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Original Article

Positive allosteric modulation of GABA$_A$ receptors attenuates high blood pressure in Schlager hypertensive mice

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Objective: BPH/2J hypertensive mice have neurogenic hypertension associated with differences in hypothalamic GABAA receptors compared with their normotensive counterparts (BPN/3J). Allopregnanolone is an endogenous neurosteroid reduced in chronic stress and when administered, decreases anxiety by positive allosteric modulation of GABAA receptors.

Methods: To determine if allopregnanolone could be a viable therapeutic for neurogenic hypertension, male BPH/2J (n=6-7) and BPN/3J (n=8-9) were equipped with radiotelemetry probes to compare cardiovascular variables before and after implantation of subcutaneous minipumps delivering allopregnanolone (5 mg/kg/day) or its vehicle for a period of two weeks. In addition to baseline recordings, the response to stress and ganglionic blockade with pentolinium was recorded, before and 7-14 days after minipump implantation. Following treatment, brains were processed for c-Fos immunohistochemistry and qRT-PCR.

Results: Administration of allopregnanolone selectively reduced mean arterial pressure (-8.0 ± 2.7 mmHg, P=0.02) and the depressor response to pentolinium (-15.3 ± 3.2 mmHg, P=0.001) in BPH/2J mice with minimal effects observed in BPN/3J mice. Following allopregnanolone the diminished expression of GABAA δ, α4 and β2 subunits in the hypothalamus (-1.6-4.8 fold, Pstrain<0.05) was abolished. Furthermore, in BPH/2J mice, allopregnanolone treatment reduced the pressor response to dirty cage switch stress (-26.7 ± 4.5%, P<0.001) and abolished the elevated c-Fos expression in pre-sympathetic nuclei.

Conclusion: The selective antihypertensive and stress inhibitory effects of allopregnanolone in BPH/2J mice suggest that allosteric modulation of GABAA receptors, in amygdalo-hypothalamic pathways, may contribute to development of hypertension in this model and may offer a potential new therapeutic avenue.

Keywords: allopregnanolone, autonomic nervous system, BPH/2J mice, GABAA, hypertension, medial amygdala, neuroendocrinology, paraventricular nucleus of the hypothalamus, sympathetic nervous system

Abbreviations: AlloP, allopregnanolone; BPH/2J, blood pressure high Schlager mice; BPN/3J, blood pressure normal Schlager; DAP, diastolic arterial pressure; DMH, dorsomedial nucleus of the hypothalamus; GABA, gammaaminobutyric acid; HR, heart rate; MAP, mean arterial pressure; MeAm, medial amygdala; PaPVN, parvocellular region of the paraventricular nucleus; PKC, protein kinase C; PVN, paraventricular nucleus; qRT-PCR, quantitative real-time polymerase chain reaction; SAP, systolic arterial pressure; SHR, spontaneously hypertensive rat; SNS, sympathetic nervous system; THDOC, tetrahydrodeoxycorticosterone

INTRODUCTION

There is considerable evidence that hyper-activity of the sympathetic nervous system (SNS) contributes to the pathogenesis of human essential hypertension. Elevated muscle sympathetic nerve activity and plasma noradrenaline have been observed in both pre-hypertension and established hypertension, suggesting the SNS may play a crucial role in both the development and maintenance of hypertension [1-4]. Evidence that chronic stress contributes to the development of hypertension is demonstrated by its association with elevations in SNS activity as well as an increased risk of the development of hypertension in subjects with chronic stress [5]. Furthermore, increased pressor responses and SNS activity in response to acute stress are long-term predictors of the future development of hypertension [6, 7] . Collectively, these observations suggest that enhanced sympatho-excitation during chronic stress can facilitate the development of hypertension.

Schlager hypertensive mice (BPH/2J) represent a good model of neurogenic hypertension since ganglionic blockade abolishes the elevated blood pressure compared

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with normotensive (BPN/3J) mice [8]. Additionally, BPH/2J mice have augmented cardiovascular responses to stress and elevated central catecholamines in regions of the brain associated with the stress response [9, 10]. The enhanced cardiovascular response to stress in BPH/2J mice coincides with greater activation in the amygdalo-hypothalamic-brainstem pathways [11]. Furthermore, the elevated activity of the medial amgydala (MeAm) and enhanced functional connectivity to regions of the brain associated with the cardiovascular response to stress is also apparent in humans with augmented pressor responses to stress [12] suggesting it may be characteristic of stress related hypertension. Most critically, BPH/2J mice have an augmented surge in mean arterial pressure (MAP) upon arousal which is associated with increased activation of these same brain regions [8], suggesting activation of these pathways may play a major role in the hypertension in this strain.

One possible mechanism driving neuronal hyperactivity in the amygdala and hypothalamus is a lack of GABAergic inhibition, leading to exaggerated responses to excitatory inputs to the nucleus. Normally, critical nuclei involved in mediating the response to stress within the amygdala, hypothalamus and brainstem are under tonic inhibition predominantly mediated by the δ- γ-aminobutyric acid-A receptor ($GABA_AR$) subtype [13-15]. This particular subunit is uniquely suited to conduct tonic GABAergic currents due to both its ubiquitous distribution and its resistance to desensitization following prolonged exposure to GABA. This tonic neuronal inhibition serves to diminish sympathetic activity and modulate cardiovascular reactivity [16]. Evidence of dysfunctional GABAergic inhibition is apparent across numerous rodent models. Both the pressor and sympatho-excitatory actions of inhibiting hypothalamic $GABA_AR$ with bicuculline are attenuated or even abolished in rats with renal wrap hypertension and in spontaneously hypertension rats (SHRs) [17, 18]. Additionally, BPH/2J mice have lower levels of message of α4, δ, and β2 $GABA_AR$ subunits in the hypothalamus compared with their normotensive counterparts [19]. Pharmacologically, this culminates in resistance to chronic treatment with the positive allosteric modulator of $GABA_AR$, diazepam, in BPH/2J but not BPN/3J mice [20]. Interestingly, these differences in subunit gene expression between strains are most prominent at an early age, prior to the development of hypertension, and progressively decline with age such that they are no longer evident at 26 weeks of age [19]. These differences in $GABA_A$ composition and subsequent functionality may partly explain exaggerated inputs to stress pathways and subsequent elevated AP in neurogenic models of hypertension.

Of the numerous neurosteroids known to modulate the $GABA_AR$, allopregnanolone (AlloP) is the most potent and is able to modulate the receptor depending of its subunit configuration [21]. The effect of AlloP on δ-$GABA_AR$ is allosteric modulation of chloride currents, mediated through enhanced chloride channel opening time and potentiation of inhibition [22]. There is potentiation and direct activation. These changes are especially evident in the δ and α4 combination which are sensitive to fluctuating levels of neurosteroids in the brain. Most importantly, this neurosteroid has been demonstrated to specifically up-regulate α4δβ2 subunits, which are down regulated in BPH/2J mice [19, 23]. Up-regulation of α4δ subunits appears to be associated with a reduction in neural activity [24]. Although a number of acute studies have demonstrated AlloP-induced changes in neural activation and sympatho-excitation [24-26], to our knowledge this has never been investigated in a chronic in vivo setting or in a hypertensive model. Therefore, in the current study, we tested the hypothesis that there are functional differences in the $GABA_AR$ that drive sympatho-excitation, and subsequently the pathogenesis of the hypertension, in BPH/2J mice. To this end, we examined the effects of chronic administration of AlloP on basal blood pressure and the cardiovascular responses to stress and ganglionic blockade.

METHODS

Animals

Experiments were performed on 12 week old conscious male normotensive (BPN3/J; n=17) and hypertensive (BPH/2J, n=13) Schlager mice. A further 12 animals did not complete the study due to 10 telemetry probe failures after implantation and 2 deaths prior to treatment. The original breeding program involved an eight way cross for 23 generations followed by inbreeding of mice selected for high, low and normal arterial pressure (AP) [27]. Mice were individually housed within a controlled temperature and humidity facility. They were placed on a light:dark cycle of 12:12 hours (1 am – 1 pm light) with access to water and mouse chow ad libitum. The experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. For further detail refer to the online-only Data Supplement.

Telemetry surgery

A radiotelemetry transmitter (TA11PA-C10, Data Sciences International) was implanted into the aorta via the carotid artery of each mouse under isoflurane open circuit anaesthesia (4% v/v induction and 1.5-2% v/v maintenance). A lateral incision and blunt dissection were used to expose the left carotid artery which was temporarily occluded using a non-absorbable silk tie (Dysilk 1-0, Dynek Pty Ltd, SA, Australia). The catheter of the telemetry device was inserted into the carotid artery and secured using silk ties and the body of the probe was positioned subcutaneously along the right flank [28]. Mobility, alertness, weight, AP and heart rate (HR) were monitored over the following ten days to ensure full recovery. Following recovery, systolic arterial pressure (SAP), diastolic arterial pressure (DAP), calculated MAP, HR and locomotor activity were recorded continuously in freely moving mice for a period of 72 hours. Cardiovascular and locomotor recordings were sampled at 1000 Hz using an analog-to-digital data acquisition card (National Instruments 6024E) as previously described [29, 30].

Protocol for chronic AlloP treatment

AlloP (5 mg/kg/day, Tocris, Ballwin, MO, USA) was dissolved in 50% w/v β-cyclodextrin (Research Biochemicals, Natick, MA, USA) in 0.9% w/v NaCl by vortexing and 20 minutes of sonication. The solution, in a volume of 100 µl was injected into 14 day osmotic minipumps (Alzet model 1002, Durect Corp, Cupertino, CA, USA) which infused the solution at a rate of 0.3 µl/hour. Anesthesia was induced in the same manner as for telemetry surgery. A 5 mm midline incision was made between the scapulae and two minipumps containing AlloP (each delivering 2.5 mg/kg/day) or its vehicle were inserted into a subcutaneous pouch such that they sat on the right and left flank of the mice. The dose

was chosen based on a pilot study (see supplemental Figure 1) in addition to a study conducted by Ferrera and colleagues who established that the dose of 5 mg/kg/day of Allop was sufficient to modulate the expression of the gene of another neurotransmitter in mice, the Y_1 subtype of NPY receptors [31]. Importantly, the dose is less than that required to induce sedative effects via $GABA_A$ receptors [32] and did not alter levels of activity during both stress tests and baseline recordings.

Assessment of cardiovascular reactivity in response to stress

All stress tests were conducted before implantation of the minipumps and 1-2 weeks following implantation. On separate days, mice were exposed to 60 minutes of aversive dirty cage switch stress and non-aversive feeding which followed a 60 minute control period. Dirty cage switch stress involved placing the mouse, in a cage previously occupied by another male mouse for a minimum of seven days, for a period of 60 min. This stimulus is associated with sustained pressor responses and increased locomotor activity [33]. Feeding involved fasting the mice overnight and presenting them with 0.5 g of almond every 10 minutes. This served as a positive appetitive stimulus. MAP, HR and locomotor activity were recorded and means were averaged over 10 minute periods for each mouse. All the data points within the 10- minute stress period, as indicated by the shaded box, were included in the analysis. Thus each 10-minute period represents the average of ~6000 individual measurements.

Cardiovascular response to ganglionic blockade

Cardiovascular parameters were measured for 30 minutes prior to and 30 minutes following intraperitoneal injection of pentolinium (5 mg/kg; Sigma-Aldrich) dissolved in 0.9% w/v NaCl. Injections were performed at least 2 hours after the onset of the active (dark) period at baseline and 1-2 weeks following implantation of minipumps.

Analysis of cardiovascular data

Cardiovascular data are expressed as mean ± standard error of the mean (SEM). The data were analysed by multi-factor, nested split-plot analysis of variance (ANOVA), which allowed for within and between animal factors [34]. Within animal factors included the time of day (active or inactive period) and the periods before and after implantation of minipumps. Between subjects factors included strain (BPH/2J and BPN/3J) and treatment (AlloP vs vehicle). A combined residual was used to pool the between- and within-animal variance, as described previously [35]. A two-tailed value of $P \leq 0.05$ was considered statistically significant.

Immunohistochemical analyses

BPH/2J and BPN/3J mice treated with vehicle or AlloP for two weeks (n=5 for all) were exposed to 60 minutes of dirty cage switch stress immediately prior to perfusion fixation. The perfusions were performed during the inactive period, 2-5 hours prior to lights out. Mice were deeply anaesthetised with sodium pentobarbitone (100 mg/kg i.p) and perfused transcardially with 25 ml 0.9% w/v NaCl and 60 ml 4% w/v paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.2 (PB) as previously described [8]. Using a cryostat, the brains were cut into 40 µm coronal sections and placed in PB. Free-floating sections were incubated in 10% normal horse serum at room temperature for one hour. Sections were then incubated in primary antibody, goat anti-c-Fos (Chemicon) diluted 1:1000 in a solution of 2% normal horse serum and 0.3% Triton X-100 (Sigma) in PB at room temperature overnight. Sections were washed in PB prior to incubation in biotinylated donkey anti-sheep immunoglobins (1:200, Jackson) in PB containing 2% normal horse serum for 1-hour. Thereafter, the sections were washed and incubated in avidin-biotin peroxidase complex (1:100, Vector) in PB for 1-hour. Following washes in 0.05 M Tris buffer (pH 7.6), sections were incubated in a solution of 40 mg nickel ammonium sulphate and 50 mg 3-3' diaminobenzidine hydrochloride per 100 ml Tris buffer for 10 mins. Following this, 15 µl of 30 % hydrogen peroxide was added for a further 6-mins. 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 the Fos-immunoreactivity in the MeAm and parvocellular region of the paraventricular nucleus (PaPVN) as detected by black stained nuclei. Fos staining was counted in four brain sections per region in each mouse within the boundaries of the MeAm and PaPVN as outlined in the mouse brain atlas [8].

Measurement of $GABA_A$ mRNA in the hypothalamus and medial amygdala

Age matched adult BPH/2J and BPN/3J mice treated with two weeks of AlloP or vehicle (n=5/group) were killed with an overdose (100 mg/kg) of pentobarbitone (Lethobarb, Virbac Animal Health, NSW, Australia) 2-4 hours after the onset of the dark period, when MAP is at its highest [36]. The PVN and dorsomedial hypothalamus (DMH) in addition to the MeAm, as defined by known anatomical boundaries [8], were removed immediately after death. Following extraction, brains were preserved in liquid nitrogen and later transferred to a -80C freezer. The RNeasy kit (Qiagen, Chadstone, Australia) was used for RNA extraction according to the manufacturer's recommendations. RNA purity was assessed using a NanoDrop and samples with a 260/280 absorbance lower than 1.9 were excluded. (Thermo Scientific) Complementary DNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit for total cDNA and the TaqMan® MicroRNA Reverse Transcription Kit for miRNA cDNA (Life Technologies). Amplification reactions used the TaqMan® Fast Advanced Master Mix (Life Technologies). TaqMan probes (Life Technologies) were used for gene expression to assess $GABA_A$ α1, α4, β2, γ2 and δ mRNA as identified in Table 1, together with reference genes. Samples were run in duplicates. A quantitative real-time PCR (qPCR) system (model ViiA™ 7 qPCR, Life Technologies) and the ΔΔCT method were used to determine the levels of

RESULTS

Cardiovascular parameters at baseline

DAP, MAP and SAP were 19-27 mmHg greater whilst HR was 94 bpm greater in BPH/2J mice than BPN/3J mice over a 24 hour period (baseline pooled from vehicle and AlloP group, (P_{strain}<0.001 for all, Table 2). In addition, locomotor activity was 1.6 fold greater in BPH/2J mice compared with BPN/3J mice (P_{strain}=0.004). Elevated cardiovascular parameters were most prominent during the night (active period) with day-night differences 2.6-4.3 fold greater in BPH/2J mice compared with BPN/3J mice (P_{strain}<0.001 for all).

Effects of chronic allopregnanolone treatment on cardiovascular parameters

Following two weeks' treatment with AlloP, 24-hour MAP was reduced in BPH/2J mice by -8.0 ± 2.7 mmHg (P_{AlloP}=0.02, Figure 1) but not after one week's treatment or at one or two

week's treatment in BPN/3J mice. The reduction in AP arose predominantly in SAP (-10.7 ± 2.4 mmHg; P_{AlloP}<0.001; Table 1) with only a modest reduction in 24-hour DAP observed (-5.7 ± 1.8 mmHg; P_{AlloP}=0.01). Percentage reductions in AP were similar across the active and inactive periods (P<0.58 for all). Additionally, no detectable changes in HR, locomotor activity or body weight were observed from week 0 in either strain or treatment group at weeks one or two.

Sympathetic contribution to blood pressure

Prior to intervention, pentolinium produced a greater depressor response in BPH/2J mice (-45.9 ± 2.0 mmHg) than BPN/3J mice (-29.7 ± 1.7 mmHg, P<0.001, Figure 2). Additionally, a bradycardic response was observed in BPH/2J mice of -105 ± 9.0 bpm which was not apparent in BPN/3J mice (+16.7 ± 16 bpm, P_{strain}<0.001). Following treatment with AlloP, the depressor and bradycardic responses to pentolinium were reduced by 15.3 ± 3.2 mmHg and 90.4 ± 23 bpm, respectively, in BPH/2J mice (P_{AlloP}=0.001 for MAP, P_{AlloP}=0.02 for HR) but remained similar in BPN/3J mice and in vehicle treated mice of either strain. No detectable difference in locomotor activity was observed in either strain or treatment groups before and following minipump implantation.

Cardiovascular response to the aversive restraint stress

Prior to intervention, the pressor response to restraint stress in BPH/2J mice (+45.7 ± 0.9 mmHg, Figure S2) was markedly greater than in BPN/3J mice (+26.3 ± 0.7 mmHg; baselines pooled from vehicle and AlloP groups). In contrast, there was a greater tachycardic response to restraint stress observed in BPN/3J mice (253 ± 15 bpm) compared with BPH/2J mice (213 ± 20 bpm, P_{strain}=0.03).

Following treatment with AlloP, the pressor response to restraint increased by +8.1 ± 1.8 mmHg in BPN/3J mice (P_{AlloP}<0.001) whilst it decreased by -5.9 ± 1.7 mmHg in BPH/2J mice (P_{AlloP}<0.03). However, the pressor response to restraint stress remained similar after minipump implantation in vehicle treated mice from both strains. There were no detectable changes in the tachycardic response to restraint stress before and after minipump implantation in either strain or treatment group.

Cardiovascular response to the aversive cage-switch stress

Prior to intervention, the pressor response to cage-switch stress was markedly greater in BPH/2J mice compared with BPN/3J mice. Dirty cage switch stress induced 85.2% greater pressor responses in BPH/2J mice (+33.3 ± 0.7 mmHg, Figure 3) compared with BPN/3J mice (+18.3 ± 0.5 mmHg, P_{strain}<0.001) prior to treatment. This was accompanied by increased locomotor activity that was 2 fold higher in BPH/2J mice (P_{strain}<0.001) and tachycardic responses which were comparable between strains (P_{strain}=0.35).

Following treatment with AlloP the pressor response to cage-switch stress was attenuated by 26.7 ± 4.5% in BPH/2J mice (+27.0 ± 1.2 mmHg) but augmented by 34.3 ± 2.3% in BPN/3J mice (+23.1 ± 0.7 mmHg) compared with pre-treatment (P_{AlloP}<0.002 for both). Furthermore, following treatment with AlloP the tachycardic response was augmented in BPN/3J mice (+71.5 ± 8.3 bpm, P_{AlloP}<0.001) but remained similar in BPH/2J mice. The pressor and tachycardic responses remained similar in vehicle treated mice before and after minipump implantation. Additionally, there was no detectable change in locomotor activity in either strain or treatment group.

Cardiovascular response to the non-aversive feeding stress

Contrary to dirty cage switch stress, BPN/3J and BPH/2J mice had similar pressor and locomotor responses to non-aversive feeding prior to intervention (P_{strain}<1 for both). Interestingly the tachycardic response was greater in BPN/3J mice compared with BPH/2J mice (+70 ± 11.6 bpm, P_{strain}<0.001). No detectable changes before and after minipump implantation were observed in the pressor, tachycardic or locomotor responses to feeding stress in either strain or treatment group.

Fos immunohistochemical analysis following cage switch stress

Fos expression in the MeAm and PaPVN was 1.3-1.5 fold greater in vehicle treated BPH/2J mice compared with BPN/3J mice (P_{strain} <0.03 for both, Figure 4 and 5). Average Fos expression per section was 63.1 and 25.4% less in BPH/2J mice treated with AlloP compared with vehicle in the PaPVN and MeAm respectively (P_{AlloP} <0.001 for both) but remained similar in vehicle and AlloP treated BPN/3J mice in both regions.

Expression of genes for $GABA_A$ receptor subunits

Vehicle treated BPH/2J mice had lower expression of the α4 (-1.6 fold), β2 (-3.7 fold) and δ (-4.8 fold) subunits of the $GABA_A$ receptor than BPN/3J mice in the PVN (P_{strain}<0.04 for all, Figure 6). AlloP treated BPH/2J mice had greater expression of the α4 (+1.8 fold), β2 (+2.5 fold) and δ (+7.6 fold) subunits of the $GABA_A$ receptor than vehicle treated BPH/2J mice (P_{AlloP}<0.03 for all). In contrast, expression of α4, β2 and δ subunits was similar in vehicle compared with AlloP treated BPN/3J mice. There was no detectable difference in the expression of α1 or γ2 subunits across either strain or treatment group

DISCUSSION

Our current findings provide evidence that differences in $GABA_AR$ function between hypertensive and normotensive Schlager mice may at least partially mediate the elevated sympathetic drive and subsequent hypertension in BPH/2J mice. This conclusion is supported by the observation that AlloP reduced resting AP, and the response to ganglion blockade, in BPH/2J mice but not BPN/3J mice. AlloP treatment was also associated with marked upregulation of the levels of message for subunits of the $GABA_AR$ associated with tonic neuronal inhibition and with reductions in neuronal activity. Importantly, after AlloP treatment the difference, in expression of the δα4β2 subunits of the $GABA_AR$ in the MeAm and hypothalamus, between BPH/2J and BPN/3J mice, was abolished. Thus, the maintenance of the hypertension in BPH/2J mice may arise through altered $GABA_AR$ structure and function contributing to reduced tonic neuronal inhibition of amygdalo-hypothalamic pathways and subsequent elevations in sympathetic activity.

In addition to their role in regulating AP at rest, the amygdala and hypothalamus are intricately involved in activating and maintaining the cardiovascular response to stress [37]. We included different types of stress in order to differentiate the various pathways within the CNS that mediate responses to stress and arousal. Cage swap stress is mainly an olfactory stimulus known to activate specific regions of the amygdala while restraint stress is an emotional aversive stress not involving olfactory pathways. Furthermore, an almond treat is a pleasurable arousal or "appetitive" arousal stimulus. Indeed, lower levels of neuronal activity in the MeAm and parvocellular region of the PVN in AlloP-treated BPH/2J mice were associated with

diminished pressor responses to aversive dirty cage-switch stress compared with mice that received vehicle. Interestingly, no differences between strains were observed in response to appetitive feeding stress, indicating that endogenous differences in the cardiovascular response to stress are limited to pathways that are activated in response to aversive stress. Furthermore, as there were no differences in the pressor response to feeding stress between BPH/2J mice receiving AlloP or vehicle, it appears as though treatment with AlloP selectively inhibits pathways that mediate the response to aversive stress and is not associated with arousal or appetitive stimulation during non-aversive stimulus. This establishes the complex manner in which treatment with AlloP affects the cardiovascular response to stress, and consequently provides some insight into the discrete pathways that may be affected. To add further complexity, AlloP resulted in increased pressor responses to aversive but not non-aversive stimulus in BPN/3J mice which is indicative of strain selective effects of the neurosteroid.

The paradoxical effects of AlloP upon the stress response between strains are suggestive of bimodal actions of AlloP. Previous literature demonstrates the bimodal effects of AlloP upon anxiety, whereby the anxiolytic effects of AlloP become deleterious above optimal dosing. The ability of AlloP to enhance the pressor response to aversive stress in BPN/3J mice but reduce it in BPH/2J mice is consistent with the proposition that endogenous levels of AlloP are greater in normotensive compared with hypertensive mice. This could explain how increasing AlloP levels in normotensive mice results in an enhanced pressor response to stress as levels of AlloP may be greater than those required to induce anxiolytic effects. Whilst neurosteroids such as tetrahydrodeoxycorticosterone (THDOC) and AlloP reduce basal activity of the HPA axis, counterintuitively, endogenous AlloP, released in response to acute stress can activate the HPA axis [38, 39]. These effects appear to be mediated by δ subunit containing receptors which, through a series of intracellular kinase processes, inactivate the major transporter involved in extruding chloride from the cell, KCC2 [38]. Consequently, GABAergic transmission switches from inhibitory to excitatory when potentiated through the subsequent collapse of the maintenance of the chloride gradient.

There is now strong evidence that the hypertension in BPH/2J mice is neurogenic. For example, in the present study we confirmed previous finding that ganglionic blockade produces a greater depressor response in BPH/2J mice compared with BPN/3J mice [8]. Previous studies have demonstrated that BPH/2J mice over-express molecules associated with catecholamine synthesis and storage and under-express molecules associated with catecholamine degradation [40]. Additionally, BPH/2J mice have altered levels of noradrenaline in regions of the brain involved in the regulation of AP [9], suggesting the hypertension is largely sympathetically mediated. Furthermore, when compared with BPN/3J mice, BPH/2J mice have over expression of renal renin and reduced activation of the microRNA 181a which binds renin message and inhibits the expression of renin. [41]. We suggest that these effects are also sympathetically mediated via the renal innervation. Therefore, it would be expected that decreases in AP, induced by AlloP in this strain, would be mediated by changes in sympathetic activity.

Our findings indicate that the antihypertensive effects of AlloP are mediated, at least in part, by inhibition of the SNS. AlloP reduced both resting AP and the depressor response to ganglionic blockade in BPH/2J mice. Importantly, both resting AP and the depressor response to pentolinium remained similar prior to and during treatment with AlloP in BPN/3J mice. Furthermore the greater reduction in SAP compared with DAP is congruent with a sympatho-inhibitory action of AlloP, potentially through increased arterial compliance [42-44]. Inhibition of the SNS would be expected to remove the tonic restraint on arterial distensibility and subsequently increase arterial compliance, producing a greater influence on SAP than DAP. The reduction in cardiovascular reactivity in response to stress following AlloP is also most likely driven by reductions in sympathetic drive as evidenced by reduced levels of activity in pre-sympathetic nuclei. However, without direct sympathetic recordings this remains to be elucidated. However, a sympatho-inhibitory effect of the neurosteroid is congruent with previous literature which has demonstrated that AlloP shifts the baroreflex function curve to a lower operating pressure and attenuates spinal reflex potentiation [24, 25].

The antihypertensive effects of AlloP likely result from a combination of acute allosteric activation of the $GABA_AR$, and more chronic effects mediated by orthosteric modulation of the $GABA_AR$. This is evident by the reduction in basal AP appearing only after two weeks' treatment with AlloP but not at one. Contrasting this, the reduction in the pressor response to aversive stress was observed after one weeks' treatment which may be indicative of a more rapid effect of AlloP upon the cardiovascular response to stress. As the antihypertensive and sympatho-inhibitory effects of AlloP are restricted to BPH/2J mice and AlloP is known to modulate the $GABA_AR$, it suggests that the differences in the biophysical and pharmacological profile of $GABA_AR$ between the two strains may be driving these effects. This conclusion is supported by the observation that AlloP selectively upregulated the levels of message for the δ, α4 and β2 subunits of $GABA_AR$ in BPH/2J but not BPN/3J mice. Peng and colleagues demonstrated that progesterone, for which AlloP is a metabolite, increased the expression of both δ and α4 subunits and attenuated the induction of spinal reflex potentiation [24]. Importantly, these effects were abolished by inhibition of the major enzyme catalysing the conversion of progesterone to AlloP, 5α reductase, with finasteride. Furthermore, the sympatho-inhibitory effects of another neurosteroid metabolised by 5α reductase, tetrahydrodeoxycorticosterone (THDOC), were abolished when $GABA_ARs$ were inhibited, further validating the GABA mediated sympatho-inhibitory actions of neurosteroids [45]. Whilst the mechanism by which administration of AlloP enables increased incorporation of δ and α4 subunits into the receptor remains largely unknown, it is postulated to involve phosphorylation by various subtypes of protein kinase C (PKC). Indeed, inhibiting PKC prior to AlloP treatment prevents the typical increase in extrasynaptic subunits following exposure to neurosteroids [46]. Interestingly, inhibition of PKC also abolishes the enhanced decay time of spontaneous inhibitory postsynaptic currents induced by AlloP [47]. Taken together these observations suggest that upregulation of δα4 $GABA_AR$ subunits is critical for reducing sympathetic potentiation and subsequently AP following treatment with AlloP.

Altered functionality of the $GABA_AR$ does not appear to be restricted to hypertensive Schlager mice but rather appears to be a characteristic of multiple forms of

hypertension. For example, Shimada and colleagues demonstrated that GABA rich chlorella produced selective hypotensive effects in hypertensive patients with no change in BP in normotensive individuals [48]. Most notably, this reduction in BP occurred after the same duration of treatment as that in the present study and, as in the current study, the most prominent changes were in SAP. Microinjection of the $GABA_AR$ agonist muscimol into the posterior hypothalamus produces a larger depressor response in SHR than in normotensive Wistar Kyoto (WKY) [49]. In addition to these changes, SHRs have been demonstrated to have reduced GABA turnover in the hypothalamus [50] and altered functioning of $GABA_AR$ [18]. Additionally, whilst the $GABA_A$ antagonist bicuculline increases the firing rate of neurons within the PVN in normotensive rats, the firing rate is inhibited or unchanged in SHRs [51]. This altered response of neurons to bicuculline culminates in a diminished pressor response in both SHRs and renal wrapped hypertensive rats [17, 18] further supporting the hypothesis of altered functionality of $GABA_AR$ in hypertension. Taken together, a lack of functional GABAergic inhibition, particularly in the hypothalamus, appears to be characteristic of hypertension across both numerous rodent models and in human hypertension. Thus, restoration of GABAergic function in the hypothalamus may represent a useful therapeutic avenue in hypertension.

The present study further highlights the importance of GABAergic inhibition in regulating AP and the CV response to stress in hypertensive rodents. To our knowledge this is the first study to explore the therapeutic potential of AlloP in a model of hypertension. Clinically there is a strong association with heightened activity of stress pathways with an exaggerated CV response to stress [12], which serves as a risk factor for the development of hypertension [6]. The attenuated pressor responses to stress following AlloP were shown to closely correlate with changes in stress induced Fos activity. Thus, impaired GABAergic inhibition may be a common characteristic of neurogenic hypertension. This conclusion is further supported by the association of the selective reduction in AP and sympathetic vasomotor drive following AlloP with enhanced expression of $GABA_A$ subunits that mediate tonic neuronal inhibition. Therefore, selectively modulating $GABA_AR$ with neurosteroids such as AlloP may offer a novel area of therapy to attenuate hyperactivity of neuronal pathways in neurogenic hypertension.

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Conflicts of Interest

There are no conflicts of interest.

REFERENCES

1. Grassi G, Cattaneo BM, Seravalle G, Lanfranchi A, Mancia G. Baroreflex control of sympathetic nerve activity in essential and secondary hypertension. Hypertension. 1998; 31 (1):68-72.
2. Anderson EA, Sinkey CA, Lawton WJ, Mark AL. Elevated sympathetic nerve activity in borderline hypertensive humans - evidence from direct intraneural recordings. Hypertension. 1989; 14:177-83.
3. Esler M, Jennings G, Biviano B, Lambert G, Hasking G. Mechanism of elevated plasma noradrenaline in the course of essential hypertension. J Cardiovasc Pharmacol. 1986; 8:S39-S43.
4. Floras JS, Hara K. Sympathoneural and haemodynamic characteristics of young subjects with mild essential hypertension. J Hypertens. 1993; 11 (6):647-55.
5. Schnall PL, Schwartz JE, Landsbergis PA, Warren K, Pickering TG. A longitudinal study of job strain and ambulatory blood pressure: results from a three-year follow-up. Psychosom Med. 1998; 60 (6):697-706.
6. Matthews KA, Katholi CR, McCreath H, Whooley MA, Williams DR, Zhu S, et al. Blood pressure reactivity to psychological stress predicts hypertension in the CARDIA study. Circulation. 2004; 110 (1):74-8.
7. Flaa A, Eide IK, Kjeldsen SE, Rostrup M. Sympathoadrenal stress reactivity is a predictor of future blood pressure: An 18-year follow-up study. Hypertension. 2008; 52 (2):336-41.
8. Davern PJ, Nguyen-Huu T, La Greca L, Head GA. Role of the sympathetic nervous system in Schlager genetically hypertensive mice. Hypertension. 2009; 54 (4):852-9.
9. Denoroy L, Sautel M, Schlager G, Sacquet J, Sassard J. Catecholamine concentrations in discrete brain nuclei and sympathetic tissues of genetically hypertensive mice. Brain Res. 1985; 340 (1):148-50.
10. Davern PJ, Jackson KL, Nguyen-Huu T, La Greca L, Head GA. Cardiovascular responses to aversive and non-aversive stressors in Schlager genetically hypertensive mice. Am J Hypertens. 2010; 23 (8):838-44.
11. Davern PJ, Jackson KL, Nguyen-Huu T, La Greca L, Head GA. Cardiovascular reactivity and neuronal activation to stress in Schlager genetically hypertensive mice. Neuroscience. 2010; 170 (2):551-8.
12. Gianaros PJ, Sheu LK, Matthews KA, Jennings JR, Manuck SB, Hariri AR. Individual differences in stressor-evoked blood pressure reactivity vary with activation, volume, and functional connectivity of the amygdala. J Neurosci. 2008; 28 (4):990-9.
13. DiMicco JA, Abshire VM. Evidence for GABAergic inhibition of a hypothalmic sympathoexcitatory mechanism in anesthetized rats. Brain Res. 1987; 402:1-10.
14. DiMicco JA, Stotz-Potter EH, Monroe AJ, Morin SM. Role of the dorsomedial hypothalamus in the cardiovascular response to stress. Clin Exp Pharmacol Physiol. 1996; 23 (2):171-6.
15. Dampney RA, Horiuchi J, Killinger S, Sheriff MJ, Tan PS, McDowall LM. Long-term regulation of arterial blood pressure by hypothalamic nuclei: some critical questions. Clin Exp Pharmacol Physiol. 2005; 32 (5-6):419-25.
16. Farrant M, Nusser Z. Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat Rev Neurosci. 2005; 6 (3):215-29.
17. Martin DS, Haywood JR. Reduced GABA inhibition of sympathetic function in renal-wrapped hypertensive rats. Am J Physiol Regul Integr Comp Physiol. 1998; 275 (5 Pt 2):1523-9.
18. Li DP, Pan HL. Role of gamma-aminobutyric acid (GABA)A and GABAB receptors in paraventricular nucleus in control of sympathetic vasomotor tone in

hypertension. J Pharmacol Exp Ther. 2007; 320 (2):615-26.
19. Marques FZ, Campain AE, Davern PJ, Yang YH, Head GA, Morris BJ. Global identification of the genes and pathways differentially expressed in hypothalamus in early and established neurogenic hypertension. Physiol Genom. 2011; 43 (12):766-71.
20. Davern PJ, Chowdhury S, Jackson KL, Nguyen-Huu T-P, Head GA. $GABA_A$ receptor dysfunction contributes to high blood pressure and exaggerated response to stress in schlager genetically hypertensive mice. J Hypertens. 2014; 32 (2):352-62.
21. Puia G, Santi MR, Vicini S, Pritchett DB, Purdy RH, Paul SM, et al. Neurosteroids act on recombinant human GABAA receptors. Neuron. 1990; 4 (5):759-65.
22. Hosie AM, Wilkins ME, da Silva HM, Smart TG. Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. Nature. 2006; 444 (7118):486-9.
23. Shen H, Gong QH, Aoki C, Yuan M, Ruderman Y, Dattilo M, et al. Reversal of neurosteroid effects at alpha4beta2delta GABAA receptors triggers anxiety at puberty. Nat Neurosci. 2007; 10 (4):469-77.
24. Peng HY, Chen GD, Lee SD, Lai CY, Chiu CH, Cheng CL, et al. Neuroactive steroids inhibit spinal reflex potentiation by selectively enhancing specific spinal GABA(A) receptor subtypes. Pain. 2009; 143 (1-2):12-20.
25. Laiprasert JD, Rogers RC, Heesch CM. Neurosteroid modulation of arterial baroreflex-sensitive neurons in rat rostral ventrolateral medulla. Am J Physiol Regul Integr Comp Physiol. 1998; 43 (4):R903-R11.
26. Heesch CM. Neurosteroid modulation of arterial baroreflex function in the rostral ventrolateral medulla. Auton Neurosci. 2011; 161 (1-2):28-33.
27. Schlager G. Genetic hypertension in mice. In: Ganten D, De Jong W, (editors). Handbook of Hypertension: Elsevier Science B.V.; 1994. p. 158-72.
28. Butz GM, Davisson RL. Long-term telemetric measurement of cardiovascular parameters in awake mice: a physiological genomics tool. Physiol Genomics. 2001; 5 (2):89-97.
29. Jackson KL, Head GA, Morris BJ, Chin-Dusting J, Jones E, La Greca L, et al. Reduced cardiovascular reactivity to stress but not feeding in renin enhancer knockout mice. Am J Hypertens. 2007; 20:893-9.
30. Head GA, Lukoshkova EV, Burke SL, Malpas SC, Lambert EA, Janssen BJ. Comparing spectral and invasive estimates of baroreflex gain. IEEE Eng Med Biol Mag. 2001; 20 (2):43-52.
31. Ferrara G, Serra M, Zammaretti F, Pisu MG, Panzica GC, Biggio G, et al. Increased expression of the neuropeptide Y receptor Y(1) gene in the medial amygdala of transgenic mice induced by long-term treatment with progesterone or allopregnanolone. J Neurochem. 2001; 79 (2):417-25.
32. Lancel M. The GABA(A) agonist THIP increases non-REM sleep and enhances non-REM sleep-specific delta activity in the rat during the dark period. Sleep. 1997; 20 (12):1099-104.
33. Lee DL, Webb RC, Brands MW. Sympathetic and angiotensin-dependent hypertension during cage-switch stress in mice. Am J Physiol Regul Integr Comp Physiol. 2004; 287 (6):R1394-8.
34. Snedecor GW, Cochran WG. Statistical Methods. 7th ed. Ames, Iowa: Iowa State University Press; 1980.
35. Korner PI, Badoer E, Head GA. Cardiovascular role of the major noradrenergic cell groups in the rabbit: analysis based on 6-hydroxydopamine-induced transmitter release. Brain Res. 1987; 435 (1-2):258-72.
36. Marques FZ, Campain AE, Yang YHJ, Morris BJ. Meta-Analysis of Genome-Wide Gene Expression: Differences in Onset and Maintenance Phases of Genetic Hypertension. Hypertension. 2010; 56:319-24.
37. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci. 2009; 10:397-409.
38. Sarkar J, Wakefield S, MacKenzie G, Moss SJ, Maguire J. Neurosteroidogenesis is required for the physiological response to stress: role of neurosteroid-sensitive GABAA receptors. J Neurosci. 2011; 31 (50):18198-210.
39. Guo AL, Petraglia F, Criscuolo M, Ficarra G, Nappi RE, Palumbo MA, et al. Evidence for a role of neurosteroids in modulation of diurnal changes and acute stress-induced corticosterone secretion in rats. Gynecol Endocrinol. 1995; 9 (1):1-7.
40. Friese RS, Mahboubi P, Mahapatra NR, Mahata SK, Schork NJ, Schmid-Schonbein GW, et al. Common genetic mechanisms of blood pressure elevation in two independent rodent models of human essential hypertension. Am J Hypertens. 2005; 18 (5 Pt 1):633-52.
41. Jackson KL, Marques FZ, Watson AMD, Palma Rigo K, Nguyen-Huu T-P, Morris BJ, et al. A novel interaction between sympathetic overactivity and aberrant regulation of renin by mir-181a in BPH/2J genetically hypertensive mice. Hypertension. 2013; 62 (4):775-81.
42. Mangoni AA, Mircoli L, Giannattasio C, Mancia G, Ferrari AU. Effect of sympathectomy on mechanical properties of common carotid and femoral arteries. Hypertension. 1997; 30 (5):1085-8.
43. Safar ME, Levy BI, Struijker-Boudier H. Current perspectives on arterial stiffness and pulse pressure in hypertension and cardiovascular diseases. Circulation. 2003; 107 (22):2864-9.
44. Boutouyrie P, Lacolley P, Girerd X, Beck L, Safar M, Laurent S. Sympathetic activation decreases medium-sized arterial compliance in humans. Am J Physiol. 1994; 267 (4 Pt 2):H1368-76.
45. Womack MD, Pyner S, Barrett-Jolley R. Inhibition by alpha-tetrahydrodeoxycorticosterone (THDOC) of pre-sympathetic parvocellular neurones in the paraventricular nucleus of rat hypothalamus. Br J Pharmacol. 2006; 149 (5):600-7.
46. Abramian AM, Comenencia-Ortiz E, Modgil A, Vien TN, Nakamura Y, Moore YE, et al. Neurosteroids promote phosphorylation and membrane insertion of extrasynaptic GABAA receptors. Proc Natl Acad Sci U S A. 2014; 111 (19):7132-7.
47. Fancsik A, Linn DM, Tasker JG. Neurosteroid modulation of GABA IPSCs is phosphorylation dependent. J Neurosci. 2000; 20 (9):3067-75.
48. Shimada M, Hasegawa T, Nishimura C, Kan H, Kanno T, Nakamura T, et al. Anti-hypertensive effect of gamma-aminobutyric acid (GABA)-rich Chlorella on high-normal blood pressure and borderline hypertension in placebo-controlled double blind study. Clin Exp Hypertens. 2009; 31 (4):342-54.
49. Wible JH, Jr., Luft FC, DiMicco JA. Hypothalamic GABA suppresses sympathetic outflow to the cardiovascular system. Am J Physiol Regul Integr Comp Physiol. 1988; 254 (4 Pt 2):R680-7.
50. Sasaki S, Kuwabara T, Yoshitomi T, Yoneda Y, Takenaka K, Takesako T, et al. Decreased hypothalamic and medullary GABA turnover in spontaneously hypertensive rats. Cardiovasc Res. 1992; 26 (3):261-4.
51. Li DP, Pan HL. Plasticity of GABAergic control of hypothalamic presympathetic neurons in hypertension.

Am J Physiol Heart Circ Physiol. 2006; 290 (3):H1110-9.

Table 1. Sequences of primers and TaqMan probes for mouse GABAA receptor subunits.

Gene	Primer/Probe Sequence
α1 (assay number Mm00439046)	
Forward:	5′-CAA GAG CAG AAG TTG TCT ATG AGT-3′
Reverse:	5′-GCA CGG CAG ATA TGT TTG AAT AAC-3′
Probe:	5′-6-FAM-ACC AGA GAG CCA GCC CGT TCA GTG-TAMRA-3′
α4 (assay number Mm00802631)	
Forward:	5′-AGA ACT CAA AGG ACG AGA AAT TGT -3′
Reverse:	5′-TTC ACT TCT GTA ACA GGA CCC C -3′
Probe:	5′-6-FAM-ACG CAG CCT GTT GTC ATA ACC ATC CAG C-TAMRA-3′
β2 (assay number Mm00433467)	
Forward:	5′-AAC TAC ATC TTC TTT GGG AGA GGA -3′
Reverse:	5′-GGT CCA TCT TGT TGA CAT CCA G -3′
Probe:	5′-6-FAM-CGC ATC TTC TCA TTG TTG GCA TTA GCA GC-TAMRA-3′
δ (assay number Mm01266203)	
Forward:	5′-GGA CGT TCT GGG CTG GC -3′
Reverse:	5′-CCT CCA TTA AGC CAT CCA GGT-3′
Probe:	5′-6-FAM-CTG CTG CCG CTC CTG CTG CTC T-TAMRA-3′
γ2 (assay number Mm0043349)	
Forward:	5′-ACT TCT GGT GAC TAT GTG GTG AT -3′
Reverse:	5′-GGC AGG AAC AGC ATC CTT ATT G -3′
Probe:	5′-6-FAM-AAG GAC ACC CAG GAC AGG ACC ACG A-TAMRA-3′
GADPH (assay number Mm99999915)	
Forward:	5′-CAA TGT GTC CGT CGT GGA TCT -3′
Reverse:	5′-GTC CTC AGT GTA GCC CAA GAT G-3′
Probe:	5′-6-FAM-CGT GCC GCC TGG AGA AAC CTG CC-TAMRA-3′
ACTH (assay number Mm00607939)	
Forward:	5′-GGC TGC TTT GCT TTC AGT TTT GTA G -3′
Reverse:	5′-CCC AGT TAC TAA GTG GTT TTT TTG CTT G-3′
Probe:	5′-6-FAM-TAC TGA GCT GCG TTT TAC ACC CTT T-TAMRA-3′

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.

Table 2: Average 24-hour SAP, DAP, HR and locomotor activity at baseline and weeks 1 and 2 during AlloP or vehicle treatment in BPN/3J and BPH/2J mice.

Vehicle		BPN/3J			BPH/2J			
	P strain	Baseline	Week 1	Week 2	Baseline	Week 1	Week 2	
SAP (mmHg)	†††	117 ± 2	120 ± 2	120 ± 2	144 ± 4	149 ± 3	144 ± 3	
DAP (mmHg)	†††	88.6 ± 2	89.9 ± 1	89.8 ± 1	109 ± 3	110 ± 3	106 ± 2	
MAP (mmHg)	†††	105 ± 2	105 ± 1	105 ± 2	126 ± 3	130 ± 3	125 ± 2	
HR (b/min)	†††	480 ± 13	476 ± 11	457 ± 10	572 ± 20	578 ± 20	531 ± 17	*
Activity (units)	††	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.5 ± 0.4	1.8 ± 0.4	1.4 ± 0.3	

Allopregnanolone		BPN/3J			BPH/2J			
	P strain	Baseline	Week 1	Week 2	Baseline	Week 1	Week 2	
SAP (mmHg)	†††	116 ± 2	118 ± 2	120 ± 1	144 ± 3	137 ± 5	132 ± 5	***
DAP (mmHg)	†††	86.8 ± 1	88.1 ± 2	88.5 ± 1	105 ± 3	102 ± 3	99.2 ± 3	*
MAP (mmHg)	†††	101 ± 1	103 ± 1	104 ± 1	124 ± 3	119 ± 4	115 ± 4	*
HR (b/min)	†††	467 ± 9	491 ± 9	479 ± 8	572 ± 24	584 ± 23	580 ± 23	
Activity (units)	†	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	

Systolic arterial pressure, SAP; diastolic arterial pressure, DAP; heart rate, HR. Values are mean ± SEM. Comparison of baseline with weeks 1 and 2 post-minipump implantation with allopregnanolone or vehicle in BPN/3J and BPH/2J represented by *P<0.05 and ***P<0.001. Between strain comparison of baseline measurements represented by †P<0.05, ††P<0.01 and ††† P< 0.001.

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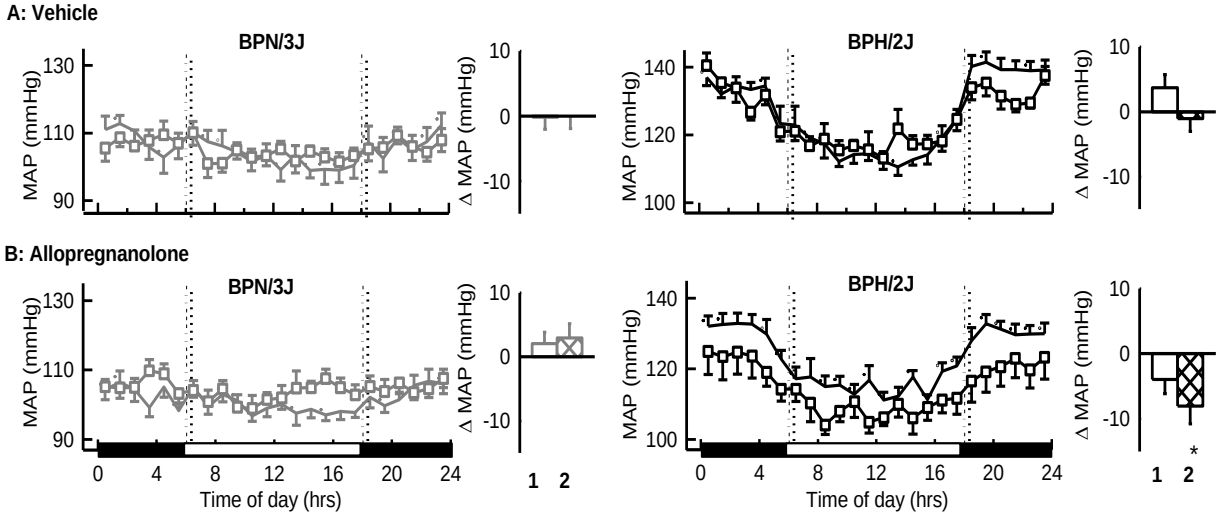

Figure 1: Effects of allopregnanolone (AlloP) and its vehicle (veh) on mean arterial pressure (MAP). Line graphs show hourly averages of MAP during the active (night, outer panels) and inactive (day, middle panel) periods in BPN/3J (left, grey n=8-9) and BPH/2J mice (right, black, n=7) at baseline (open symbol) and two weeks following minipump implantation (filled symbol). Histograms are mean ± SEM, indicating within animal variance, from baseline following one week (unhatched) or two weeks (hatched) treatment with veh (top panels) or AlloP (bottom panels) in BPN/3J and BPH/2J mice. Statistical analysis was conducted using multi-factor split-plot ANOVA and Bonferroni-adjusted pairwise comparisons both within strains and treatment groups. Difference from baseline * $P<0.05$.

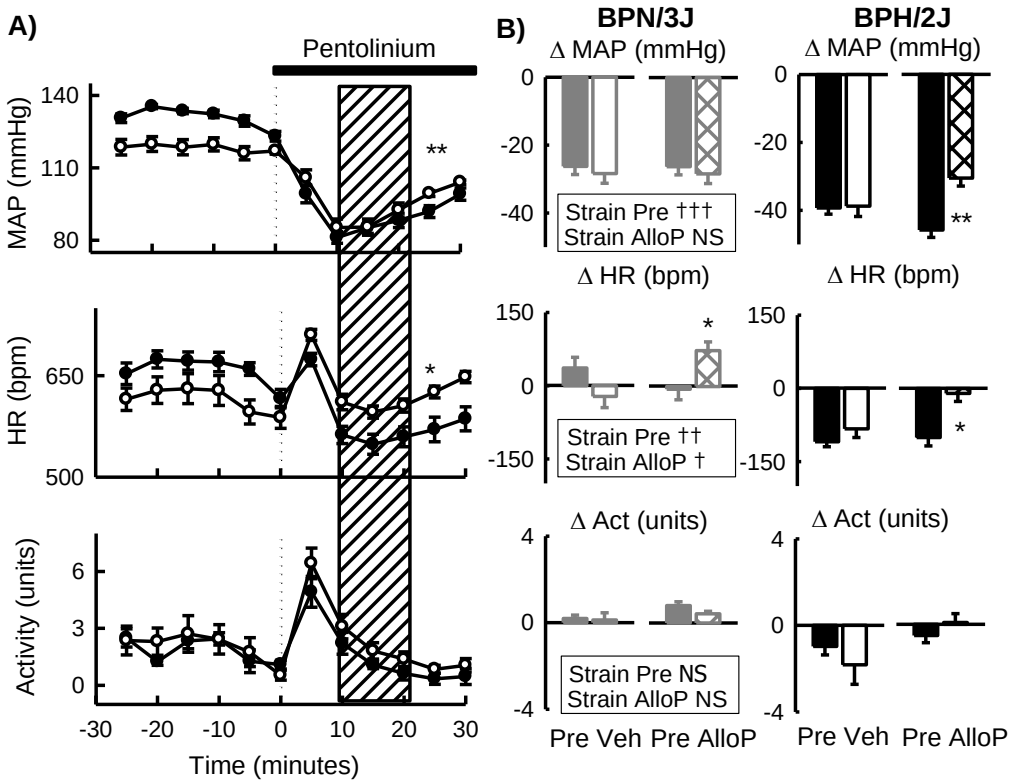

Figure 2 Effects of ganglion blockade. A) Line graphs represent the pooled mean arterial pressure (MAP, mmHg), heart rate (HR, beats/min) and activity (Act, units) responses to pentolinium (5 mg/kg) in BPH/2J mice prior to (n=6, filled circles) and during treatment with allopregnanolone (n=6, unfilled circles). B) Bar graphs represent average changes in MAP, HR and locomotor activity in response to pentolinium in BPN/3J and BPH/2J mice prior to (Pre, filled bars) and during treatment with vehicle (Veh, unfilled bars, n=8 for both) and allopregnanolone (AlloP, hatched bars, n=6-7). The changes represent the difference between the 30 minute control period and the shaded period 10-20 minutes following the injection. Values are mean ± SEM. Statistical analysis was conducted using multi-factor split-plot ANOVA and Bonferroni-adjusted pairwise comparisons both within strains and treatment groups. NS $P>0.05$, † $P<0.05$, †† $P<0.01$, ††† $P<0.001$ represent between strain comparisons before (Strain Pre) and during allopregnanolone treatment (Strain AlloP). *$P<0.05$ and ***$P<0.001$ represent comparisons within strains before and after minipump implantation.

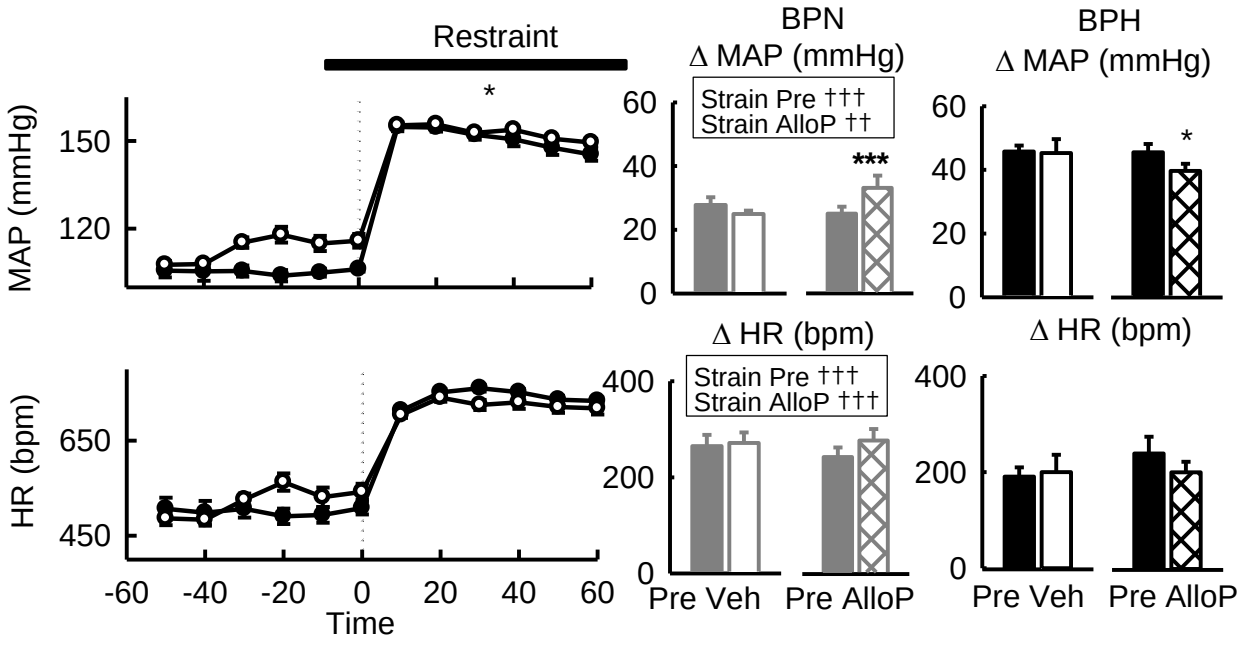

Figure 3 Cardiovascular response to restraint stress before and following treatment with allopregnanolone and its vehicle. Line graphs represent average mean arterial pressure (MAP), heart rate (HR, beats per minute) responses before and during restraint stress in BPH/2J mice prior to (n=7, filled circles) and during allopregnanolone treatment (n=7, AlloP, unfilled circles). Each circle represents mean ± SEM, averaged across a 10-minute period. Bar graphs represent average MAP and HR responses to restraint stress at baseline (pre, filled bars) and average response during vehicle (Veh, unfilled bars) or AlloP (hatched bars) treatment in BPN/3J (grey) and BPH/2J mice (black). Averages were calculated over 60 minutes of rest and 60 minutes of restraint stress in each animal. Values are mean ± SEM. Statistical analysis was conducted using multi-factor split-plot ANOVA and Bonferroni-adjusted pairwise comparisons both within strains and treatment groups. **††** $P<0.01$, **†††** $P<0.001$ represent between strain comparisons prior (Strain Pre) and during AlloP treatment (Strain AlloP). *$P<0.05$ and ***$P<0.001$ represent comparisons within strains before and after treatment.

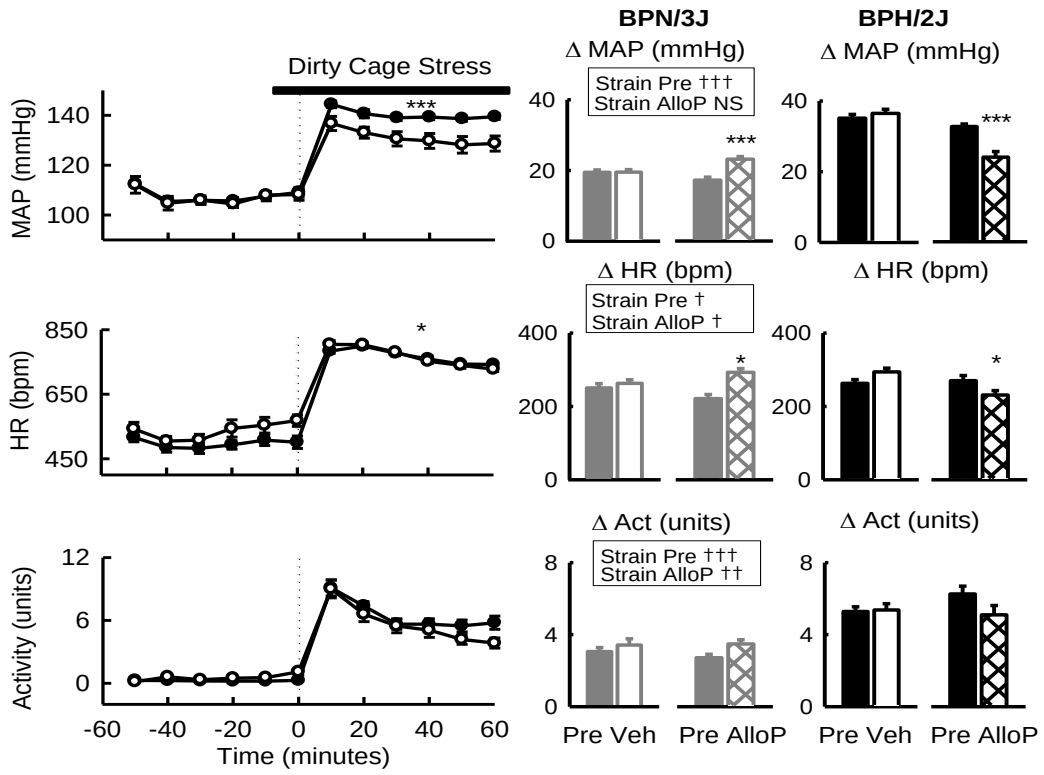

Figure 4 Cardiovascular response to dirty cage stress before and following treatment with allopregnanolone and its vehicle. Line graphs represent average mean arterial pressure (MAP), heart rate (HR, beats per minute) and locomotor activity (Act) responses before and during dirty cage switch stress in BPH/2J mice prior to (n=7, black filled circles) and during treatment with allopregnanolone (n=7, AlloP, unfilled circles). Each circle represents mean ± SEM, averaged across a 10-minute period. Bar graphs represent average MAP, HR and locomotor activity response to the stimuli at baseline (pre, filled bars) and average response during vehicle (Veh, unhatched bars, n=7-8) or AlloP (hatched bars, n=7-9) treatment in BPN/3J (grey) and BPH/2J (black) mice. Averages were calculated over 60 minutes of rest and 60 minutes of feeding stress in each animal. Values are mean ± SEM. Statistical analysis was conducted using multi-factor split-plot ANOVA and Bonferroni-adjusted pairwise comparisons both within strains and treatment groups. NS P>0.05, † P<0.05, †† P<0.01, ††† P<0.001 represent between strain comparisons before (Strain Pre) and during allopregnanolone treatment (Strain AlloP). ***P<0.001 represents comparisons within strains before and after minipump implantation.

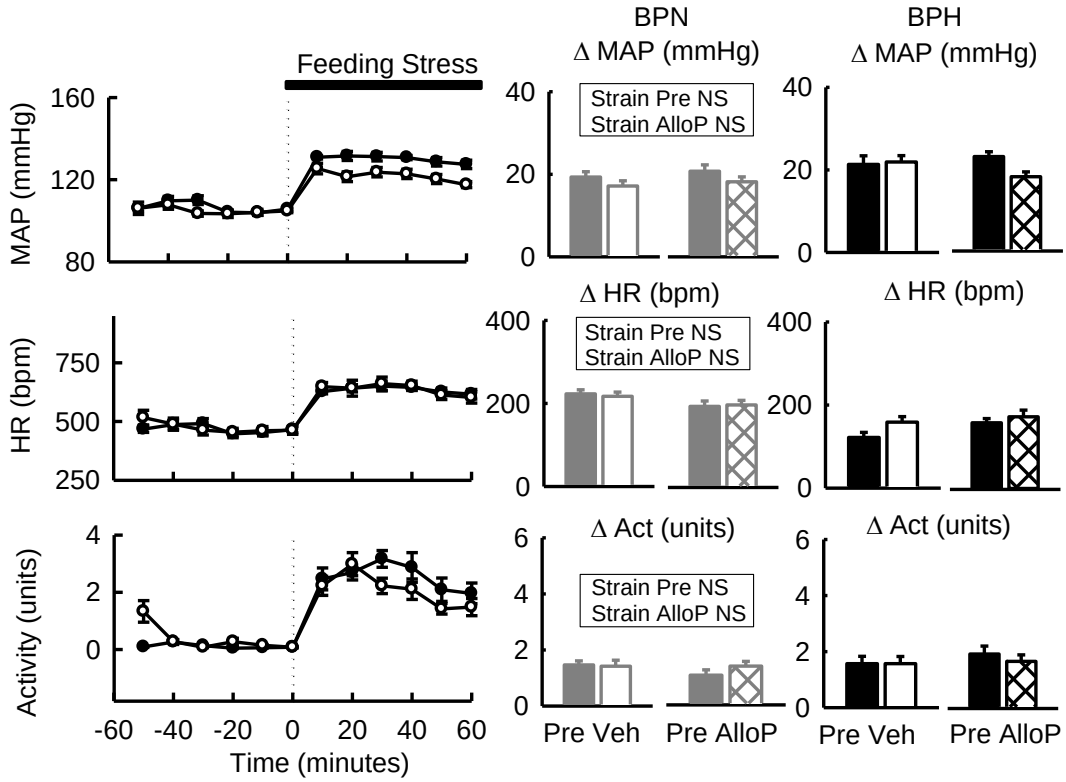

Figure 5

Cardiovascular response to feeding before and following treatment with allopregnanolone and its vehicle. Line graph represent average mean arterial pressure (MAP), heart rate (HR, beats per minute) and locomotor activity (Act) responses before and during feeding stress in BPH/2J mice prior to (filled circles) and during allopregnanolone treatment (AlloP, unfilled circles, n=6). Each circle represents mean ± SEM, averaged across a 10-minute period. Bar graphs represent average MAP, HR and locomotor activity response to the stimuli at baseline (pre, filled bars) and average response during vehicle (Veh, unfilled bars, n=7-8) or AlloP (hatched bars, n=7-9) treatment in BPN/3J and BPH/2J mice. Averages were calculated over 60 minutes of rest and 60 minutes of feeding stress in each animal. Values are mean ± SEM. Statistical analysis was conducted using multi-factor split-plot ANOVA and Bonferroni-adjusted pairwise comparisons both within strains and treatment groups. NS represents between strain comparisons prior (Strain Pre) and during AlloP treatment (Strain AlloP). *P<0.05 represent comparisons before and after treatment.

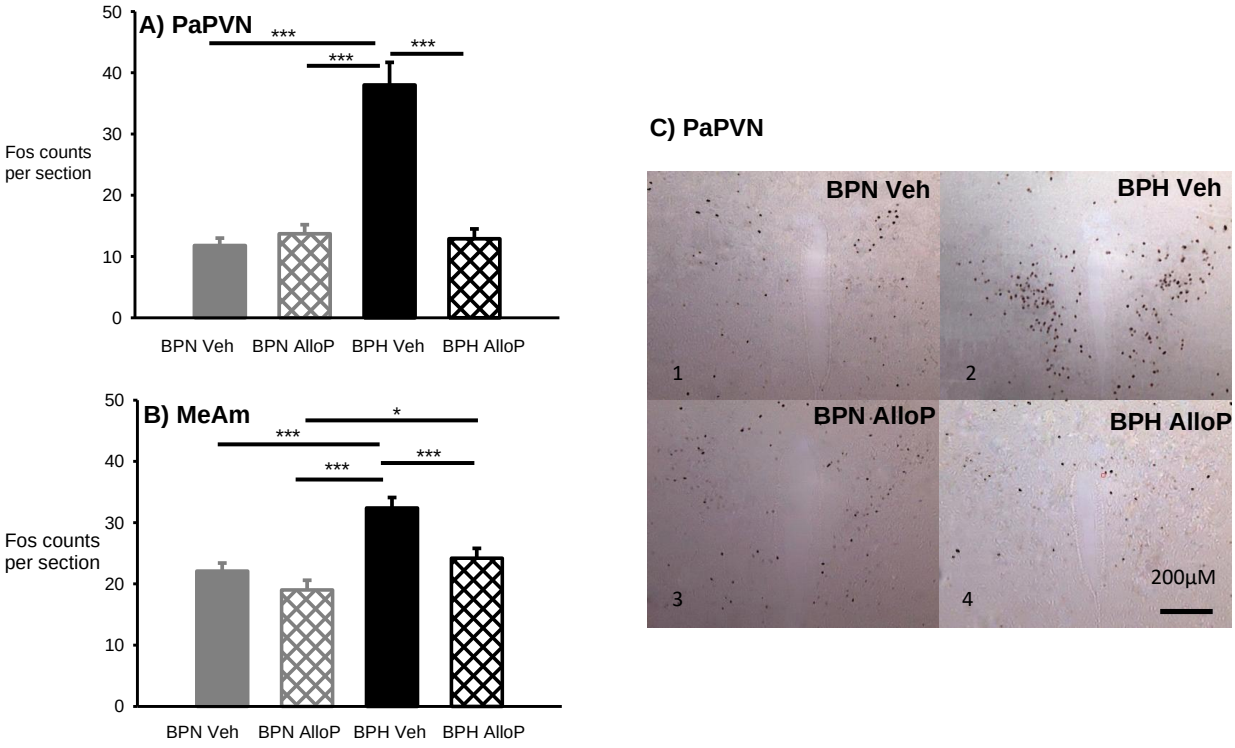

Figure 6

Fos counts in A) the parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus and B) the medial amygdala (MeAm) in hypertensive (BPH, black bars) and normotensive (BPN, grey bars) mice treated with allopregnanolone (AlloP, hatched bars) or vehicle (Veh, filled bars). Bars represent mean ± SEM Fos counts. * $P<0.05$ and*** $P<0.001$ represents comparison between strains and treatment groups using t tests. Photomicrographs depict dirty cage switch stress induced Fos expression within the PVN in BPN/3J (1 and 3) and BPH/2J (2 and 4) mice treated with vehicle (top panels) or AlloP (bottom panels). Sections were cut coronally through the PVN and imaged using a Motic BA400 microscope.

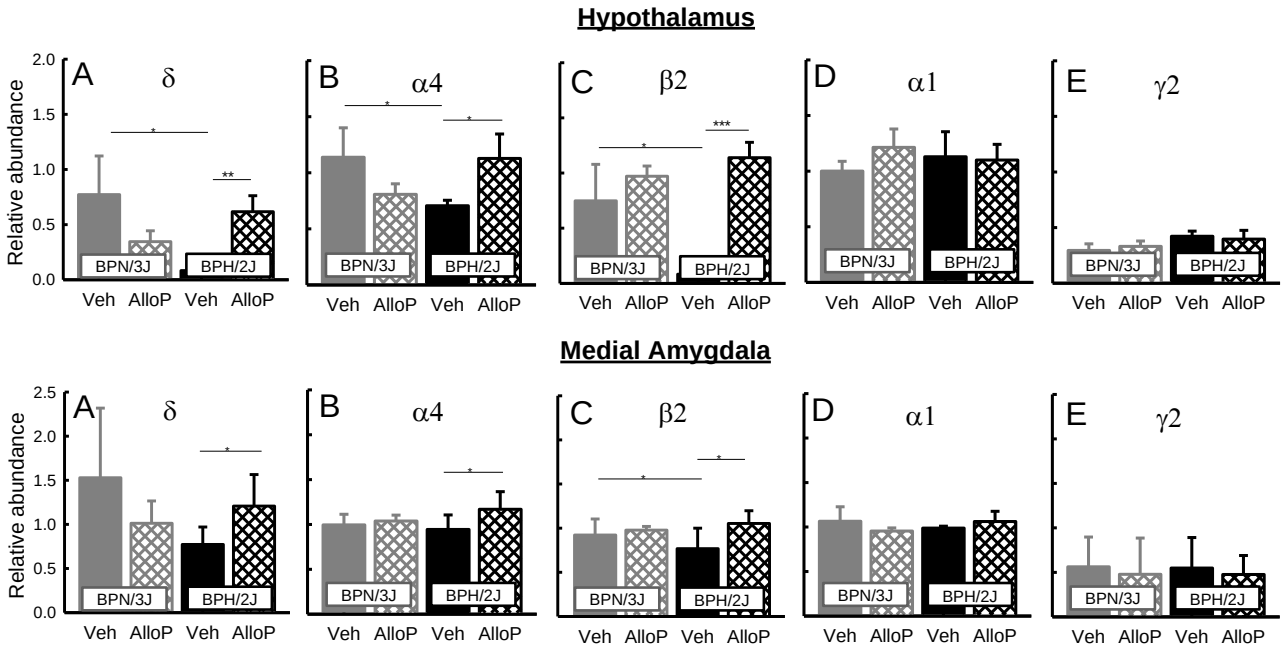

Figure 7

Relative levels of message of $GABA_A$ subunits in BPH/2J and BPN/3J mice treated with allopregnanolone and its vehicle in the hypothalamus and medial amygdala. Bars represent the mean ± SEM mRNA abundance (2-Δ ΔCT) of **A)** $GABA_A$ δ, **B)** $GABA_A$ α4, **C)** $GABA_A$ β2, **D)** $GABA_A$ α1 and **E)** $GABA_A$ γ2 subunits in BPN/3J (grey) and BPH/2J (black) mice following two weeks treatment with vehicle (Veh, filled bars) and allopregnanolone (AlloP, hatched bars) in the paraventricular nucleus of the hypothalamus. Reference mRNA (ACTB and Gapdh) levels were not different between the two groups. * $P<0.05$, ** $P<0.01$ *** $P<0.001$ represents comparison between strains and treatment groups using t tests.