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Research paper

Altered GABA$_A$ α5 subunit expression in the hypothalamic paraventricular nucleus of hypertensive and pregnant rats

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HIGHLIGHTS

- Common to hypertension and pregnancy is exaggerated sympathetic activity.
- Characterisation of the GABA$_A$ receptor expression in the PVN.
- Significant decrease in α5 subunit GABA$_A$ receptor in the PVN.
- Altered α5 subunit GABA$_A$ expression may be a contributor to sympatho-excitation.

ARTICLE INFO

ABSTRACT

A characteristic of both hypertension and pregnancy is increased sympathetic nerve activity. The level of sympathetic activation is determined, in part, by a tonic GABAergic inhibition arising from the hypothalamic paraventricular nucleus (PVN). In hypertension, decreases in GABAergic inhibition and increases in glutamatergic excitation within the PVN contribute to this sympatho-excitation. In late-term pregnancy however, the sympatho-excitation appears to be mediated by decreases in GABAergic inhibition only. This study examined whether changes in subunit expression for GABA$_A$ receptors in the PVN could provide a molecular basis for the sympatho-excitation characteristic of hypertension and pregnancy. Hypertension and pregnancy were accompanied by significant decrease in the GABA$_A$ receptor α5 subunit in the PVN. We suggest that decreases in the α5 subunit of the GABA$_A$ receptor may be important in mediating the sympatho-excitation observed in both hypertension and pregnancy.

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1. Introduction

The hypothalamic paraventricular nucleus (PVN) is a critical site for central integration of sympathetic outflow to the cardiovascular system [27]. Furthermore, presympathetic PVN neurons have consistently been identified as making a significant contribution to the sympatho-excitation evident in (patho) physiological conditions [3,7,15].

The PVN contains both GABA and NMDA receptors and the normal tonic inhibition of vasomotor sympathetic nerve activity is dependent upon activation of GABA$_A$ with suppression of NMDA receptor activity [18]. In the PVN of the spontaneously hypertensive rat (SHR), the activity of the GABA$_A$ receptor is attenuated and

NMDA receptor is potentiated, whereas in late-term pregnant rats only GABA$_A$ receptor attenuation is evident [15,16,19,23].

GABA$_A$ receptors comprise five subunits assembled from a pool of 19 with the individual GABA$_A$ receptor subunits having distinct anatomical distributions throughout the brain [26]. GABAergic inhibition within the PVN can be phasic (synaptically-mediated) or tonic (extrasynaptic) and these actions are mediated by receptors with physiological and pharmacologically distinct properties as well as subcellular localization. Receptors mediating tonic inhibition are primarily composed of the α5 or δ subunits that preferentially target the receptor to the extra synaptic membrane [6]. Studies investigating the role of the α5 subunit in mediating tonic inhibition have found that within the supraoptic nucleus (SON) of rats, the α5 subunit contributes to tonic inhibition to a much greater extent than the δ subunit with α5 subunit expression being 400 times greater than that of the δ subunit [14]. Although the SON and PVN are two anatomically distinct nuclei, their similar neurochemistry and cellular make up suggests that the control of tonic inhibition could be controlled in a similar manner.

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Hypertension and pregnancy are two conditions that display significant elevations in sympathetic nerve activity (SNA) resulting, in part from disruption in the neurochemical milleu of the PVN [7,9,13]. In hypertension, this sympatho-excitation results in an increase in blood pressure, whereas in pregnancy the sympatho-excitation contributes to hypervolaemia [9,10,13].

In normotensive rats, excitatory networks within the PVN are heavily inhibited by GABAergic inputs [18]. In the SHR, these inhibitory networks are compromised leading to increases in the activity of the presympathetic neurons [19]. In addition in pregnancy, which is characterised by increase in cardiac output, blood volume and plasma and brain concentration of hormones including oestrogen and progesterone, again inhibitory networks within the PVN are compromised [4,11–13]. Progesterone and its metabolites are known modulators of GABA$_A$ receptors, particularly those located extrasynaptically [4,21,28]. Application of progesterone into cardiovascular centres in the virgin rat brain has been shown to produce cardiovascular effects that mimic those of the pregnant rat [11,29].

This study sought to ascertain whether the (patho)physiological states of hypertension and pregnancy share a common molecular GABA$_A$ receptor subunit alteration that might help to explain the characteristic sympatho-excitation present in both of these conditions.

2. Material and methods

2.1. Ethics statement

A total of 35 animals were used in this study. All experiments were approved by the Local Ethics Committee of Durham University and performed in accordance with UK Animals (Scientific Procedures) Act, 1986. Animals were housed under a 12 h light: dark cycle and allowed *ad libitum* access to food and water. Age matched (14 week old) female Wistar control, (Harlan, n = 14) and female SHR (Harlan, n = 10) and timed pregnant Wistar (Harlan) or mated in house (n = 11) were used. The presence of a vaginal plug was defined as day 1 of pregnancy and late-term animals were sacrificed on day 19 [30]. All surgical procedures were carried out on anaesthetised animals, which were killed with an overdose of sodium pentobarbitone at the termination of the experiment.

2.2. Measurement of arterial blood pressure

Arterial blood pressure (aBP) was measured in a subset of each group of rats (female Wistar control, SHR and late-term pregnant Wistar, n = 3 each group) prior to perfusion. Under isofluorane (5% in 6 L/min O$_2$) anaesthesia a cannula was inserted into the aorta, this anaesthetic was then withdrawn and rats infused with a combination of α-chloralose (75 mg/kg) and urethane (700 mg/kg). Arterial blood pressure was then recorded using a pressure transducer and converted to a digital signal (PowerLab 8/30; ADInstruments). The signal was displayed using LabChart (v 7) on an iMac computer. Once a stable recording of aBP was achieved the rats then progressed to the perfusion protocol with the terminal anaesthesia being the α-chloralose-urethane mixture.

2.3. Tissue preparation for immunohistochemistry

Three groups of rats (female Wistar control, n = 4; SHR, n = 4 and late term pregnant Wistar, n = 4) underwent perfusion-fixation with paraformaldehyde (PFA) following a protocol we have previously described [1]. The brains were removed, post-fixed overnight (4% PFA) at 4 °C and cryprotected in 20% sucrose until sectioned.

2.4. Immunohistochemistry for GABA$_A$ receptor α5 subunit

Immunohistochemistry was carried out on 40 μm thick free-floating sections, prepared with a freezing microtome. Every second section underwent immunohistochemical staining and the adjacent section counterstained with cresyl violet (0.5%) to aid in anatomical identification of PVN subnuclei (Figure Bii). Sections were incubated in 3% hydrogen peroxide (H$_2$O$_2$) for 5 min, followed by antigen retrieval using Liberate Antibody Binding Solution (L.A.B, Polysciences, Inc) for 5 min. The sections were blocked for 60 min at room temperature (RT) in PB pH 7.4 containing 0.4% Triton-X-100, 10% normal goat serum (NGS). The antibody diluent for both primary and secondary antibodies was PB, pH7.4 containing 1% NGS and 0.4% Triton-X-100. After blocking, sections were incubated overnight at RT in primary α5 subunit antibody (gift of Professor W Sieghart) at a concentration of 3 μg/ml. Following this, the sections were rinsed and washed in PB (3 × 5 min) and then incubated in biotinylated goat anti-rabbit secondary antibody (1:200) for 2 h (RT). This was followed by Vectastain ABC reagent (Vector) for 1 h, made up per manufacturer's instructions. The peroxidase staining was revealed with 0.05% 3′3-diaminobenzidine (DAB) and 0.0033% H$_2$O$_2$. Sections were then mounted on gelatinized slides and air-dried. The sections were dehydrated through increasing concentrations of alcohol, cleared in xylene and a coverslip positioned using DPX mounting medium.

2.5. Retrograde labeling of PVN-spinally projecting presympathetic neurons

To aid in anatomical identification of PVN subnuclei and location of PVN-spinally projecting neurons the retrograde tracers cholera toxin B subunit (CTB) or Fluorogold (FG) were injected into T2 segmental level of the spinal cord. Briefly, seven female Wistar control rats were anaesthetised with medetomidine (0.25 ml/100 g) and ketamine (0.06 ml/100 g) and either 2 μl of CTB (0.5%) or FG (2%) were pressure injected into the left side spinal cord inter-mediolateralis. On completion of surgery analgesia in the form of buprenorphine (0.01 ml/100 g) was administered. The animals recovered for 7–14 days before being perfused-fixed as previously described.

2.6. Visualisation of CTB-labeled PVN-spinally projecting neurons

Free-floating 40 μm sections were rinsed in 0.1 M PB followed by a 60 min incubation (RT) in blocking buffer (10% normal horse serum [NHS]-0.4% Triton-X-100). The sections were then placed in incubation buffer (1% NHS-0.1 M PB) containing anti CTB (1:200,000) for 2 days (RT). After removal of primary antibody the sections were rinsed in PB (2 × 5 min) and then ExtraAvidin-HRP (1:1500) added for a 4 h RT incubation. After a further rinse the sections were incubated in 0.05% DAB and 0.0033% H$_2$O$_2$ PB solution. The sections then under went a final rinse before mounting on gelatinised slides, dehydrated and coverslipped as previously described.

2.7. Image, acquisition, visualization and analysis

Sections were examined using a Zeiss Axioskop 2 microscope under bright field illumination for the presence of GABA$_A$ receptor α5 subunit within the PVN. Epifluorescence was used to examine the relationship between FG-PVN spinally projecting neurons and the α5 subunit.

All images were captured using either a Hamamatsu Orca 285-CCD camera or Mircopublisher 3.3 RTV camera controlled by Improvision Volocity software (v 6.2.1). Final images were imported into Adobe Photoshop (CS4 extended v 11.02), which was

used to adjust brightness and contrast. Images were grouped into plates and labeled in Photoshop.

For receptor subunit analysis, the pixel size of the image was calibrated using a 1 mm graticule and using an on-screen axis, a 75 μm × 75 μm square was drawn. This was placed randomly within the anatomical boundaries of the PVN subnuclei throughout its rostro-caudal extent [25]. Cell counts of neurons expressing the receptor subunits were performed using the on-screen counting tool. Cell counts were performed on one of each sub nucleus per animal at the same rostrocaudal level. A random selection of the sections for the different groups underwent blind counting to ensure no observer bias. No correction was made for double counting and only cells that expressed morphological characteristics of a neuron (soma or associated with identifiable projections) were counted.

For PVN-FG spinally projecting neurons and receptor subunit of interest, images were acquired by capturing a series of z-stacks sampled at 0.3 μm using standard wide field fluorescence. The z-stack was then deconvolved using iterative restoration and the appropriate point spread function to produce a confocal-like image. The image was then converted to a 3-D Opacity rendered view and then displayed as a 3-D non-transparent solid of the surface element (Isosurface) [1]. Quantification to identify the number of FG-spinally projecting neurons expressing receptor subunits was carried out.

2.8. Antibody specificity

The specificity of α5 was determined on production by preabsorption with their respective synthetic peptide and confirming the absence of staining by immunohistochemistry [26]. As a further check control sections were incubated with the omission of primary or secondary antibodies and with this regime no staining was observed.

2.9. Microdissection of PVN and cortex and isolation for immunoblot analysis

Rats from the three groups (n = 6 for each group) were anaesthetized with isofluorane as previously described and then rapidly killed by an intracardiac injection of sodium pentobarbitone. The brains were quickly removed, flash frozen in −20 °C isopentane for 3 min and stored at −20 °C until ready for use. The brains were mounted onto a freezing microtome and two 600 μm sections were taken through the PVN. Using the third ventricle as an anatomical landmark, bilateral PVN micropunches were taken for each brain and placed in sample buffer [30 mM NaH_2PO_4, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue, 7.5% (w/v) sodium dodecyl sulphate (SDS) made up in dH_2O] containing a protease inhibitor cocktail (1:100). The samples were vortexed vigorously for 10 min to solubilise the proteins and then stored at −20 °C until required.

To ascertain whether any changes in $GABA_A$ receptor subunit expression detected for the PVN were specific to this region, the level of protein expression for the α5 subunit was analysed from cortex tissue isolated from the same animals. From the two 600 μm sections where the PVN was punched, the cortex (bilateral) dorsal to the corpus callosum was removed. These were homogenized using a glass–glass dounce in homogenization buffer (50 mM Tris base, 5 mM EDTA, 5 mM EGTA, 150 μM protease inhibitor cocktail, pH 7.4).

2.10. Immunoblot

The total protein concentration from the cortex samples was determined using a Lowry assay [20]. Protein concentration was not determined for PVN micropunches due to low protein levels, but

differences in protein levels was corrected by probing with β-actin. Immunoblots of PVN micropunches were performed as previously published [2]. Briefly, 10 μl aliquots of each micropunch were subjected to electrophoresis on a 10% acrylamide gel and transferred to nitrocellulose. The primary antibodies to $GABA_A$ receptor α5 subunit (1 μg/ml) was exposed to the nitrocellulose overnight at 4 °C followed by HRP-linked goat anti-rabbit secondary (1:2000) for 1 h. Protein bands were then visualised using luminol (68 mM luminol, 1.25 mMp- couramic acid, 30% H_2O_2) and exposed to Hyperfilm^{TM}.

2.11. Statistical analysis

All cell counts were subjected to a one-way analysis of variance (ANOVA) with Bonferroni post-hoc test and expressed ± SEM. For receptor protein expression images were quantified using ImageJ and band density (standardized against β-actin) analysed as for cell counts. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Physiological parameters of female wistar control, SHR and late-term pregnant rats

Female Wistar control rats had a mean weight of 199 ± 2 g (n = 14). Female SHR weighed 178 ± 2 g (n = 10) and pregnant Wistar group had a mean weight of 299 ± 7 g (n = 11). For the pregnant animals, between 6 and 14 (mean 10 ± 0.7) fetuses were *in utero*. The foetus mean weight was 2.16 ± 0.15 g placing them between gestation dates 19–20 [30].

The mean aBP of female SHR, 150 ± 5 mmHg (n = 3) was significantly greater than the female Wistar control (72 ± 5 mmHg P < 0.001, n = 3). Furthermore, the mean blood pressure of the late term pregnant rats was also significantly higher than the Wistar control (90 ± 2 mmHg vs. 72 ± 5 mmHg, P < 0.05, n = 3) (Fig. 1A).

3.2. Distribution of PVN-CTB labeled spinally projecting neurons

Injection of CTB (n = 3) into the spinal cord T2 region of the female Wistar control rat resulted in the labeling of ipsilateral PVN neurons throughout its rostrocaudal extent (Fig. 1Bi). The majority of CTB-labeled neurons were found in the mid-to caudal levels of the PVN (Bregma −1.80 mm to −2.12 mm). Cholera toxin B subunit labeled neurons were also evident in the contralateral PVN. No CTB-labeled neurons were found in other hypothalamic nuclei outside the boundaries of the PVN. The CTB labeled a mean of 1090 ± 92, range 998–1182 neurons on the ipsilateral side.

Both the soma and dendritic projection contained the CTB and these labeled neurons were predominantly found in the 5 parvocellular regions of the PVN (Fig. 1Bi,Bii). Some labeled neurons were observed in the magnocellular subnuclei as previously reported [22,27].

3.3. $GABA_A$ receptor α5 subunit

Only the female Wistar control and late-term pregnant rats showed a rostrocaudal distribution of $GABA_{A\alpha}$5-IR neurons within the PVN (Fig. 2A, C, D). There was an absence of $GABA_{A\alpha}$5-IR neurons throughout the PVN of the SHR (Fig. 2B, D). Furthermore, late-term pregnancy was associated with a significant decrease in α5-expression in the medial (56 ± 4%, P < 0.01), ventral (60 ± 2%, P 0.01) and dorsal cap (52 ± 6%, P < 0.05) parvocellular region when compared to the non-pregnant controls (Fig. 2D).
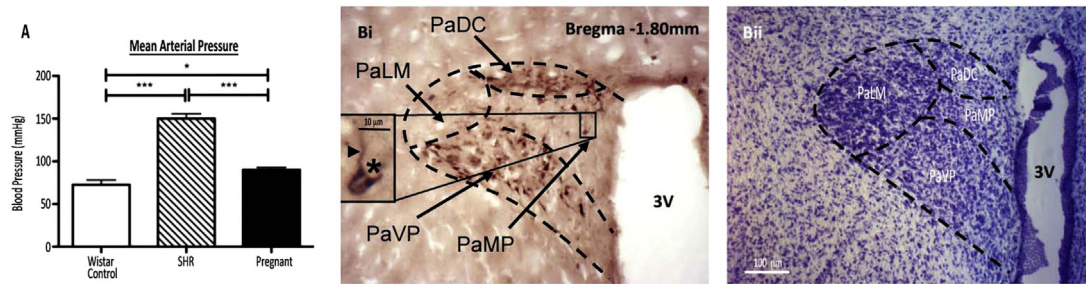
Fig. 1. (A) Mean arterial blood pressure of female Wistar control, female SHR and late-term pregnant rats. SHR's had a significantly higher blood pressure than both the Wistar control ($P < 0.001$) and pregnant ($P < 0.001$) rats. Pregnant rats also had a significantly higher blood pressure than the Wistar control ($P < 0.05$). CTB-spinally projecting neurons and corresponding cresyl violet conterstained section at bregma 1.80 mm outlining parvocellular sub nuclei (Bi-Bii). Inset image (Bi) shows magnified view of boxed area. Scale bar Bi-Bii = 100 μm, inset = 10 μm. PaDC—dorsal cap, PaVP—ventral parvocellular, PaLM—lateral magnocellular, PaMP—medial parvocellular, 3V—3rd ventricle.
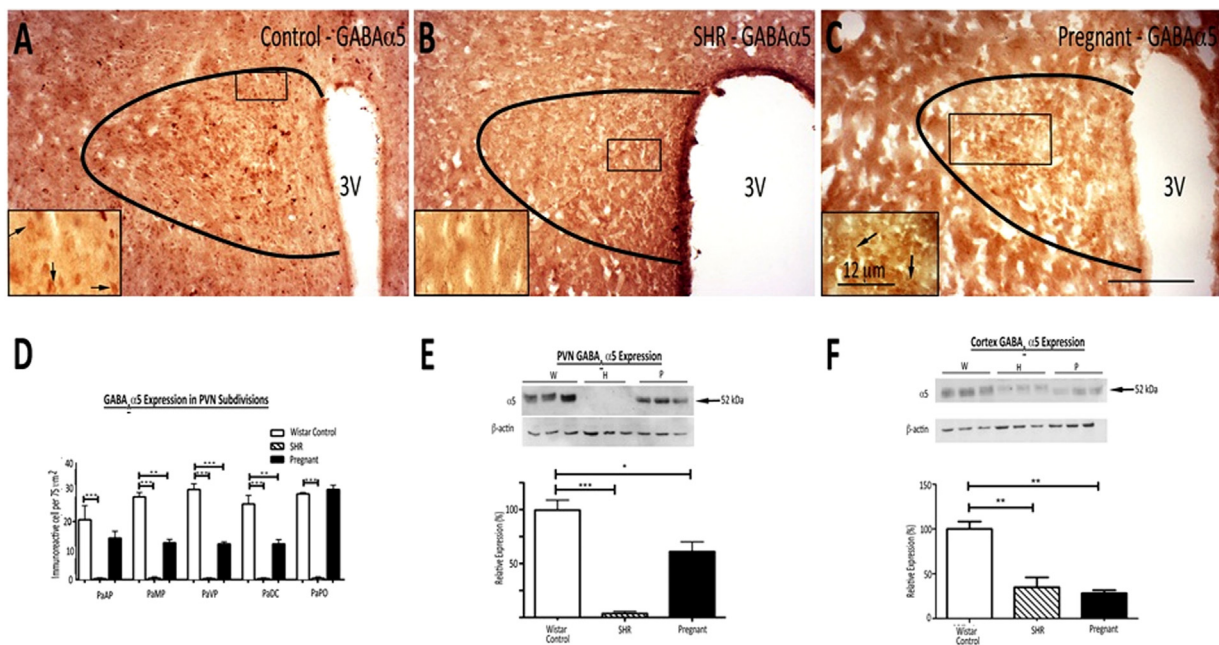

Fig. 2. Photomicrographs of GABA$_A$ receptor α5 subunit expression within the PVN at bregma 1.80 mm of female Wistar control (A), female SHR (B) and late-term pregnant rats (C). Boxed areas show examples of somatic labeling (arrows). Mean cell counts for GABA$_{A\alpha}$5 (D) in the five parvocellular subnuclei of the three groups.
A significant decrease in GABA$_{A\alpha}$5 expression was observed in all of the parvocellular subnuclei of the SHR and pregnant compared with the Wistar control ([**]$P < 0.01$, [***]$P < 0.001$). GABA$_{A\alpha}$5 protein expression in the PVN (E) and cortex (F) of female non pregnant Wistar control, female SHR and late-term pregnant rats. A significant decrease in expression of GABA$_{A\alpha}$5 (E) was observed between SHR and Wistar control ($P < 0.001$). In pregnant rats, GABA$_{A\alpha}$5 protein expression was also significantly reduced relative to the Wistar control ($P < 0.05$). GABA$_{A\alpha}$5 protein expression in the cortex (F) was significantly decreased in the SHR ($P < 0.01$) and pregnant ($P < 0.01$) compared to the Wistar control. Scale bar A–C = 80 μm boxed inserts scale bar = 12 μm. PaAP—anterior parvocellular, PaMP—medial parvocellular, PaVP—ventral parvocellular PaDC—dorsal cap, PaPO—posterior parvocellular, W—Wistar control, H—SHR, P—pregnant.

3.4. GABA$_A$ receptor α5 subunit protein expression in the PVN

Compared to the female Wistar control, there was a $97 \pm 2\%$ ($P < 0.001$) reduction in protein expression level of the GABA$_{A\alpha}$5 subunit in the SHR. Similarly a $38 \pm 8\%$ ($p < 0.05$) reduction was observed in the late-term pregnant rat for this subunit (Fig. 2E).

3.5. GABA$_A$ receptor α5 subunit expression in the cortex

Within the cortex, there was a significant reduction in the protein expression levels for GABA$_{A\alpha}$5 in SHR ($65 \pm 10\%$, $P < 0.01$) and late-term pregnant ($71 \pm 3.5\%$, $P < 0.05$) compared to the female Wistar control rats.

3.6. Relationship between GABA$_{A\alpha}$5 receptor subunit and PVN-spinally projecting neurons

To determine whether spinally projecting presympathetic neurons express the GABA$_{A\alpha}$5 subunit, and therefore could be affected by its down regulation in hypertension and pregnancy, PVN-spinally projecting neurons were labeled with FG (Fig. 3A). Neurons immunoreactive for both GABA$_{A\alpha}$5 and FG were found throughout the PVN (Fig. 3B, C). Those FG positive neurons not co-expressing the GABA$_{A\alpha}$5 subunit lay close to GABA$_{A\alpha}$5-IR only neurons (Fig. 3C). Semi-quantitative analysis revealed $73.3 \pm 2\%$ of FG labeled neurons were also positive for GABA$_{A\alpha}$5-IR (Fig. 4).

4. Discussion

We have demonstrated that within the PVN, the expression of the GABA$_A$ receptor α5 subunit is reduced in both hypertensive and late-term pregnant rats. Expression of the GABA$_A$ receptor α5 subunit is associated with identified PVN-presympathetic spinally projecting neurons that are known to generate sympathetic tone [18]. The presence of α5 containing GABA$_A$ receptors on presympathetic PVN neurons suggests this subunit is part of the mechanism required for normal tonic inhibition of these neurons. Therefore,
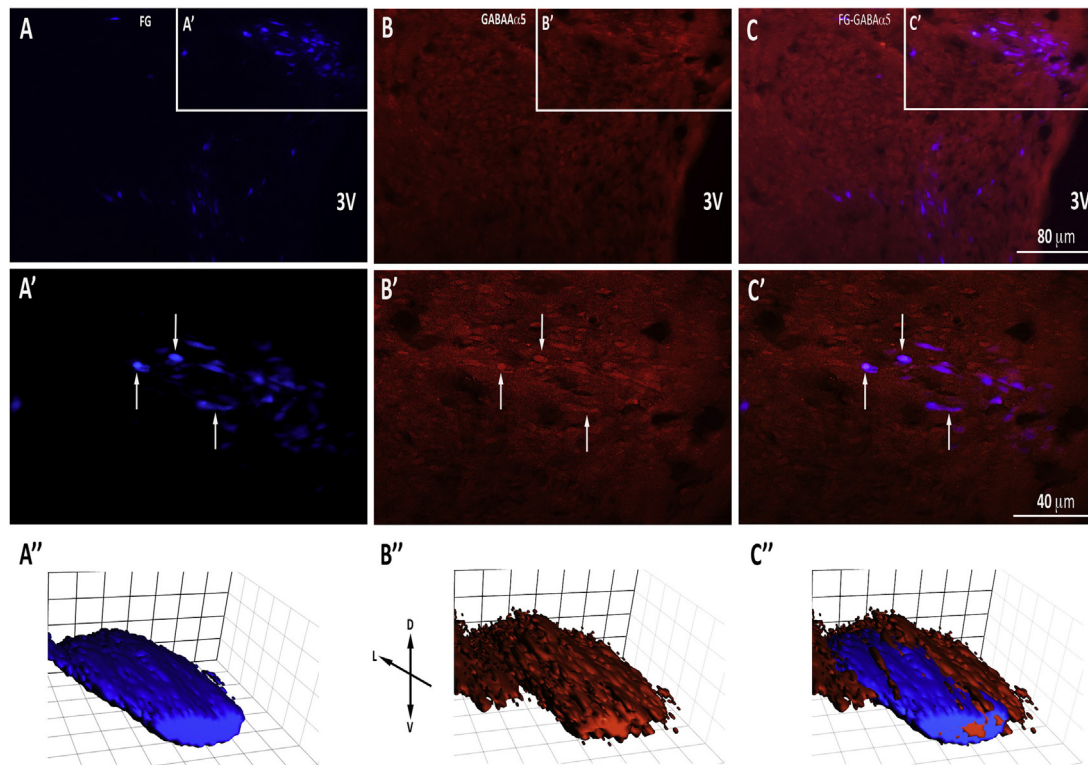
Fig. 3. Photomicrographs showing the relationship between spinally projecting PVN neurons and GABA$_{A\alpha}$5 subunit (A–C) at bregma 1.80 mm level of the PVN of female Wistar control rat. The GABA$_{A\alpha}$5 was found closely apposed to the spinally projecting FG-labeled neurons (A'–C'). Isosurface view (A"–C") revealed the GABA$_{A\alpha}$5 subunit to surround the FG labeled spinally projecting neurons (arrows). Scale bars A–C = 80 μm, A'–C' = 40 μm, A"–C" 1 unit = 2.6 μm^2. 3V—3rd ventricle, FG—Fluorogold.
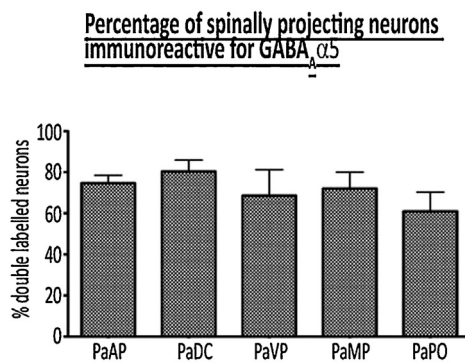

Fig. 4. Percentage of spinally projecting neurons immunoreactive for GABA$_{A\alpha}$5 subunit in the five parvocellular subnuclei of the PVN in female Wistar control rats. For GABA$_{A\alpha}$5 there was no difference in neuronal expression of the subunit within the different subnuclei. PaAP—anterior parvocellular, PaDC—dorsal cap, PaVP—ventral parvocellular, PaMP—medial parvocellular, PaPO—posterior parvocellular.

the significant decrease in expression of these subunit-containing receptors in hypertension and late term pregnancy may contribute to the dysfunctional mechanism generating sympatho-excitation.

Most studies have labeled PVN-presympathetic spinally projecting neurons in the male rats [27,31]. Unsurprisingly for female rats, the characteristics of these neurons are similar to those of the male. Furthermore, for the female Wistar rat at least 70% of the PVN-presympathetic neurons in the PVN express the GABA$_A$ receptor α5 subunit. Thus for normal cardiovascular control and the generation of sympathetic tone this subunit might be important. This also means any change in expression of this subunit could contribute the PVN-spinally projecting neurons generating an altered sympathetic output. Thus the decrease of the GABA$_A$ receptor α5 subunit in the hypertensive and late-term pregnant animal may

play a role in the sympathetic excitation characteristic of these conditions. Activation of PVN-spinally projecting neurons results in adjustments in sympathetic nerve activity especially renal and cardiac [5]. It is important to note, not all PVN-spinally projecting neurons were labeled and it is likely those parvocellular neurons expressing the α5 subunit would have a role in sympathetic modulation as presympathetic neurons projecting to other central targets [28] or as interneurons. Therefore, these neurons are also susceptible to fluctuations in the expression of the α5 subunit.

4.1. Expression of GABA$_A$ receptor α5 subunit

Differential expression levels of the GABA$_A$ receptor α5 subunit for both SHR and late-term pregnant rat were observed in comparison with the female Wistar control animals. Protein expression of the GABA$_A$ receptor α5 subunit was almost totally lost (97%) in the PVN of the SHR. Furthermore in the SHR, not only was a complete absence of GABA$_A$ receptor α5-IR within the parvocellular regions observed, but also in the adjacent regions surrounding the PVN known to contain GABAergic interneurons [1]. Application of muscimol or bicuculline to manipulate the GABAergic system within the PVN of the SHR suggests the normal regulation of this system is altered [17,18]. Our results provide a possible molecular subunit alteration to help understand the altered mechanism.

Focusing on late-term pregnant rats, the expression levels of the GABA$_A$ receptor α5 subunit are reduced in the PVN but the decrease in protein expression was not as profound when compared to the SHR. There was also a reduction in neurons immunoreactive for the α5 subunit in the parvocellular nuclei. Very little functional work is available relating to the contributors of sympatho-excitation in the late-term pregnant rat. However, unilateral microinjection of bicuculline into the PVN of the late term rat produces attenuated pressor response, tachycardia and sympatho-excitation compared

with non-pregnant controls [12,15,24]. Therefore, we could assume GABAergic inhibition is reduced in the late-term pregnant rats and the subunit down regulation we have reported may contribute to this effect.

4.2. Physiological consequences for sympathetic outflow

In pregnancy the marked elevations in sympathetic nerve activity (SNA) are less than that observed in hypertension [9,13,29], and may be reflected in the less significant down regulation of the α5 subunit compared with hypertension. Previous studies have shown that blocking the α5 subunit of the GABA$_A$ receptor increases basal membrane activity [8]. The down regulation of α5 subunit expression that we observe in this study therefore has the potential to contribute to the elevated activity of presympathetic neurons and subsequently SNA in both hypertensive and pregnant animals.

5. Conclusion

We suggest that normal cardiovascular control requires a contribution from the GABA$_A$ receptor α5. For both hypertension and late-term pregnancy down regulation of the GABA$_A$ receptor α5 subunit might be part of the explanation for sympatho-excitation with the retention of some GABA$_A$ receptor α5 subunit expression reflecting the plasticity evident in pregnancy such that sympathetic nerve activity can revert to normal post-partum.

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