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CARDIOVASCULAR REACTIVITY AND NEURONAL ACTIVATION TO STRESS IN SCHLAGER GENETICALLY HYPERTENSIVE MICE

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Abstract—Schlager inbred hypertensive mice (BPH/2J) have been suggested to have high blood pressure (BP) due to an overactive sympathetic nervous system (SNS). The brain nuclei associated with the hypertension are also those involved in the integration of the cardiovascular responses to stress. Therefore, in the present study, we hypothesize that BPH/2J mice likely have a greater response to stress that is associated with greater neuronal activation in the limbic system, hypothalamus and medulla in regions known to regulate sympathetic activity. Male hypertensive BPH/2J and normotensive BPN/3J mice were implanted with telemetry devices and exposed to dirty cage-switch, an acute model of aversive stress. Stress exposure caused a 60% greater pressor response in BPH/2J compared with BPN/3J mice and an increase in activity, by contrast the level of tachycardia was less in BPH/2J mice. Stress-induced cardiovascular responses were also associated with greater neuronal activation, as detected by c-Fos expression, in BPH/2J compared with BPN/3J mice in the medial nucleus of the amygdala (MeAm), dorsomedial hypothalamus (DMH) ($P<0.001$) and marginally in the rostral ventrolateral medulla (RVLM; $P=0.7$). These findings suggest that hypertension in the BPH/2J mice is associated with greater sympathetic vasomotor responses to central pathways mediating the arousal responses to acute aversive stress in particular the amygdala, hypothalamus and rostral ventrolateral medulla.

Key words: brain, aversive stress, sympathetic nervous system, blood pressure, heart rate, c-Fos-immunohistochemistry.

The sympathetic nervous system (SNS) is now recognized to participate in the long-term regulation of blood pressure (BP) and contribute to supporting BP during hypertension. Importantly, the SNS is a major contributor to the circadian pattern in BP and heart rate (HR) in humans (Panza et al., 1991; Somers et al., 1993) and young borderline hypertensives are known to have an overactive SNS (Esler, 1995), elevated renal noradrenaline spillover (Esler, 1995) and high release rates of subcortical noradrenaline possibly from the hypothalamus (Ferrier et al., 1993). Furthermore, morning surge in BP is markedly greater in hypertensive patients and "white coat" hypertensives have an even greater morning BP surge moving from a normal level of BP during the night to an intermediate phenotype during the day (Head and Lukoshkova, 2008; Head et al., in press). It has been suggested that these responses are likely mediated by central SNS hyper-responsivity (Smith et al., 2002). Recent developments in treating resistant hypertension have involved inhibiting the SNS (Krum et al., 2009; Wustmann et al., 2009). Accordingly, the SNS may therefore be important in pathological states such as hypertension, heart failure, diabetes and obesity (Prior et al., 2010) not only for its influence on basal BP, but also for its role in the increased risk of arrhythmias, sudden cardiac death and overall mortality (Farrell et al., 1991; Lohmeier, 2001). In view of the important role of sympathetic activation in stress responses (Esler, 1998; Head and Burke, 2004) and human hypertension (Panza et al., 1991; Somers et al., 1993), it is critical to investigate the central mechanisms that contribute to the overactivity in an appropriate animal model.

The Schlager inbred hypertensive (BPH/2J) mouse strain has recently been recognized as a neurogenic form of hypertension which may serve as a useful model to understand the mechanisms by which an overactive SNS leads to hypertension (Davern et al., 2009b). Early studies with these mice found that they exhibited a reduction in noradrenaline content in the hypothalamus, amygdala and cerebellum (Schlager et al., 1983). Denoroy and colleagues identified greater noradrenaline content in the preoptic area; but reduced levels in the paraventricular nucleus of the hypothalamus (PVN; Denoroy et al., 1985). BPH/2J mice have a 41% greater noradrenaline content in the superior cervical ganglion, but normal levels of catecholamines and metabolites in the adrenal medulla compared with random-bred mice (Denoroy et al., 1985) consistent with a neurogenic form of hypertension. Recently, using radiotelemetry, we demonstrated that BPH/2J mice display exaggerated cardiovascular reactivity associated with circadian patterns compared with normotensive BPN/3J mice across 24 h (Davern et al., 2009b). Importantly, BPH/2J mice have markedly greater neuronal activation associated with these circadian patterns in the hypothalamus in regions critical for cardiovascular autonomic regulation and in the amygdala suggesting that stress related pathways may also be overactive (Davern et al., 2009b). More-

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Abbreviations: ANOVA, analysis of variance; AP, area postrema; BP, blood pressure; BPH/2J, Schlager hypertensive mice; BPN/3J, Schlager normotensive mice; BST, bed nucleus of the stria terminalis; CeAm, central nucleus of the amygdala; CVLM, caudal ventrolateral medulla; DMH, dorsomedial hypothalamus; HR, heart rate; MAP, mean arterial pressure; MeAm, medial nucleus of the amygdala; MnPO, median preoptic nucleus; NTS, nucleus of the solitary tract; OVLT, vascular organ of the lamina terminalis; PVN, paraventricular nucleus of the hypothalamus; RPa, raphe nucleus; RVLM, rostral ventrolateral medulla; SFO, subfornical organ; SNS, sympathetic nervous system; SON, supraoptic nucleus.

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over, our data indicate a contribution by the SNS in maintaining BP in these mice as the ganglionic blocker, pentolinium completely abolished the BP difference between BPH/2J and normotensive BPN/3J mice (Davern et al., 2009b).

Given that the areas of the CNS which are associated with the magnification of the hypertension during the active period are also the same regions involved in the integration of stress and arousal, we hypothesise that BPH/2J mice are likely to have a greater response to stress associated with greater neuronal responsiveness in specific regions of the limbic, hypothalamic and medullary regions that are known to regulate the SNS. Therefore, in the present study, we investigated cardiovascular reactivity in BPN/3J and BPH/2J mice before and during exposure to cage-switch, a model of acute aversive stress known to produce a robust hypertension (Lee et al., 2004; Davern et al., 2009a) and utilized c-Fos-immunohistochemistry to detect neuronal activation.

EXPERIMENTAL PROCEDURES

Animals

Experiments were carried out in 18±1 weeks old conscious male normotensive (BPN/3J; $n=20$) and hypertensive (BPH/2J, $n=16$) Schlager mice bred at the Baker IDI Heart and Diabetes Institute. Animals were kept on a 12:12-h light/dark cycle (6 AM-6 PM light). All mice were allowed access *ad libitum* to water and mouse chow (Specialty Feeds, Glen Forrest, Western Australia, 19% protein, 5% fat, 5% fibre, 0.2% sodium). The experiments were previously approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals.

Measurement of blood pressure, heart rate and locomotor activity in freely moving mice

Mice were anaesthetised and implanted with radiotelemetry transmitters (BPN/3J $n=13$ and BPH/2J $n=9$) with the catheter inserted into the carotid artery and transmitter body implanted along the right flank. Following a 10-day recovery period, mice were housed individually for the remaining duration of the study. During the recording session, measured continuously at rest and throughout the stress protocol, pulsatile arterial pressure and gross locomotor activity were monitored and sampled at 1000 Hz using an analog-to-digital data acquisition card and the beat-to-beat mean arterial pressure (MAP) and HR were detected on-line and analyzed as described previously (Jackson et al., 2007). Dirty cage-switch stress was conducted during the inactive period (day time) over a 60 min period by removing mice from their original cage and randomly placing them into a cage previously occupied by a different male mouse. A further analysis was performed to calculate MAP and HR during the stress when the activity signal was below the "no-movement" threshold for ≥6 s using a method described earlier (Davern et al., 2009a).

c-Fos immunohistochemical analysis of cage-switch test

Immediately following 60 min of dirty cage-switch stress, five mice from each group were perfused. Control animals also included five mice from each group, but by contrast these mice were not exposed to stress, and perfusions were time matched with the experimental group. All brains were processed for c-Fos-immunohistochemistry using a previously described method from our laboratory (Davern et al., 2009b).

Briefly, coronal sections (40 μm) were incubated in 10% normal horse serum at room temperature for 60 min. Sections were incubated in primary antibody, sheep anti-c-Fos (Chemicon, Temecula, CA, USA) diluted 1:2000 in 2% normal horse serum and 0.3% Triton X-100 (Sigma, Castle Hill, NSW, Australia) in PB at room temperature overnight. Sections were washed in PB prior to incubation in biotinylated donkey anti-sheep immunoglobins (1:200, Jackson, West Grove, PA, USA) in PB containing 2% normal horse serum for 60 min. Thereafter, the sections were washed and incubated in avidin-biotin peroxidase complex (1:100, Vector, Burlingame, CA, USA) in PB for 60 min. Following washes in 0.05 M Tris buffer (pH 7.6), sections were incubated in a solution of 40 mg nickel ammonium sulfate and 50 mg 3–3′ diaminobenzidine hydrochloride per 100 ml Tris buffer for 10 min, 15 μl of 30% hydrogen peroxide was added for further 6 min. Following final washes sections were mounted on gelatin coated microscope slides.

Bright-field illumination using a Motic BA400 microscope and Motic images plus 2.0 were used to assess sections that exhibited c-Fos-immunoreactivity identified by black stained nuclei. The Mouse Brain Atlas (Paxinos and Franklin, 2001) was used to identify comparable sections through each brain region in a blind analysis of up to four sections per animal for each brain site in BPN/3J ($n=5$) and BPH/3J ($n=5$) mice. Counts from the vascular organ of the lamina terminalis (OVLT; bregma: +0.65 to +0.5 mm) only included sections with both lateral aspects and the dorsal cap located immediately dorsal to the optic chiasm (0.06 mm^2). The median preoptic nucleus (MnPO; bregma: +0.25 to +0.05 mm) included combined counts of both the ventral and dorsal aspects at the level where the anterior commissure traverses the midline (0.13 mm^2). These same sections were used for the bed nucleus of the stria terminalis (BST) and counts were made from the area medial to the internal capsule between the lateral ventricle and anterior commissure (0.15 mm^2). Counts for the subfornical organ (SFO) were from sections close to the rostral/caudal midpoint (bregma: −0.6 mm; 0.06 mm^2). The PVN (bregma: −0.8 to −0.95 mm) included combined counts from the parvocellular, magnocellular and ventral regions and the dorsal cap (0.12 mm^2). Lateral to the optic tract, the supraoptic nucleus (SON) was counted from sections at the level of rostral PVN (bregma: −0.7 to −0.95 mm; 0.08 mm^2). The central nucleus of the amygdala (CeAm; bregma: −0.8 to −1.1 mm) included the lateral division, medial division and capsular region and counts were combined for the entire circular-shaped region between the external capsule and globus pallidus (0.56 mm^2). The medial nucleus of the amygdala (MeAm; bregma: −1.45 to −1.7 mm) was defined by the optic tract (medial) and its most ventral point was used to identify the dorsal aspect of the MeAm and counts were made medial of this apex and from a vertical point at the base of the brain (0.63 mm^2). The medial aspect of the dorsomedial hypothalamus (DMH; bregma: −1.85 to −1.95 mm) was defined by the 3rd ventricle, the mammillothalamic tract was used to identify the lateral/dorsal point, the fornix identified the lateral/ventral point and counts are made between these borders (0.33 mm^2). The raphe nucleus (RPa; bregma: −5.5 to −6.5 mm) was readily identifiable by its location between the pyramidal tracts at the level of the 4th ventricle in the hindbrain (0.06 mm^2). The rostral ventrolateral medulla (RVLM; bregma: −6.5 to −6.7 mm) was counted from sections ventral to the rostral aspect of the ambiguus nucleus at the level of the 4th ventricle and immediately rostral to the caudal ventrolateral medulla (CVLM; 0.10 mm^2). The CVLM (bregma: −7.5 to −7.7 mm) also included counts ventral to the ambiguus nucleus but at the level of the area postrema (AP; 0.08 mm^2). Counts of the nucleus of the solitary tract (NTS) (bregma: −7.5 to −7.7 mm) included combined numbers from subnuclei that traversed the midline (0.38 mm^2). These were evident at the level of the area postrema and these same sections were also used to make counts of the area postrema (0.13 mm^2).