Original Article

$GABA_A$ Receptor Dysfunction Contributes To High Blood Pressure And Exaggerated Response To Stress In Schlager Genetically Hypertensive Mice

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Objective:Schlager BPH/2J hypertensive mice have high blood pressure likely due to overactivity of the sympathetic nervous system regulated by neurons in amygdala-hypothalamic pathways. These areas are normally under tonic inhibition by GABA containing neurons that may be deficient in Schlager hypertensive mice as suggested by microarray analysis. In the present study, cardiovascular effects of chronic activation of $GABA_A$ receptors were examined in BPH/2J mice.
Methods: Male normotensive BPN/3J and hypertensive BPH/2J mice were administered diazepam in drinking water for 7 days. Blood pressure, heart rate and locomotor activity were recorded by telemetry.
Results: Diazepam (2.5 mg/kg) reduced blood pressure of BPN/3J mice during the night time by -7.1±2.0 mmHg (P=0.001) but had no effect in BPH/2J mice (+2±2 mmHg) and no effect on heart rate or locomotor activity in either strain. Diazepam reduced the responses to restraint stress in BPN/3J mice by 20 % (P=0.01) and there was no association between Fos-immunoreactive neurons and neurons expressing GABAA receptors or neuropeptide Y in the medial amygdala and paraventricular nucleus of the hypothalamus. By contrast diazepam had no effect on the pressor response to stress in BPH/2J mice and ~50 % of stress-activated neurons in these regions also expressed $GABA_A$ receptors and ~45 % were neuropeptide Y-containing.
Conclusion: These findings show that BPH/2J mice are resistant to the effects of diazepam and suggests that $GABA_A$ receptor dysfunction in BPH/2J mice may be contributing to the neurogenic hypertension by not suppressing arousal induced sympathetic activation within amygdala and hypothalamic nuclei.Key words: Hypertension; rilmenidine; BPH/2J mice; sympathetic nervous system; rostroventrolateral medulla.

INTRODUCTION

The Schlager BPH/2J hypertensive mouse strain has recently been recognised as a neurogenic model of hypertension [1]. This is based on the ability of the ganglion blocker pentolinium to abolish the blood pressure (BP) difference between normotensive BPN/3J and BPH/2J mice while blockade of the renin angiotensin system had an equal hypotensive effect in both strains [2]. Thus it is likely that there is a greater sympathetic contribution to the hypertension in BPH/2J mice. The use of radiotelemetry devices recording for 24 hours identified that BPH/2J mice not only display hypertension but also have a greater day-night difference in BP compared with BPN/3J mice [3]. Interestingly, the greater BP surge in BPH/2J mice was associated with activation of neurons in the amygdala and hypothalamus indicating brain regions important for cardiovascular regulation of stress [3]. Indeed BPH/2J mice also respond markedly more than BPN/3J mice to aversive stress demonstrated by 70-90 % greater pressor responses and only small effects (+20 %) induced by non-aversive stress such as feeding indicating typical levels of arousal [4]. Moreover, aversive stress has been shown to involve an amplification of amygdala-hypothalamic-brainstem pathways that may be associated with sympathetic activation [5].

The excessive activation of neurons in the amygdala and downstream hypothalamus and associated greater responses to stress may be due to a loss or dysfunction of inhibitory GABAergic or neuropeptide Y (NPY) influences since both are recognised as major regulators of these pathways [6, 7]. Kunkler and Hwang suggested that lower $GABA_A$ receptor binding in the amygdala and hypothalamus of spontaneously hypertensive rats (SHR) may contribute to hypertension in this strain [8]. Later studies in the same strain demonstrated that activation of GABA receptors in the medial amygdala (MeAm) inhibits the pressor response to restraint stress [9]. Studies of some neurogenic diseases in humans describe a loss of GABAergic neurons in the amygdala that cause a reduction in inhibitory inputs leading to hyper-excitability of the amygdala circuitry [10]. Recently, microarray analysis was used to detect changes in the brains of BPN/3J and BPH/2J mice and identified altered expression of $GABA_A$ receptor subunits in the hypothalamus of BPH/2J mice particularly those related to the benzodiazepine binding site [11, 12]. NPY also elicits anxiolytic effects via Y1 receptors in the MeAm which is known to be located on GABAergic neurons [13, 14]. NPY gene expression in the hypothalamus of BPH/2J mice with established hypertension is 2 fold less as measured by microarray and confirmed by semiquantitative real-time PCR [12]. Taken together we hypothesise that hypertension and exaggerated responses to stress may be due to reduced function of GABA and NPY in the amygdala and hypothalamus. This may be due to down regulation of receptors (as in SHR), a

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deficit in neurotransmitter release or activity or altered receptor function. Diazepam, a benzodiazepine, acts via the $GABA_A$ receptor complex and has been shown to protect against neuronal loss in the amygdala [10]. Moreover, chronic diazepam treatment alters Y1 receptors in the amygdala suggesting that the interaction between GABA and NPY may be important in the regulation of anxiety [15]. Diazepam is therefore a useful probe of the function of the GABA and NPY systems which may be influencing hypertension in BPH/2J mice. In the present study we therefore investigated the contribution of $GABA_A$ receptors to the hypertension in BPH/2J mice using chronic diazepam treatment and radiotelemetry devices to record 24 hour BP and cardiovascular responses before and during exposure to stressful stimuli. Immunohistochemistry was used to label stress-activated neurons (expressing Fos) in the amygdala and hypothalamus and their localisation with $GABA_A$ receptors and NPY-containing neurons.

METHODS

Animals

Experiments were carried out in 18-19 week old conscious male normotensive (BPN/3J, n=18) and hypertensive (BPH/2J, n=19) Schlager mice from inbred colonies raised at the Alfred Medical Research and Education Precinct Animal Centre (Generation 15-20) from breeders purchased at generation 20-36 from Jackson laboratories. The original breeding selection program, took place in the 1970's for at least 23 generations and then brother sister mating followed to create these inbred strains [16]. Mice were housed individually within a controlled temperature and humidity facility on a 12:12 hr light and dark cycle (1 am-1 pm light) with ad libitum access to water and chow pellets (19 % protein, 5 % fat, 5 % fibre, 0.2 % sodium; Specialty feeds, Glen Forrest, Western Australia). The experiments were previously approved by the Alfred Medical Research and Educational Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals.

Mice were implanted with a radiotelemetry transmitter (TA11PA-C10, Data Sciences International) under isoflurane open circuit anaesthesia (4 % induction and 1.5-2 % maintenance). The catheter was inserted into the common carotid artery and the transmitter was implanted along the right flank [17]. During a 7 to 10 day recovery period: mobility, alertness, weight, BP and heart rate (HR) recordings were monitored to ensure full recovery.

Protocol for Chronic Diazepam Treatment

Following a 10 day recovery period, systolic arterial pressure (SAP), diastolic arterial pressure (DAP), calculated mean arterial pressure (MAP), HR, activity responses and respiratory rates were recorded in freely moving mice in their home cage continuously across a 48 hour period. During the recording session, pulsatile arterial pressure and gross locomotor activity were sampled at 1000 Hz using an analog-to-digital data acquisition card (Jackson et al., 2007) and the beat-to-beat MAP and HR were detected online and analysed as previously described [18].

Some mice were then treated with diazepam (2.5 mg/kg/day) in drinking water based on previous studies in mice [19, 20] and our pilot experiments while another group remained on water alone. Diazepam doses were adjusted according to the volume ingested for each individual mouse during the previous period every 3 days throughout the 7 day treatment, then cardiovascular parameters and locomotor activity were again recorded for another 48 hours. Time control experiments were also performed in mice (BPN/3J, n=5 and BPH/2J, n=6) where basal levels of cardiovascular variables and the responses to stress were examined before and after a control period of "no diazepam treatment".

Assessment of Cardiovascular Reactivity in Response to Stress Tests

Mice underwent three behavioural stress tests randomised on separate days during the inactive light period including a non-aversive appetitive stress and aversive stresses in the form of dirty cage switch and restraint as previously described [4, 21]. During 60 min of behavioural stress tests: MAP, HR and activity response recordings were analysed at 10 min intervals and baseline cardiovascular data were recorded for a 60 min control period immediately prior to stress exposure. Briefly, a feeding response involved presenting fasted mice with palatable food (pieces of almond and chow pellets) in their home cages and eating patterns were observed in 1 min intervals for a period of 60 min. Dirty cage switch tests were conducted by removing a mouse from their original cage and randomly placing it into a cage previously occupied by a different male mouse. Restraint stress involved guiding the mouse into a cylindrical Plexiglass restrainer with a sliding back plate to confine the mouse without applying physical pressure. More detail is provided in previous publications [1, 2, 4, 5, 22-24].

Sympathetic Contribution to Blood Pressure Assessed by Ganglion Blockade

Following a 10 day recovery period from surgery to implant telemetry devices, recordings of SAP, DAP and calculated MAP, HR and locomotor activity were obtained in freely moving mice in their home cage. Cardiovascular parameters were measured for 30 min prior to and for 30 min after i.p. injections of pentolinium (5 mg/kg; Sigma-Aldrich) during the active (night) period at least 2 hours after lights off. The dose used in the present study was based on previous studies [2]. Pentolinium was dissolved in isotonic 0.9 % saline (vehicle) and freshly prepared each day.

Cardiovascular Variability and Cardiac Baroreceptor Sensitivity

Beat-to-beat data were analysed separately to calculate power spectra using a program written in Labview [25]. The auto- and cross-power spectra were calculated for multiple overlapping (by 50 %) segments of MAP and HR using a Fast Fourier transform as adapted for conscious mice [22]. The cardiac baroreflex sensitivity was estimated as the average value of the transfer gain in the frequency band between 0.3 and 0.5 Hz [22]. Baroreflex slope was considered significant if the coherence between MAP and HR across several overlapping segments in the analysed frequency band was >0.4. Data periods with low locomotor activity were chosen (4 from each circadian period) from 48 hour recordings minimizing the influence of physical activity.

Cardiovascular data analysis

Cardiovascular data collected over 48 hours were analysed using a split plot, repeated measure analysis of variance (ANOVA) that allowed for within-animal and between-animal (group) contrasts. Within animal contrasts indicated differences in the day versus night and effect of diazepam compared with control mice that were not administered diazepam.

Immunohistochemical Analysis of Brains from Mice Exposed to Restraint Stress

Water only control BPN/3J and BPH/2J mice (n=5 per group) and mice treated with chronic diazepam (n=6 per group) were exposed to 60 min of restraint stress during the inactive day time period immediately prior to perfusion. Mice were deeply

anaesthetized with sodium pentobarbitone (100 mg/kg) given intraperitoneally and perfused transcardially with 20 ml 0.9 % saline and 50-100 ml 4 % paraformaldehyde (PFA) solution dissolved in 0.1 M phosphate buffer, pH 7.2 (PB). Brains were removed and post-fixed in 4 % PFA/PB for 1 hour and then transferred to 20% sucrose in PB and stored at 4^0C overnight.

Coronal brain sections were cut on a cryostat (40 μm) and collected in PB at room temperature. Free-floating sections were initially incubated in 10 % normal horse serum in PB for 1 hour prior to consecutive overnight incubation in each of the primary antibodies which were diluted in a solution of 2 % normal horse serum and 0.3 % Triton x-100 (Sigma) in PB. Sections were washed three times for 5 min intervals with PB following either incubation in primary antibodies or after a 1 hour incubation in the appropriate fluorophore diluted 1:500 in PB.

Immunohistochemistry for the protein product (Fos) of the immediate early gene c-fos was used to examine neuronal expression as it is a well established powerful tool for identifying neurons activated by external stimuli. Therefore, sections were initially incubated in the primary antibody: goat anti Fos (Santa Cruz, 1:1000) followed by donkey anti-goat Alexa 488 fluorophore (Invitrogen). The second primary antibody: rabbit anti $GABA_A$ receptor (Sigma) was diluted 1:1000 followed by donkey anti-rabbit Alexa 546 fluorophore (Invitrogen) and the third primary antibody: sheep anti NPY (Millipore, 1:500) followed by donkey anti-sheep Alexa 647 fluorophore (Invitrogen). Following final washes, the sections were mounted on gelatin subbed slides and coverslipped with DePeX (BDH Chemicals Ltd).

Immunohistochemical Data Analysis

The MeAm and PVN were identified using a process previously described [5] and counts of Fos labeled cells were made in a minimum of four sections per brain region in each animal. The counts were made by an observer who was unaware of which group the sections were from (blind analysis). An Olympus BX61 fluorescence microscope with tri-filters was used to photograph and quantify double and triple labeling for $GABA_A$ receptors and NPY labeled neurons. Statistical evaluation for each of the counts for Fos, $GABA_A$ receptors and NPY was performed by one-way ANOVA and Bonferroni post-hoc t-tests. All variables are presented as mean ± SEM. Values were considered significant when $P<0.05$.

RESULTS

Basal Cardiovascular, Locomotor and Respiratory Parameters

The SAP, DAP, MAP, HR and respiratory rate, averaged over the 24 hour period, during the night (active) and also during the day (inactive) were greater in BPH/2J compared with BPN/3J mice ($P<0.05$ for all). There was no difference in activity between groups at either of these times. Day to night time differences indicate that BP, HR, activity and respiration were greater during the dark period and also more pronounced in the BPH/2J mice compared with the BPN/3J mice (see Supplemental Data Table 1).

Dose Response to Oral Diazepam Treatment

A preliminary dose response curve was performed in 5 mice starting with 2.5 mg/kg/day for 3 days and then increasing to 5.0, 10 and 20 mg/kg/day, each for 3 days followed by a 1 week washout. Diazepam decreased BP and HR in BPN/3J but increased BP and HR in BPH/2J (P between groups<0.001). There was no clear dose response relationship for BP indicating that maximum effects were observed at 2.5 mg/kg/day which was subsequently used for the main study (see Supplemental Data Figure S1). Overall during the 7 day experimental treatment, BPN/3J mice drank an average of 2.8 ± 0.6 mls/day while BPH/2J mice drank 2.7 ± 0.7 mls/day. The calculated dose for BPN/3J mice was 2.9 ± 1.8 mg/kg/day and BPH/2J mice was 2.7 ± 1.2 mg/kg/day which was not statistically different from the advocated dose of 2.5 mg/kg/day.

Effect of Chronic Diazepam Treatment on Cardiovascular Parameters

Chronic treatment over 7 days with 2.5 mg/kg per day of diazepam in drinking water, decreased MAP in BPN/3J mice during the dark active period (-7.1±2.0 mmHg compared with control, P=0.0013, Fig. 1) but had no effect on MAP during the inactive period. Whereas in BPH/2J mice, diazepam treatment had no effect on MAP during either the active or inactive periods (Fig. 1). There was no change in BP observed in control (no treatment) animals that continued on drinking water over the same period during either the active or inactive periods (see Supplemental Data Figure S2). The level of HR and locomotor activity in either BPN/3J or BPH/2J mice was not affected by diazepam treatment during both active and inactive periods (Fig. 1). While there was also no effect on locomotor activity in time control animals (no treatment), HR was slightly reduced during both the day and night time phases during the second recording in BPN/3J mice (P=0.048 and P=0.036, respectively) and in BPH/2J mice during the day time (inactive) period only (P=0.005) (see Supplemental Data Figure S2).

Sympathetic Contribution to Blood Pressure Assessed by Ganglion Blockade

While the depressor response to sympathetic blockade by pentolinium during the active (night) phase was reduced in BPN/3J mice treated with diazepam ($P<0.05$), diazepam had no effect on the reduction in MAP induced by pentolinium in BPH/2J mice. There was no effect of pentolinium on HR or locomotor activity on water or diazepam treated mice in either strain (Fig. 2).

Cardiovascular Variability and Cardiac Baroreceptor Sensitivity

Following diazepam treatment mid frequency MAP power was reduced by 50 % in BPN/3J mice ($P<0.05$) with no change observed in BPH/2J mice before and following diazepam. While high frequency baroreflex gain was augmented by diazepam treatment in BPN/3J mice only ($P<0.05$), there were no changes in HR power before and after treatment with diazepam in either strain (see Supplemental Data Table S2).

Cardiovascular Response to Non-Aversive Behavioural Test: Feeding Stress

Repeated presentation of palatable food (almond and chow pellets) over a 60 min period caused an immediate and sustained pressor and tachycardic response in all mice (Fig. 3). The pressor response to feeding was greater in BPH/2J mice compared with BPN/3J mice (P_{strain} <0.001), but there was no effect of diazepam treatment (Fig. 3). The tachycardia response to feeding was also greater in BPH/2J (P_{strain}=0.004) and increased slightly after treatment with diazepam ($P_{treatment}$=0.02). Activity was greater in BPH/2J mice compared with BPN/3J mice (P_{strain}<0.001) and this response was not affected by diazepam treatment (Fig. 3). Time control mice that continued on normal drinking water had slightly less pressor responses ($P_{treatment}$=0.03) and tachycardia responses to feeding (BPN/3J only reached significance, Ptreatment=0.049) compared with control mice not treated with diazepam but were consistently greater in BPH/2J compared with BPN/3J mice (see Supplemental Data Figure S3).

Cardiovascular Response to Aversive Behavioural Test: Dirty Cage Switch Stress

While the pressor response to dirty cage switch was markedly greater in BPH/2J than BPN/3J mice (P_{strain}<0.001, Fig. 4), treatment with diazepam only reduced the rise in BP in normotensive BPN/3J mice (-3.9 mmHg, $P_{treatmen}$t=0.01) with no effect in BPH/2J mice. HR responses to dirty cage switch were similar between both groups of mice and there was no effect of diazepam. While BPH/2J mice were markedly more active than BPN/3J mice (P_{strain}<0.001) during dirty cage switch, there was no effect of diazepam treatment in either group (Fig. 4). Pressor and locomotor activity responses to dirty cage switch in time control mice that continued on normal drinking water were similar to control mice not treated with diazepam but there was an effect of HR (Ptreatment<0.001) (see Supplemental Data Figure S4).

Cardiovascular Response to Aversive Behavioural Test: Restraint Stress

Restraint stress for 60 min produced a markedly greater pressor response in BPH/2J mice than BPN/3J mice (P_{strain}<0.001, Fig. 5). As observed with dirty cage switch, diazepam treatment reduced the increase in MAP induced by restraint in BPN/3J mice (-3.4 mmHg, P=0.01) but not in hypertensive BPH/3J mice (+2.2 mmHg, Fstrain x treatment=0.048). The HR response to restraint was also greater in BPH/2J (P_{strain}=0.044, Fig. 5) and treatment with diazepam induced a markedly greater tachycardic response in both BPN/3J and BPH/2J mice compared with control ($P_{treatment}$<0.001). Time control mice that continued on normal drinking water had similar pressor and tachycardia responses to restraint compared with control mice not treated with diazepam (see Supplemental Data Figure S5).

Immunohistochemical Analysis of Fos, $GABA_A$ receptors and NPY-containing Neurons in Response to Restraint Stress

The mean number of activated neurons (Fos positive nuclei) in the MeAm and PVN observed in response to 60 mins restraint stress was less in the BPN/3J strain compared with BPH/2J in both water control mice and mice treated with diazepam (P<0.05 for all, Table 1). Chronic treatment with diazepam over 7 days reduced the number of neurons activated in response to restraint stress in the MeAm and PVN of both strains (P<0.01 for all). While there were similar numbers of neurons expressing $GABA_A$ receptors and NPY-containing neurons in water control mice from both strains, greater numbers of $GABA_A$ receptor positive neurons and NPY-containing neurons were detected in hypertensive BPH/2J mice compared with BPN/3J treated with diazepam (P<0.01 for both, Table 1). The effect of diazepam treatment in BPN/3J mice compared with BPN/3J water control mice was a reduction in the number of NPY-containing neurons in the PVN (P<0.001) only. While there was no effect of diazepam treatment on NPY-containing neurons in the PVN of BPH/2J mice, there was greater labelling of NPY in the MeAm (P<0.001) and greater numbers of $GABA_A$ receptor positive neurons were observed in both the MeAm (P<0.001) and PVN (P<0.01, Table 1) compared with water control mice from the same strain.

Effect of Diazepam Treatment on the Distribution of Stress-activated Neurons Expressing $GABA_A$ receptors and NPY

There was a substantial overlap within the MeAm and PVN of BPH/2J mice treated with diazepam between neurons expressing Fos in response to restraint stress and neurons expressing $GABA_A$ receptors. These double-labeled neurons represented 51 % of all MeAm and PVN activated neurons in these hypertensive mice. Additionally, many neurons in the MeAm (87%) and PVN (83%) of BPH/2J mice identified as expressing $GABA_A$ receptors also contained NPY and represented 45 % and 43 % of all activated neurons in these brain regions respectively. By contrast, in the MeAm and PVN, there was little or no evidence of an association between activated neurons and $GABA_A$ receptor positive neurons in BPN/3J mice following chronic administration of diazepam. However, neurons expressing $GABA_A$ receptors in BPN/3J mice were mostly co-localised with NPY-containing neurons (Table 1, Fig. 6).

DISCUSSION

In the present study we determined whether the hypertension and exaggerated responses to stress in BPH/2J mice may be related to altered function of GABA and NPY in the amygdala and hypothalamus. The major findings were that chronic treatment with diazepam caused a decrease in nocturnal BP and a reduction in the pressor response to aversive stress (dirty cage switch and restraint) in normotensive BPN/3J mice but no effect in hypertensive BPH/2J mice. Importantly, ~50 % of neurons activated in response to restraint (as detected by Fos-immunoreactivity) in the MeAm and PVN of BPH/2J mice treated with diazepam were also labeled for $GABA_A$ receptors and many (~45 %) were found to contain NPY. This suggests that $GABA_A$ receptors are present but are not activated by diazepam to cause local inhibition in these brain regions in BPH/2J hypertensive mice. As such, resistance to diazepam treatment in BPH/2J mice has led to neurons in the MeAm and PVN to remain active in response to aversive stressor arousal. By contrast, there was little or no overlap of labeling of activated neurons (Fos) with $GABA_A$ receptor positive neurons in BPN/3J mice, but many neurons expressing GABAA receptors were also NPY-immunoreactive. Thus in normotensive mice while $GABA_A$ receptors and NPY-immunoreactive neurons were present, they were not located on stress-activated neurons (expressing Fos), presumably because their activity has been inhibited by diazepam. Indeed we identified fewer activated neurons in the MeAm and PVN of normotensive BPN/3J mice signaling effective inhibition by diazepam treatment via GABAA receptors. Previously BPH/2J mice were shown to have greater Fos expression in the MeAm and PVN compared with BPN/3J mice in response to aversive stress [5]. Our findings suggest that BPH/2J mice exhibit “resistance” to GABAA receptor activation, by benzodiazepines, while BPN/3J mice respond to the treatment as might be expected. Additionally, the depressor response to pentolinium was reduced by diazepam treatment in BPN/3J mice whereas the exaggerated sympathetic nervous system effect on BP in BPH/2J mice remained unaffected by diazepam and was confirmed by mid frequency MAP power. Thus the BP of BPH/2J mice particularly during nocturnal arousal may be higher due to the absence of $GABA_A$ inhibitory drive to presympathetic neurons arising from the amygdala and hypothalamus. Therefore, it is likely that a GABAergic mechanism is a major contributor to the hypertension in these mice.

Interestingly, we found that diazepam similarly reduced the number of Fos containing neurons following restraint in all animals of both strains. Thus there is an effect of diazepam in the BPH/2J mice suggesting that the “resistance” is not due to differences in bioavailability but related to different receptor mechanisms. Secondly, neurons expressing $GABA_A$ receptors are elevated in BPH/2J mice with diazepam but are unchanged in BPN/3J mice where we observed the reduction in BP and the pressor response to stress. The suggestion that $GABA_A$ receptors may contribute to neurogenic hypertension first

came from a study identifying lower levels of densities of $GABA_A$ receptor binding in the hypothalamus and amygdala in young spontaneously hypertensive rats (SHR) compared with normotensive rats [8]. A more recent study using radiotelemetry to measure BP, found that acute treatment with the same dose of diazepam used in the present study (2.5 mg/kg) did not affect basal BP in SHR nor in normotensive rats [26]. This is consistent with our findings of a lack of effect during the inactive period of diazepam in either BPH/2J or BPN/3J mice. While the effect of diazepam was not studied during the active dark phase of rats, diazepam abolished the pressor response of Sprague-Dawley normotensive rats to open field but had no effect in SHR [26]. This is similar to our findings with a lesser pressor response to restraint stress in BPN/3J and no effect in BPH/2J mice. We demonstrated modest effects of diazepam treatment on restraint stress to the change in BP in BPN/3J mice and reduction in BP during the dark phase was only 7mmHg. Indeed there were no differences in the number of neurons labeled for $GABA_A$ receptors between control strains that were not administered diazepam and no differences in BPN/3J mice untreated or treated with diazepam but there were greater numbers of $GABA_A$ receptor positive neurons in BPH/2J mice following diazepam treatment. Even so, the magnitude of the BP responses and receptor labeling does not necessarily tell us the contribution of the $GABA_A$ receptor to the hypertension of BPH/2J but the lack of effect of diazepam in BPH/2J indicates a major difference between the strains in the functioning of the $GABA_A$ receptor. Interestingly deficits in the BP response to benzodiazepines appear to be common in both rat (SHR) and mouse (BPH/2J) forms of "neurogenic" hypertension. Furthermore, the effect of diazepam was not sedative as the pressor response to feeding was not affected and locomotor activity and responsiveness were normal. The clear question arising from these studies is the underlying mechanism.

The mechanism may be due to i) loss of local GABA interneurons which can lead to hyperexcitability of the amygdala circuitry [27]; ii) loss of their connections such as that which occurs following chronic stress (observed as reduced spine density on stellate neurons in MeAm [28]); or iii) loss of effective neurotransmission due to differences in GABAA subunit assembly which can also occur in the PVN of rats exposed to chronic stress [29]. The $GABA_A$ receptor is a heteromeric pentamer with α1-6, β1-4, γ1-3 subunits among others that when assembled in different combinations allows a vast number of structurally unique $GABA_A$ receptors. The benzodiazepine, diazepam, is commonly prescribed for the treatment of anxiety disorders [30] and is a positive allosteric modulator of benzodiazepine receptors augmenting the Cl- flux to enhance inhibitory neurotransmission [31]. The α and β subunits form the GABA binding pocket while the α and γ form the benzodiazepine binding pocket [32]. These have more recently been sub-classified with diazepam binding involving the α1, α2, α3, α5 and γ2 subunits of $GABA_A$ receptors [33]. It is known that loss of GABAergic influence in the amygdala results in reduced inhibitory inputs leading to 'hyperexcitability' of the amygdala circuitry [10] but there has been little investigation of the cardiovascular impact of such changes. Differences in gene expression of $GABA_A$ subunits determined initially using microarray and confirmed by RT-PCR [11, 12] suggest that there may be altered subunit assembly in BPH/2J compared with BPN/3J mice and this may include changes between young and adult hypertensive mice. However, this requires further confirmation. We observed lower levels of δ and α4 mRNA in adult BPH/2J that are not seen in pre-hypertensive BPH/2J mice and are therefore one of the major gene expression changes related to the development of hypertension [12].

NPY has been shown to generate anxiolytic effects similar to benzodiazepines acting on $GABA_A$ receptors in response to anxiety and fear by activating Y1 receptors in the MeAm [13-15]. Indeed in the MeAm there is a close and complex association between NPY-IR fibres and GABAergic neurons [15] and GABA allosteric modulators can also alter NPY-Y1 receptor mRNA. An earlier study demonstrated that diazepam counteracts the anxiogenic effect of Y1 antagonists [34] and exposure to 1 hour of restraint stress increased the expression of the Y1 receptor gene in the MeAm and PVN in mice [35]. Long term treatment with modulators of the $GABA_A$ receptor complex can induce compensatory effects by changing the firing rate of NPY-containing neurons which may lead to changes in Y1 receptor gene transcriptional activity [34]. NPY has also been shown to inhibit $GABA_A$ transmission on neurons located in the bed nucleus of the stria terminalis [36] (a region of the extended amygdala) and also in the PVN mediated by NPY-Y1, 2 and 5 receptors [37]. While there was a reduction of NPY-containing neurons in the PVN of BPN/3J mice treated with diazepam compared with water controls of the same strain, there was a marked increase in the MeAm of BPH/2J mice. Our immunohistochemical findings support the assertion that while GABA and NPY systems are coupled in the adaptation of the response to stress the interaction differs between strains. Even so, the exaggerated BP responses in BPH/2J mice are likely influenced by diminished local inhibition via an interaction or reconfiguration of $GABA_A$ receptors and NPY in the MeAm and/or PVN contributing to the maintenance of neurogenic hypertension in these mice.

Our study does not determine the precise location of the GABAergic/NPY deficit in BPH/2J mice. The immunohistochemical differences in response to diazepam were observed not only in the MeAm but importantly in the PVN. Benzodiazepines inhibit hypothalamic presympathetic neurons by potentiation of GABAergic synaptic input [38]. There is prominent inhibitory innervation of the PVN where over half the synapses are GABAergic [39]. Thus the cardiovascular effects of systemic diazepam could be resulting from actions in either region or other regions of the hypothalamus such as the dorsomedial nucleus or in the extended amygdala such as the bed nucleus of the stria terminalis which are known to be important in mediating the cardiovascular response to stress [40].

PERSPECTIVES

The present study demonstrates the importance of a GABAergic inhibitory mechanism in regulating BP responses across 24 hours in normotensive BPN/2J mice. Dysfunction of this GABAergic mechanism was identified in neurons located in the MeAm and PVN of hypertensive BPH/2J mice as diazepam treatment was ineffective in both inhibiting the activation of these neurons induced by restraint and reducing the BP response. There is also a likely interaction between $GABA_A$ receptors and neurons containing NPY arising from the MeAm contributing to the neurogenic hypertension in these mice likely via a lack of typical inhibitory drive either directly or indirectly via the PVN to presympathetic neurons. Further studies targeting the differential resistance of $GABA_A$ receptor activation in this mouse strain in particular the subunit assembly may be critical to determining the mechanism driving this form of neurogenic hypertension and may be highly relevant to eliciting treatments for human patients with centrally mediated hypertension.

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Conflicts of Interest
There are no conflicts of interest.

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Table 1. Immunohistochemical fluorescent labeling of neurons

MeAm																	
Controls water only	BPN/3J				BPH/2J			P		Controls water only	BPN/3J				BPH/2J			
Fos	24.3	±	1.4		31.5	±	2.3	**		Fos + GABAA recs	0	±	0	0%	0	±	0	0%
GABAA receptors	2.2	±	0.7		4.7	±	1.9	NS		GABAA recs + NPY	2.2	±	0.7	100%	4.7	±	1.9	100%
NPY	11.5	±	1.6		12.5	±	1.1	NS		Fos + GABAA recs + NPY	0	±	0	0%	0	±	0	0%
Diazepam treatment	BPN/3J			P	BPH/2J					Diazepam treatment	BPN/3J				BPH/2J			
Fos	15.4	±	0.8	†††	24.6	±	0.5	***	††	Fos + GABAA recs	0	±	0	0%	12.6	±	0.8	51%
GABAA receptors	4.9	±	0.4	NS	12.6	±	0.8	***	†††	GABAA recs + NPY	4.9	±	0.3	100%	11.0	±	0.6	87%
NPY	9.7	±	0.7	NS	22.0	±	1.3	***	†††	Fos + GABAA recs + NPY	0	±	0	0%	11.0	±	0.6	45%
PVN																		
Controls water only	BPN/3J				BPH/2J			P		Controls water only	BPN/3J				BPH/2J			
Fos	27.7	±	2.2		34.2	±	3	*		Fos + GABAA recs	0	±	0	0%	0	±	0	0%
GABAA receptors	4.3	±	0.7		6.9	±	1.9	NS		GABAA recs + NPY	4.3	±	0.7	100%	6.9	±	1.9	100%
NPY	17.7	±	2.6		15.6	±	1.2	NS		Fos + GABAA recs + NPY	0	±	0	0%	0	±	0	0%
Diazepam treatment	BPN/3J			P	BPH/2J					Diazepam treatment	BPN/3J				BPH/2J			
Fos	15.4	±	1.1	†††	22.4	±	0.9	*	†††	Fos + GABAA recs	0	±	0	0%	11.5	±	1.2	51%
GABAA receptors	6.0	±	0.5	NS	11.5	±	1.2	**	††	GABAA recs + NPY	5.0	±	0.3	83%	9.6	±	0.6	83%
NPY	9.9	±	0.7	†††	19.2	±	1.2	***	NS	Fos + GABAA recs + NPY	0	±	0	0%	9.6	±	0.6	43%

Number of neurons labeled for Fos, GABAA receptors and neuropeptide Y (NPY) and the number and percent of activated neurons as detected by Fos also labeled for GABAA receptors (Fos + GABAA recs), the number and percent of GABAA receptors also containing NPY (GABAA recs + NPY), and the number and percent of activated neurons as detected by Fos labeled for GABAA receptors and also containing NPY (Fos + GABAA recs + NPY) in the medial amygdala (MeAm) and paraventricular nucleus of the hypothalamus (PVN) of BPN/3J and BPH/2J control mice (water only, n=5 per group) and mice treated with diazepam for 7 days (n=6 per group). All mice were exposed to 60 min restraint stress. Mean±SEM. *P<0.05, **P<0.01, ***P<0.001 compared with BPN/3J mice and ††P<0.01, †††P<0.001 effect of diazepam treatment compared with water control.

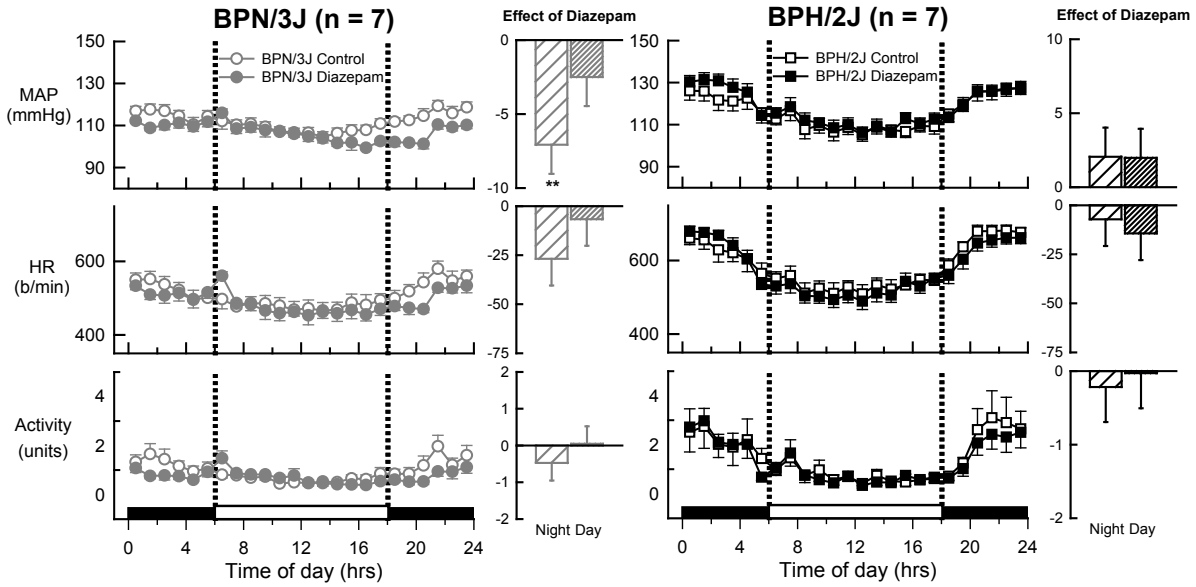

Figure 1. Line graphs represent average hourly values of mean arterial pressure (MAP, mmHg), heart rate (HR, beats/min) and activity (units) in BPN/3J mice (grey lines, circle; n=7) and BPH/2J mice (black lines, square; n=7) in control (open symbol) and diazepam treatment (closed symbol) plotted over 24 hours. Bar graphs represent average absolute changes in MAP and HR and locomotor activity during the active (night) and inactive (day) due to diazepam treatment. Values are mean ± SEM. **P<0.01.

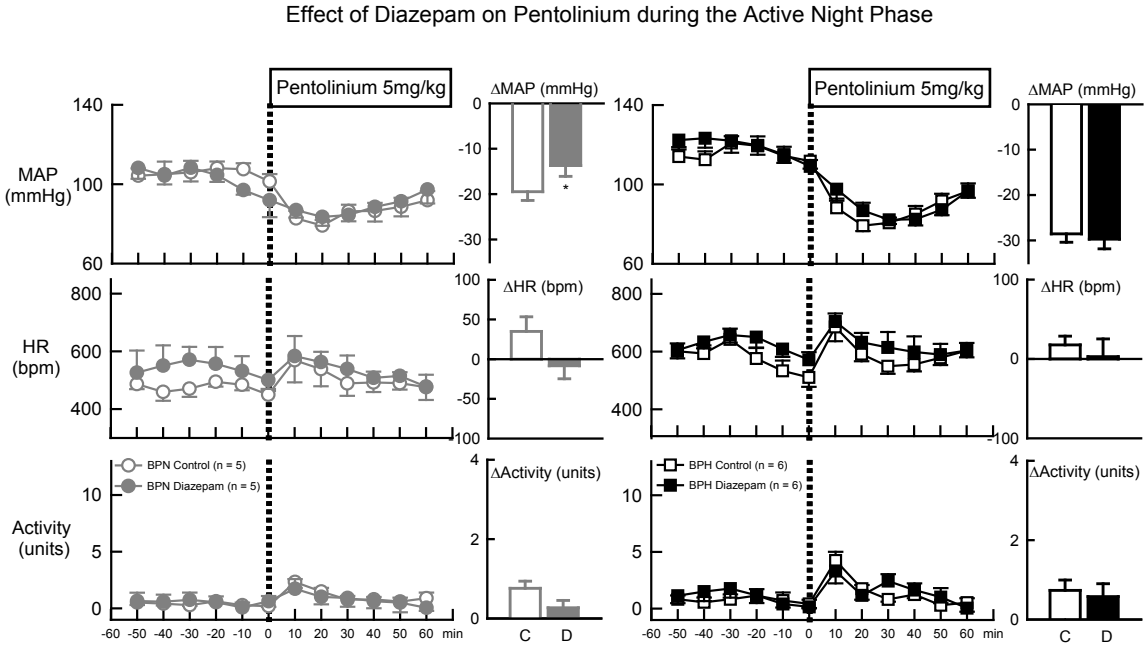

Figure 2. Line graphs represent the mean arterial pressure (MAP, mmHg), heart rate (HR, beats/min) and activity (units) responses to pentolinium 5 mg/kg in BPN/3J mice (grey lines, circle; n=5) and BPH/2J mice (black lines, square; n=6). Mice were treated with water (open symbol) and diazepam (closed symbol) during the active (night). Bar graphs represent average changes in MAP and HR and locomotor activity in response to pentolinium in BPN/3J (left, grey) and BPH/2J (right, black) mice treated with water (W, open bars) and diazepam (D, filled bars). The changes represent the difference between the control period and the period after injection. Values are mean ± SEM. *,P<0.05.

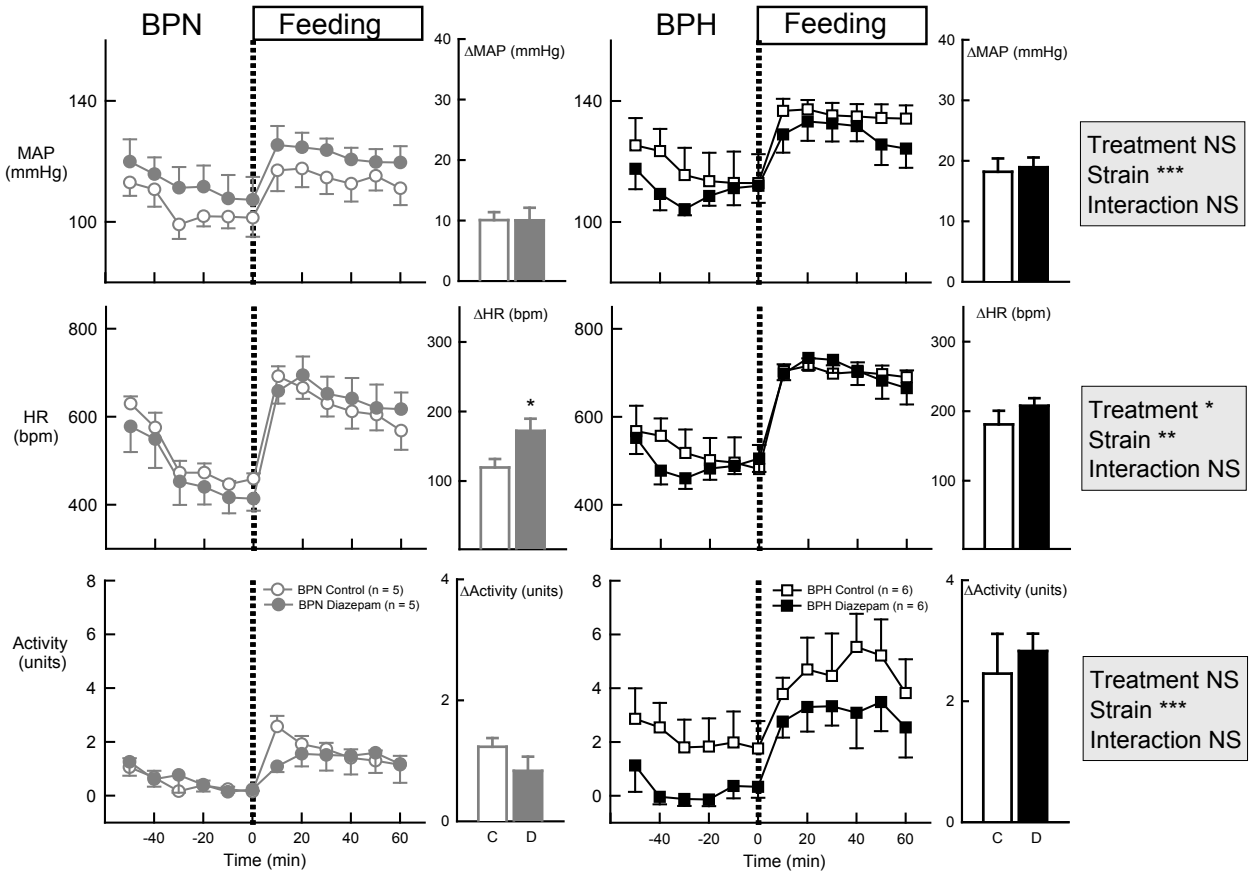

Figure 3. Line graphs represent the mean arterial pressure (MAP, mmHg), heart rate (HR, b/min) and locomotor activity before and during feeding in BPN/3J mice (left panel, grey, n=5) and BPH/2J mice (right panel, black, n=6). Each point represents 10 minute average values. Mice were prior to treatment (control) (C, open symbol) or after diazepam (D, closed symbol). Bar graphs represent difference in response to feeding averaged over 60 minutes in MAP and HR and locomotor activity during control (unfilled bar) and after diazepam treatment (filled bar). Values are mean ± SEM. Box shows the significance from the ANOVA of treatment (diazepam), strain and the interaction. *, P<0.05; **, P<0.01; ***, P<0.001; NS, not significant.

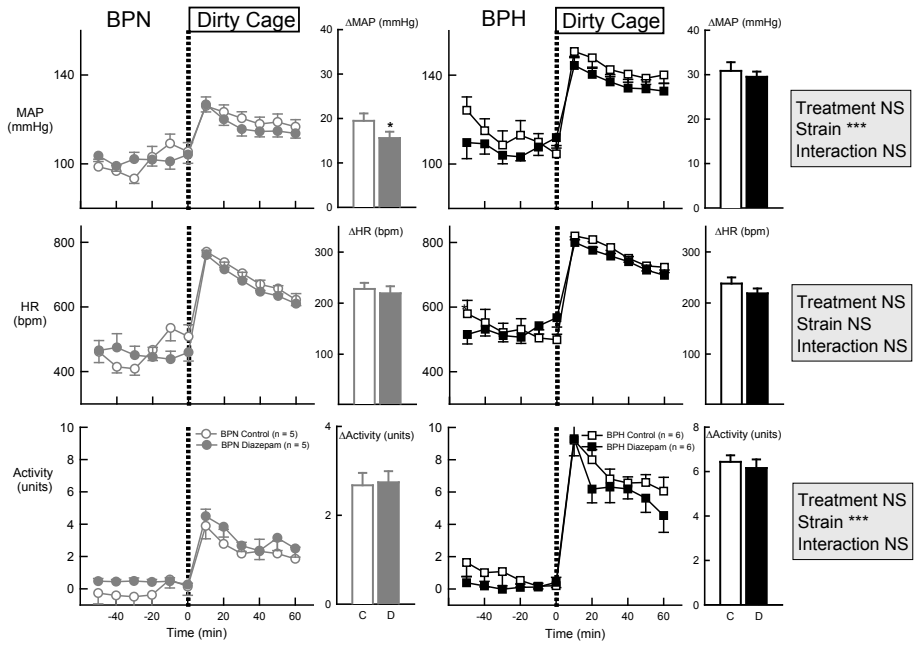

Figure 4. Line graphs represent the mean arterial pressure (MAP, mmHg), heart rate (HR, b/min) and locomotor activity before and during dirty cage switch in BPN/3J mice (left panel, grey, n=8) and BPH/2J mice (right panel, black, n=8). Each point represents 10 minute average values. Mice were prior to treatment (control) (C, open symbol) or after diazepam (D, closed symbol). Bar graphs represent difference in response to dirty cage switch averaged over 60 minutes in MAP and HR and locomotor activity during control (unfilled bar) and after diazepam treatment (filled bar). Values are mean ± SEM. Box shows the significance from the ANOVA of treatment (diazepam), strain and the interaction. **, P<0.01; ***, P<0.001; NS, not significant.

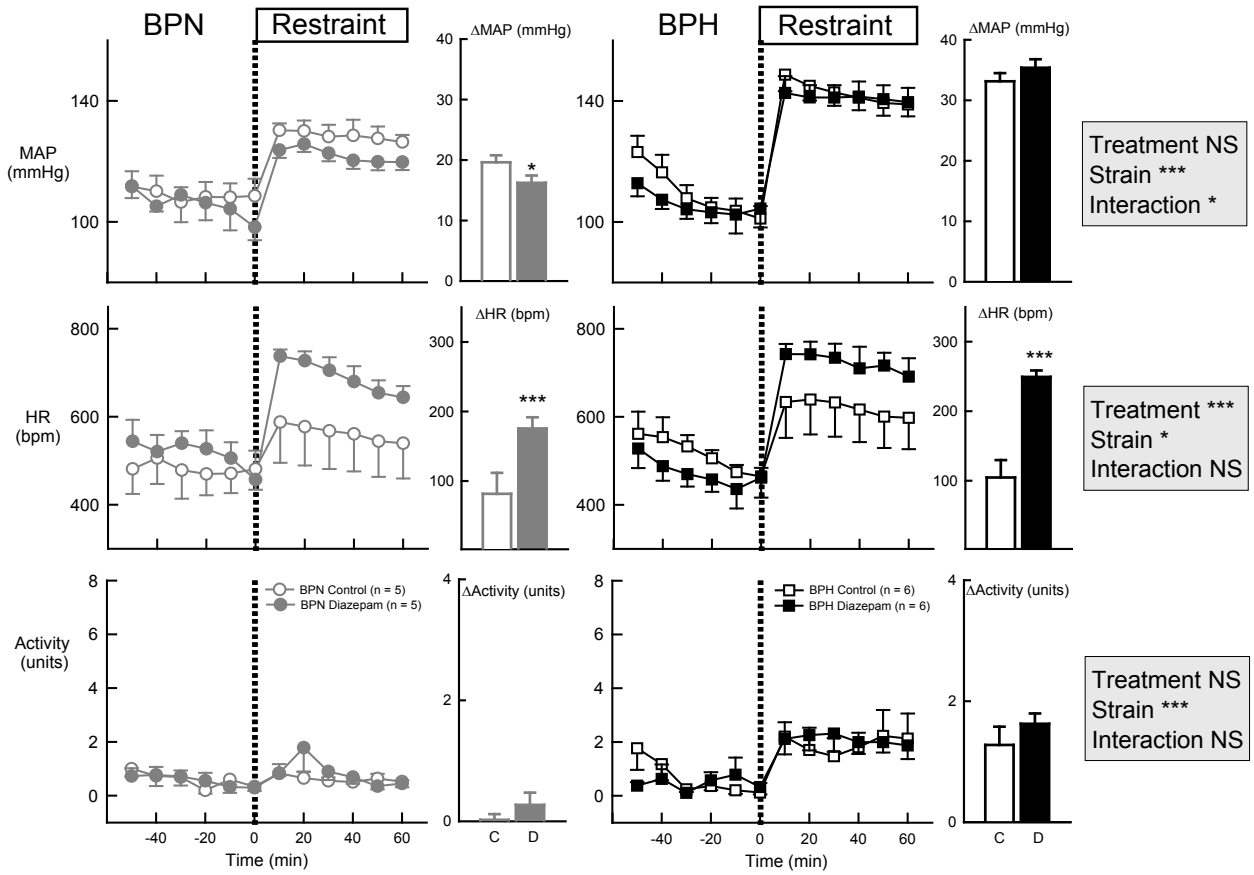

Figure 5. Line graphs represent the mean arterial pressure (MAP, mmHg), heart rate (HR, b/min) and locomotor activity before and during restraint in BPN/3J mice (left panel, grey, n=5) and BPH/2J mice (right panel, black, n=6). Each point represents 10 minute average values. Mice were prior to treatment (control) (C, open symbol) or after diazepam (D, closed symbol). Bar graphs represent difference in response to restraint averaged over 60 minutes in MAP and HR and locomotor activity during control (unfilled bar) and after diazepam treatment (filled bar). Values are mean ± SEM. Box shows the significance from the ANOVA of treatment (diazepam), strain and the interaction. *, P<0.05; ***, P<0.001; NS, not significant.

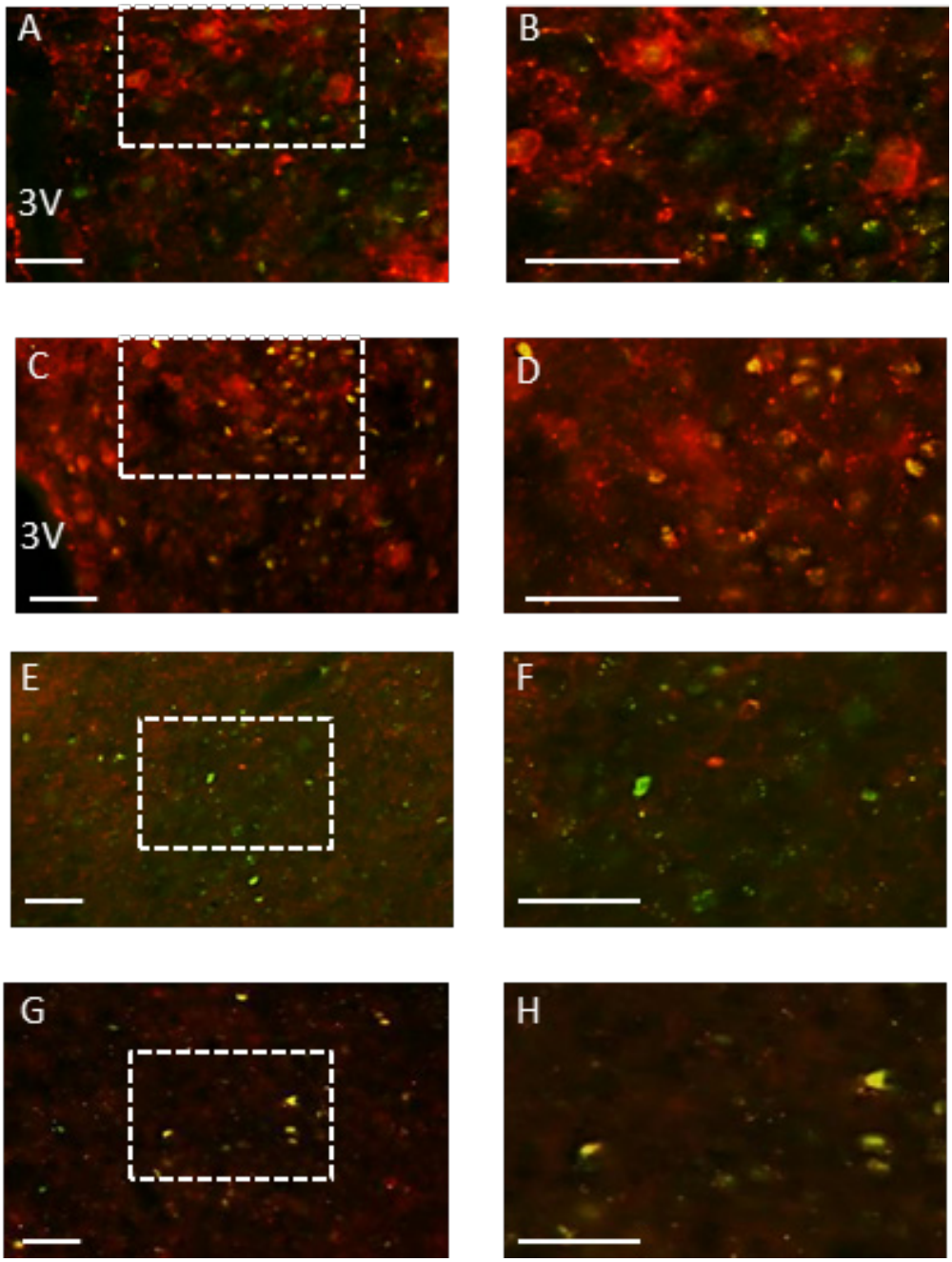

Figure 6. Photomicrographs of coronal sections through the PVN (A-D) and MeAm (E-H) of normotensive BPN/3J (A, B, E, F) and hypertensive BPH/2J mice (C, D, G, H) immediately following 60 mins restraint stress. In BPN/3J mice there was no evidence of neurons activated by restraint stress (Fos, green) co-localising with either GABAA receptors (orange) or NPY (far red) in the PVN (A, B) or the MeAm (E, F); whereas all neurons containing NPY also expressed GABAA receptors in the PVN (A, B) and MeAm (E, F). In BPH/2J mice many neurons expressing $GABA_A$ receptors were also immunoreactive for NPY and each of these were triple labeled with Fos (yellow) in the PVN (C, D) and MeAm (G, H). Abbreviations: MeAm, medial amygdala; PVN, paraventricular nucleus of the hypothalamus. Magnification bar = 100 µm.