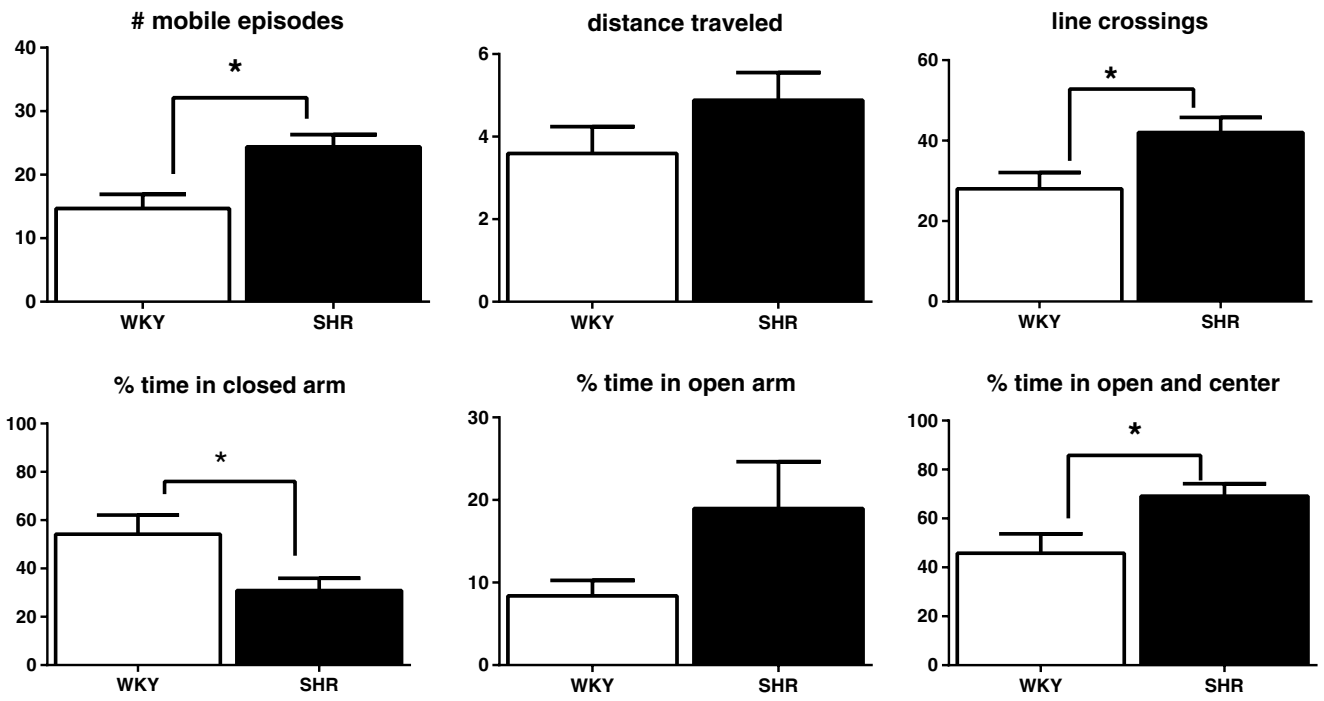

Fig. 1 Comparison of the average behaviors on elevated plus maze (EPM) between WKYs and SHRs (n = 10/group). Mean+/-SEM. * indicates significant difference between strains, $P < 0.05$

percent total time; P = 0.02). Additional parameters quantified included percent time in open arms (SHR:19 ± 7% vs WKY:8.5 ± 2%, percent total time; P = 0.09) and percent time in center (SHR:50 ± 5% vs WKY:37 ± 7%, percent total time; P = 0.12).

Evaluation of the cardiovascular response before and during EPM testing is shown in Fig. 2 (n = 7/group). Baseline MAP (SHR:148 ± 5 vs WKY: 102 ± 4, mmHg, P < 0.001) and HR (SHR:349 ± 18 vs WKY:304 ± 11, bpm, P < 0.05) were significantly higher in the SHR compared to WKY. In both strains placement on the EPM from their home cage induced a significant rise in MAP and HR (see Fig. 2a, shaded region). After 5 min on the EPM, when the animals were returned their home cage, MAP and HR began to decline. When the entire testing period was considered (before, during and after EPM testing) there was a significant difference between strains for MAP (P < 0.001) but not HR (P > 0.68). When the absolute ΔHR from baseline was calculated (see Fig. 2b), no significant effect of strain was identified (P > 0.4) and the interaction between time and strain was not significant (P > 0.1). Alternatively, when the ΔMAP was evaluated a significant effect of strain was identified (P = 0.03; see Fig. 2b) and there was a significant interaction between strain and time (P < 0.008). Further analysis identified that ΔMAP was significantly different between strains at minutes 2 and 3 during EPM and during the first minute after being taken off the EPM. When the ΔHR and ΔMAP during EPM were quantified as the AUC, the ΔMAP-AUC was significantly smaller in the SHRs compared to the WKY (SHR:59 ± 12 vs WKY: 108 ± 13, au, P < 0.05; see Fig. 2c) but the ΔHR-AUC was not significantly different between strains (SHR:287 ± 47 vs WKY: 445 ± 80, au, P = 0.13).

MEMRI analysis

The manganese-enhanced baseline mean intensity (corresponding to activity level) and number of activated voxels

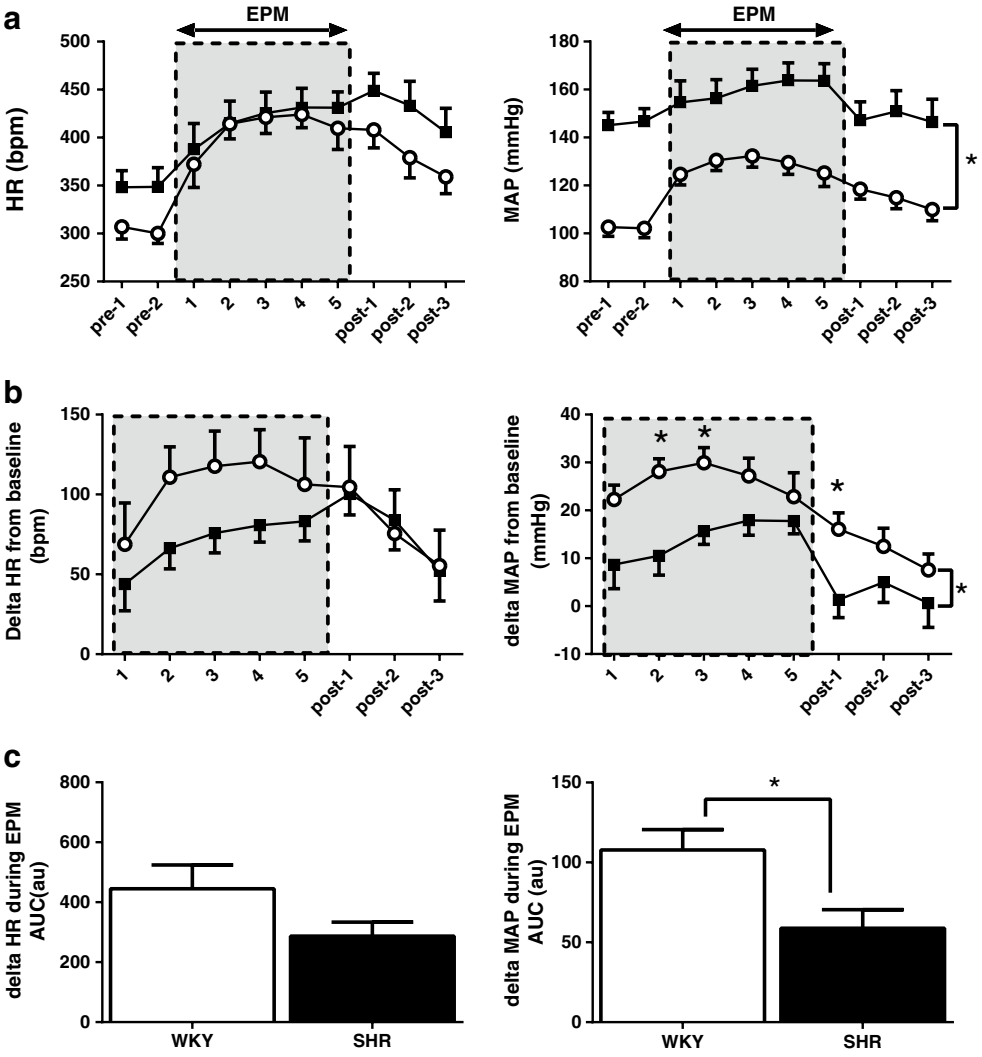

Fig. 2 Cardiovascular changes from baseline during and immediately following placement on the elevated plus maze (EPM) between SHRs and WKYs (n = 7/group). **a** Absolute heart rate (HR) and mean arterial pressure (MAP) values. **b** Change (delta) in HR and MAP from baseline. **c** Average change in HR and MAP during EPM testing alone measured as area under the curve (AUC, arbitrary units, au). Mean+/-SEM. * indicates significant difference between strains, P < 0.05

(corresponding to area of activation) from 44 brain ROIs were evaluated in adult WKY (n = 11) versus SHR (n = 13) rats. Figure 3 illustrates baseline signal intensity (SI, in arbitrary units, a.u.) in a range of ROIs as measured by MEMRI in WKY and SHR. Composite maps of WKY and SHR were processed for MEMRI signal intensity and overlaid on a segmented and annotated atlas of the rat brain as per Paxinos and Watson (2013). Figure 4a illustrates several forebrain ROIs in which mean manganese-enhanced SI was higher in the WKY compared to the SHR, including regions of the hippocampus, habenula, and several subnuclei of the amygdala. Figure 4b shows values from select regions in which the mean intensity (left) and/or the mean number of voxels (right) were significantly higher in the WKY compared to the SHR, suggesting that these regions may be involved in anxiety and/or autonomic responsiveness during anxiety provoking behavior. Regions that showed a significant increase in both intensity and number of voxels in the WKY compared to the SHR included: (i) lateral amygdaloid nucleus (1.1 ± 0.05 vs 0.3 ± 0.1 AU, P < 0.001; 295 ± 51 vs 28 ± 18, P < 0.001, intensity and voxels, respectively); (ii) CA2 (1.1 ± 0.04 vs 0.6 ± 0.1 AU, P < 0.05; 404 ± 61 vs 61 ± 33, P < 0.001); (iii) habenula (1.2 ± 0.04 vs 1.06 ± 0.04 AU, P < 0.01; 183 ± 24 vs 96 ± 26, P < 0.05); (iv) central gray (0.98 ± 0.1 vs 0.55 ± 0.2 AU, P < 0.05;389 ± 79 vs 148 ± 74, P < 0.05); and (v) the interpeduncular nucleus (1.42 ± 0.06 vs 1.1 ± 0.06 AU, P < 0.01; 230 ± 15 vs 142 ± 18, P < 0.001).

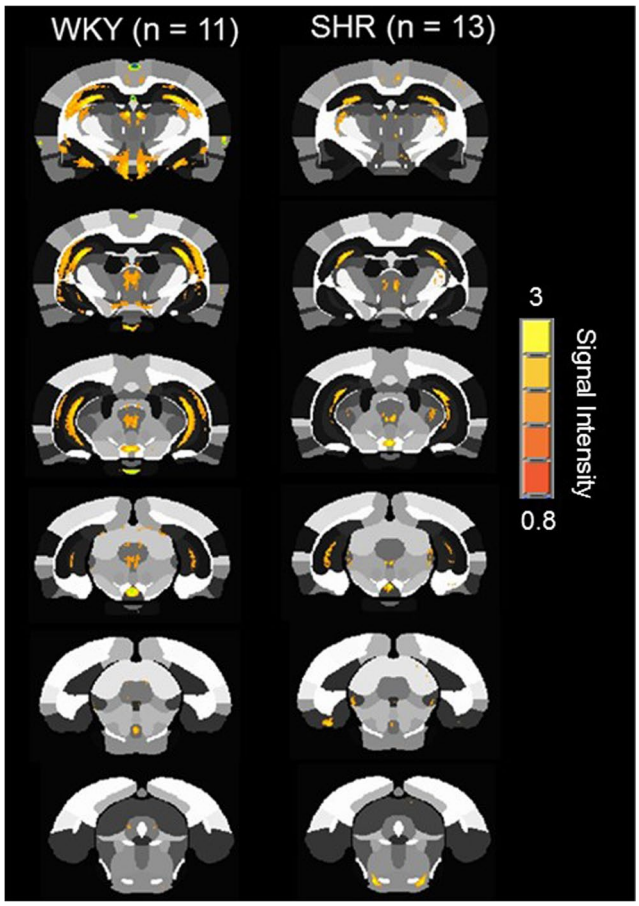

Fig. 3 Baseline MEMRI signal in the brain of WKY and SHR. Composite maps of WKY and SHR were processed for MEMRI signal intensity and overlaid on a segmented and annotated atlas of the rat brain. Images are thresholded at z > 0.9. Panel on the lower right shows color scale for signal intensity (SI) values (in a.u) above threshold

Figure 5a shows several ROIs in which mean manganese-enhanced SI was higher in the SHR compared to the WKY, including regions in the hypothalamus, locus coeruleus and several olivary nuclei. Figure 5b shows the averages from select regions in which the mean intensity (left) and/or the average number of voxels (right) was significantly higher in the SHR compared to the WKY, suggesting that these regions may be involved in hypertension and/or increased mobility during anxiety provoking behavior. Regions that were identified to show a significant increase in both intensity and number of voxels in the SHR vs WKY included (i) pontine nuclei (1.1 ± 0.02 vs 0.99 ± 0.02 AU, P < 0.05; 430 ± 63, vs 161 ± 32, P < 0.01; intensity and voxels, respectively); and (ii) pontine reticular nucleus caudal (1.1 ± 0.01 vs 0.99 ± 0.01 AU, P < 0.05; 403 ± 60 vs 112 ± 9, P < 0.001). Supplemental Table 1 summarizes results other relevant ROIs investigated in WKY and SHR.

Discussion

The present study was undertaken with two aims. The first aim was to monitor MAP and HR during EPM testing in two stains of rats with known differences in anxiety and underlying cardiovascular disease. Based on previous research demonstrating that different forms of stressful stimuli elicit exaggerated autonomic responses in the SHR (Grundt et al. 2009; Martin et al. 2016; McDougall et al. 2000, 2005), we hypothesized that the rise in MAP and HR would be greatest in the SHR during EPM testing, despite behavioral indicators of low anxiety. Contrary to our hypothesis however, the autonomic response during EPM testing was generally greater in the WKY compared to the SHR. This suggests that for this form of stress, autonomic reactivity follows the behavioral response and is not altered by underlying cardiovascular disease. The second aim was to evaluate baseline activity in different brain regions, using MEMRI, to determine whether elevated or suppressed activity might be linked to differences in anxiety and autonomic reactivity. Accordingly, in the more anxious WKY, indicators of elevated activity were identified in several regions commonly associated with both anxiety and autonomic control during stress, including the amygdala, central gray, habenula and interpeduncular nucleus (Duncan et al. 1996; Salomé

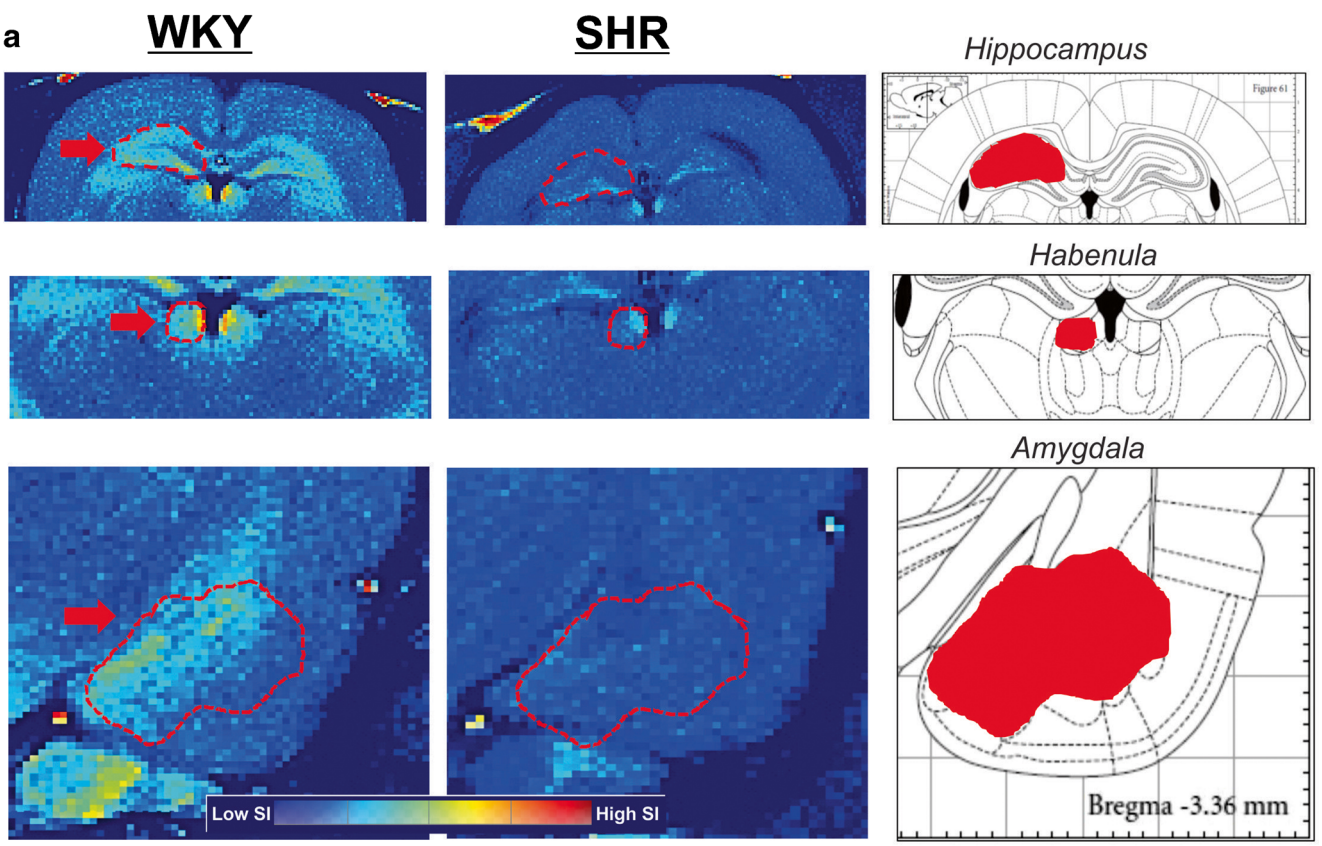

Fig. 4 **a** Comparison of manganese-enhanced signal intensity (SI, in a.u.) in WKY and SHR hippocampus (top panel), habenula (middle panel) and several amygdaloid nuclei (bottom panel). Red arrow and dotted red line approximate the appropriate regions of interest (ROIs), as per coordinates in far left panel. Far left panel illustrates respective ROIs as per Paxinos and Watson brain atlas. **b** Summary of mean intensity (left panel) and #voxels (right panel) in several ROIs in WKY vs SHR. (Mean+/-SEM; n = 11 vs 13, respectively). * indicates significant difference between strains, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

et al. 2004; Silveira et al. 1993). Conversely, indicators of heightened brain activity in the SHR were observed in the anterior hypothalamus, supraoptic nucleus (SON), locus coeruleus and pedunculopontine tegmental nucleus, regions associated with system arousal, cardiovascular control and motor activity (Padley et al. 2007; Salomé et al. 2004). Heightened activity in these regions is in agreement with the increases in mobile episodes and baseline high blood

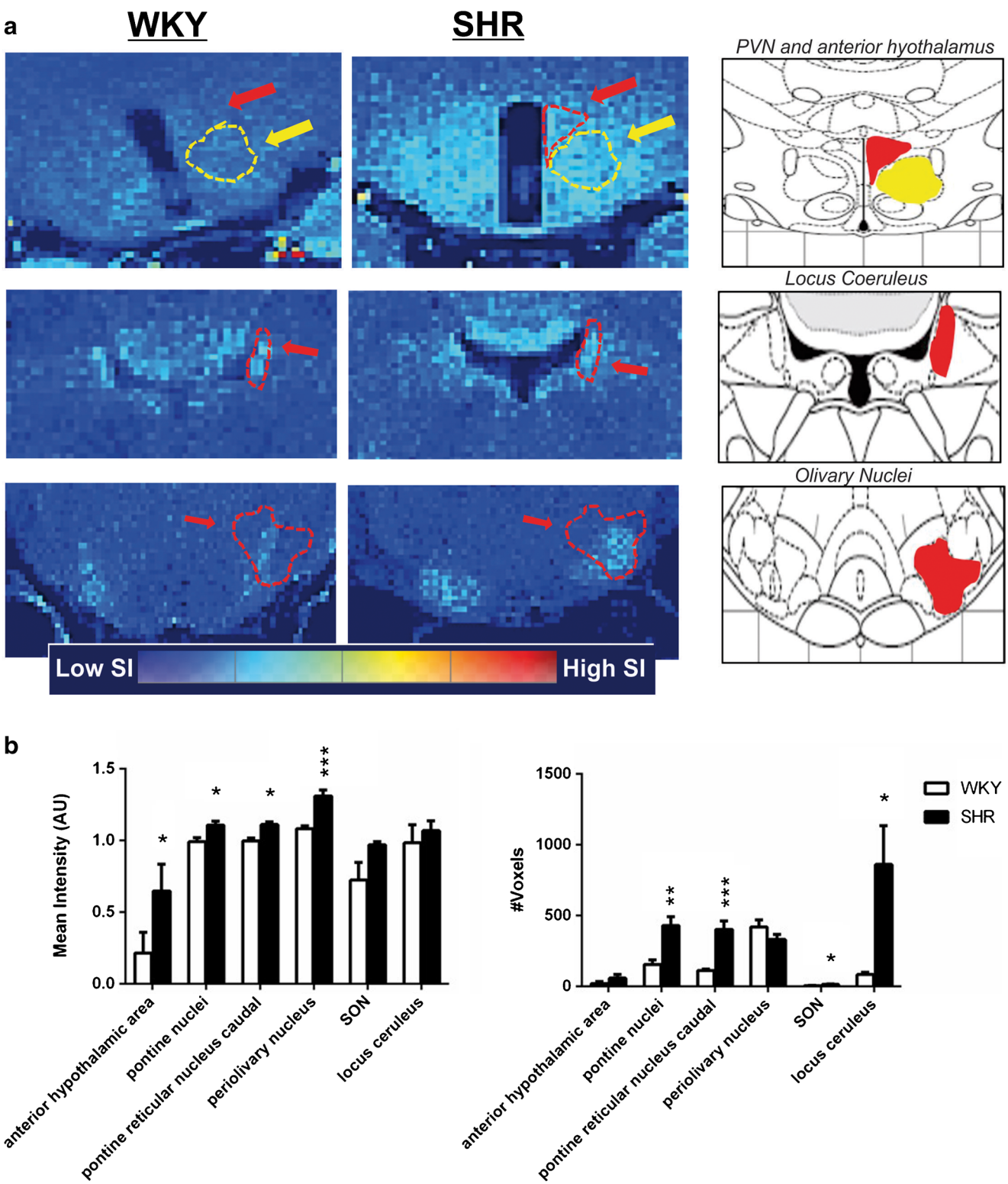

Fig. 5 **a** Comparison of manganese-enhanced signal intensity (SI, in a.u.) in WKY and SHR PVN (red dotted line and arrow) and anterior hypothalamus (yellow dotted line and arrow, top panel), locus coeruleus (red dotted line and arrow, middle panel) and several olivary nuclei (red dotted line and arrow, bottom panel). Red arrow and dotted red line approximate the appropriate regions of interest (ROIs), as per coordinates in far left panel. Far left panel illustrates respective ROIs as per Paxinos and Watson brain atlas. **b** Summary of mean intensity (left panel) and #voxels (right panel) in several ROIs in WKY vs SHR (Mean+/-SEM, n = 11 vs 13, respectively). * indicates significant difference between strains, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

pressure in the SHR compared to the WKY. Finally, MEMRI also uncovered an overall decrease in hippocampal activity in the SHR vs WKY, suggesting differences in memory or cognitive function (Meneses et al. 2011). Thus, coupling MEMRI with behavioral and autonomic monitoring provides a new perspective on the role of different brain regions in systems physiology.

Over the last 20 years, numerous studies have identified that individuals with certain forms of chronic mental stress, like post-traumatic stress disorder, anxiety and/or depression, have an increased incidence of cardiovascular disease (Bacon et al. 2014; Burg and Soufer 2016; Debert et al. 2010; Ginty et al. 2013; Meng et al. 2012) and exaggerated autonomic responses to acute stressful stimuli (Beckham et al. 2002). Alternatively, individuals with cardiovascular disease often have high depression and anxiety scores (Davies et al. 1997; Sunbul et al. 2014) as well as heightened cardiovascular responses to stress (Garafova et al. 2014). In recent years however, there have been several studies demonstrating that sustained symptoms of anxiety may actually be associated with lower blood pressure (Bhat et al. 2017; Tikhonoff et al. 2014). Accordingly, the WKY is a well-established model of heightened anxiety when compared to other rat strains, including the SHR (Nam et al. 2014; Pollier et al. 2000; Ramos et al. 1997), and is normotensive, with a low resting HR (Hayward et al. 2012). In the present study, we demonstrated for the first time that parallel to behavioral indicators of heightened anxiety, the autonomic response during EPM testing is also greater in the WKY when compared to SHRs. This is in contrast to previous studies demonstrating that WKYs have reduced autonomic reactivity to acute stressors, like air jet stress or restraint stress, when compared to the SHR, (McDougall et al. 1985, 2000). Since these are primarily physical forms of stress, our findings raise the possibility that normotensive individuals with elevated anxiety may be more reactive to psycho-social or interoceptive stressors, while less anxious individuals with cardiovascular disease may be more sensitive to physical or exteroceptive stressors (Furlong and Carrive 2007; Schaap et al. 2013). Thus, classifying stressor type may be important for predicting cardiovascular disease risk. Importantly, different regions of the brain have been identified to be involved in selectively eliciting autonomic responses to physical/exteroceptive vs psycho-social/interoceptive stress (Furlong and Carrive 2007; Iwata et al. 1986; McDougall et al. 2004). Thus, there may be differences governing response characteristics in different disease states.

Next, we utilized MEMRI to identify regional differences in baseline brain activity that could be linked to differences in behavioral or autonomic reactivity between the WKY and SHR. MEMRI is a powerful technique based on passive diffusion of $MnCl_2$ into active cells during the period of twenty-four hours (Lee et al. 2005), thus allowing determination of baseline regional activity in the brain without the confounding effects of anesthesia present during fMRI, which is of particular importance in measurements of cardiovascular and behavioral variables. Furthermore, the use of MEMRI avoids the potential confounds introduced by cardiovascular disease models in alterations in the neurovascular coupling which fMRI is strongly dependent on. To our knowledge, this is only one of three studies to-date to apply this methodology to these two rat strains (Zubcevic et al. 2014a, 2017).

One striking difference in baseline activity identified by MEMRI was a significant increase in the amygdala of the WKY vs SHR. The amygdala is one of the primary brain regions for chronic stress processing (Anand and Shekhar 2003; Roozendaal et al. 2009) and higher levels of anxiety in human subjects have been linked to heighted activity in the amygdala (Pfleiderer et al. 2014). The amygdala is regulated by an extensive GABAergic network both at rest (Shekhar et al. 2003) and during chronic stress (Liu et al. 2014). Thus, the elevated resting activity in amygdala may be linked to inherent differences in GABAergic regulation, which could contribute to a heightened behavioral and autonomic anxiety in the WKY on the EPM compared to SHR (Ball et al. 2017; O'Mahony et al. 2011). In fact, there is evidence that GABAergic signaling in the WKY is perturbed compared to both the SHR (Kunkler and Hwang 1995) and the normotensive, but less anxious Wistar strain (Lei et al. 2009).

One possible source of altered GABAergic inhibition in the amygdala of the WKY may be inputs from the SON and paraventricular nucleus of the hypothalamus (PVN). For example, both oxytocin and vasopressin are produced in the SON/PVN which have projections to the amygdala (Hernández et al. 2016). Oxytocin and vasopressin are involved in modulating anxiety-like behaviors, but in opposing ways, with vasopressin increasing anxiety and oxytocin decreasing anxiety (Granjeiro et al. 2014; Hernández et al. 2016; Radke et al. 2017; Zhang et al. 2016). The opposing actions of these two neuropeptides on behavior are hypothesized to be mediated in-part by activation of different components of the inhibitory networks within the amygdala (Huber et al. 2005). In the present study, parallel to heightened activity in the amygdala of the more anxious WKY, we documented decreased activity in the SON/PVN when compared to the SHR. In accordance with the known actions of these two peptides, this could reflect decreased oxytocinergic input to the amygdala, an associated reduction in inhibitory tone in the amygdala, and an elevation in behavioral anxiety in the WKY when compared to the SHR. Alternatively, consistent with increased vasopressinergic activity in hypertension (Pietranera et al. 2004; Yi et al. 2012), elevated activity in the SON/PVN in the SHR may also reflect increased vasopressinergic inputs to other regions of the brain involved in autonomic control (Bowen et al. 2014), contributing to elevated blood pressure in the SHR.

Circuits originating in the amygdala may also be involved in eliciting the exaggerated autonomic we identified in the WKY during EPM testing. The amygdala is known to send efferent projections to the baroresponsive neurons in the nucleus of the solitary tract (NTS) and rostral ventrolateral medulla (RVLM), two regions involved in autonomic control (Saha 2005). These amygdaloidal efferent projections are suggested to play a vital role in the reflex changes in sympathetic nerve activity involved in AP regulation during stress/anxiety responses (Saha et al. 2005; Wallace et al. 1992). Projections to the NTS are thought to be mainly GABAergic (Saha 2005), thus potentially contributing to dampening of the baroreflex during stress (Moreira et al. 2011; Paton et al. 2008). In addition, RVLM-projecting amygdaloidal neurons are reportedly activated in response to decreased AP (Saha et al. 2005), suggesting role in control of sympathetic drive.

Another potentially interesting and novel finding of the present study was identification of heightened baseline activity and volume of activation of in the habenula, interpeduncular and ventral tegmental nuclei in the WKY vs SHR, as observed by MEMRI. The habenula is thought to be involved in pain processing, feeding, reward, stress-responses, memory/learning and regulation of circadian cycle (Boulos et al. 2017; Molas et al. 2017). Dysregulation in this region has been associated with a range of neurologic and psychiatric disorders related to some of these functions (Fakhoury 2017). The role of habenula in AP control is unknown. However, the medial habenula reportedly receives dopaminergic inputs from the ventral tegmental area and noradrenergic inputs from the locus coeruleus, and projects to central gray via the interpeduncular cholinergic projections (Molas et al. 2017). The lateral habenula has been shown to receive inputs from the hippocampus, thalamus and hypothalamus, while itself projecting to ventral tegmental area, dorsal raphe and substantia nigra (Lecourtier and Kelly 2007; Molas et al. 2017). Interestingly, we observed neuronal activity differences in all these regions in the WKY vs the SHR. Dorsal raphe has been implicated in control of AP, as increased GABAergic activity in dorsal raphe reportedly reduces HR and AP (Robinson et al. 1986), while stimulation of dorsal raphe increased AP largely in the SHR (Wolf et al. 1981). The ventral tegmental region projects to amygdala, among other brain regions, and its activation reportedly modulates behavior, including locomotor activity, rewards mechanisms, cognition and stress responses (Holly and Miczek 2016; Morales and Margolis 2017), but also the associated cardiovascular responses via vasopressinergic regulation of AP (van Den Buuse and Catanzariti 2000). In the SHR, dysregulation in several neurotransmitter signaling systems and receptors in the ventral tegmental region has been linked to hypertension (De Brito Gariepy and Couture 2010) and may play an important role in integration of stress and environmental stimuli as it relates to cardiovascular control (van den Buuse 1997).

In the present study, regions of the hippocampus were also identified to have reduced activity and volume of activation in the SHR vs WKY. Indicators of dysregulation in hippocampus of the SHR have previously been reported (Meneses et al. 2011; Sabbatini et al. 2002). It has been suggested that hypertension causes small vessel damage, leading to neuronal damage in several regions including the hippocampus (Yang et al. 2016), and a recent fMRI study reported major differences in hippocampal functional connectivity in the SHR compared to the normotensive rats (Huang et al. 2016). Others have suggested that increased inflammatory profile and dysfunctional interstitial fluid drainage in the hippocampus of SHR may be associated with cognitive decline, vascular dementia, blood brain barrier damage and possibly even beta amyloid accumulation, similar to that seen in Alzheimer's disease (Tayebati et al. 2016). Conversely, improvement in hippocampal dopaminergic signaling by electrical transcranial stimulation positively affected learning and memory, and reduced hyperactivity in the SHR, a recognized model of ADHD (Leffa et al. 2016; Meneses et al. 2011).

Alternatively, one highly activated region in the SHR compared to the WKY, as measured by MEMRI, which is involved in regulation of arousal and autonomic function, is the locus coeruleus (LC). Increase in LC activity is shown to result in increased alertness (Zitnik 2016), and increased sympathetic and reduced parasympathetic activity (Berecek et al. 1987a, b; Mather et al. 2017). Indeed, increased LC activity, as measured by MRI, has been reported to be negatively associated with indicators of parasympathetic drive (Mather et al. 2017). Alternatively, LC-dependent secretion of norepinephrine in the hypothalamus has been linked to hypertension (Gong et al. 2015; Kawasaki et al. 1991). Thus, increased LC activity, as measured by MEMRI in our present study, may contribute to increase in AP in the SHR.

Finally, it is acknowledged that there are several limitations of the current study. First, we only used one test to measure anxiety in the WKYs. The WKY is a well-established model of heightened anxiety and other investigators have confirmed this using multiple behavioral anxiety measures, including EPM, open field testing and social anxiety tests (Nam et al. 2014; Pollier et al. 2000; Ramos et al. 1997). In this study, we chose the EPM testing paradigm because it provided an environment in which we could also evaluate the cardiovascular response for the first time, while also confirming that our population of WKYs were more anxious than the SHR population. Second, we did not measure performance on the EPM in the same animals that underwent MEMRI testing. It is possible that such an approach would have provided a more refined definition of the role of specific brain regions in varying levels of anxiety. Such an

approach, however, would still only be correlational, like the current study. Additional studies are needed to specifically define the role some of the novel brain regions identified in the current study in anxiety. Third, it is acknowledged that as a smooth muscle calcium channel blocker, systemic delivery of $MnCl_2$ can reduce blood pressure. However, changes in blood pressure have been shown to be very transient (Wolf and Baum 1983) and in the present study it would be anticipated that both groups of animals would have been exposed to similar drops in AP as they were given the same dose of $MnCl_2$. Furthermore, the optimal time for the passive diffusion of $MnCl_2$ to deep structures in the brain is over several hours (Lee et al. 2005). Thus, it is unlikely that the regional differences in brain activity reported here were related to the transient AP changes.

In summary, we provide a possible functional link between interoceptive stress and autonomic reactivity in the WKY. The WKY exhibits higher anxiety scores and we have now identified for the first time that this is also linked to higher autonomic reactivity on the EPM compared to the SHR. This suggests that heightened autonomic reactivity during a stressful stimulus is a true indicator of the level of perceived stress. Furthermore, our study correlates the neuronal activity of brain regions associated with stress, anxiety, memory and locomotion with behavioral traits in the WKY and SHR. Thus, MEMRI provides a powerful functional and potentially predictive biomarker of disease, and may be a way of uncovering novel mechanistic links between anxiety and activity in the autonomic nervous system. Future studies should employ functional MRI in combination with optogenetic and/or chemogenetic (DREADDs) tools to evaluate specific neuronal pathways associating sympathetic drive and behavioral anxiety in the WKY.

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Compliance with ethical standards

Conflict of interest Author J Zubcevic declares that she has no conflict of interest.
Author J Watkins declares that she has no conflict of interest.
Author P Perez declares that he has no conflict of interest.
Author L Colon-Perez declares that he has no conflict of interest.
Author M Long declares that she has no conflict of interest.
Author M Febo declares that he has no conflict of interest.
Author L Hayward declares that she has no conflict of interest.

Ethical approval As stated at the beginning of the Methods section, all studies involving the use of animals were approved by the University Institutional Animal Care and Use Committee and all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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