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Microglia in the RVLM of SHR have reduced P2Y12R and CX3CR1 expression, shorter processes, and lower cell density

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ABSTRACT

The RVLM of spontaneously hypertensive rats (SHR) contains over-active C1 neurons, which model the pathology of essential hypertension. Hypertension involves chronic low-grade neuroinflammation. Inflammation in the brain is produced and maintained primarily by microglia. We assessed microglial gene expression (P2Y12R and CX3CR1) and morphology in the RVLM of SHR compared to normotensive Wistar-Kyoto rats (WKY). The gene expression of the metabotropic purinergic receptor P2Y12 and the fractalkine receptor CX3CR1 was downregulated in the RVLM of SHR compared to WKY (by 37.3% and 30.9% respectively). P2Y12R and CX3CR1 are required for normal microglial function, and reduced P2Y12R expression is associated with changes in microglial activity. Histological analysis showed a 22.9% reduction in microglial cell density, along with 18.7% shorter microglial processes, a phenotypic indicator of activation, in the RVLM of SHR compared to WKY. These results indicate a subtle loss of function, or a mild state of inflammation, in the RVLM microglia of SHR.

1. Introduction

Essential hypertension involves the chronic over-activity of neurons in specific brainstem nuclei. The mechanistic causes of this over-activity are unclear, but can include neurotransmitter-mediated dysregulation, brainstem tissue hypoperfusion, and neuroinflammation.

One of the brainstem nuclei regulating blood pressure is the rostral ventrolateral medulla (RVLM). This is a critical relay nucleus in the sympathetic baroreflex, which maintains arterial blood pressure through a series of feedback loops (Pilowsky & Goodchild, 2002; Ito & Sved, 1997; Lipski et al., 1996). In models of essential hypertension, neurons in the RVLM projecting to the spinal cord are persistently over-active, leading to a sustained increase in blood pressure via the excessive constriction of resistance arterioles and release of noradrenaline from the adrenal medulla (Minson et al., 1996; Geraldes et al., 2014). Hypertension is associated with chronic neuroinflammation, particularly in the RVLM and other cardiovascular control sites (Agarwal et al., 2011; Dange et al., 2015), but it is unclear whether this inflammation is a cause or consequence of excessive neuronal activity. Experimentally-induced inflammation in the RVLM can lead to hypertension (Wu et al., 2012), but it can also be produced by hypertension (Oliveira-Sales et al., 2010).

Inflammation in the brain is produced and maintained by microglia, the resident macrophages of the central nervous system (CNS) that can display a range of morphological and phenotypic states. Microglia respond to neuronal activity in vivo (Li et al., 2012) and their actions can have profound effects on neuronal activity in cardiovascular control sites. For example, when microglia are depleted, experimental hypertension is attenuated, and pre-activated microglia produce a pressor response when transferred into normotensive brains (Shen, 2015). Microglia express a range of functional receptors for neurotransmitters and other signalling factors which allow them to interact with their local environment and maintain neuronal homeostasis. Potential mechanisms for microglia-neuron interactions include two Gi-coupled receptors, P2Y12R and CX3CR1. P2Y12R is a purinergic receptor exclusively expressed on microglia in the CNS (Butovsky, 2014). It is required for process extension and directional motility in response to an ATP or ADP gradient caused by injury (Swiatkowski et al., 2016) or excitotoxicity (Kato et al., 2016). P2Y12R is downregulated in lipopolysaccharide-induced inflammation (Haynes et al., 2006) and P2Y12R

Abbreviations: RVLM, rostral ventrolateral medulla; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; CNS, central nervous system; PFA, paraformaldehyde; SBP, systolic blood pressure; LPS, lipopolysaccharide

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Table 1
primer sequences used for qPCR.

Gene target	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
HPRT	CGACCCTCAGTCCCAGCGTCGT	GGGCCACAATGTGATGGCCTCCCA
P2Y12R	GCACCAGATGCCAGTCTGCAAG	GGCACCTCCATGGTCCTGGTT
CX3CR1	GGACTGGGTGAGTGGCTGGC	GTGAGGTCCTGAGCAGCTGGG
CD32	GCTGTTCCTACCGCTCCCC	GCCTTCGGAAGACCTGCATGAGA
CD206	GTGGATCCCTTTCCACGGCCA	AACCAGGGAGGCACCCATTCG
CX3CL1	GCCATCATCCTGGAGACGAGACAG	TCCAGGTGCTTCATGGCGTCT
IL-6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC
iNOS	CCAGGTGCTATTCCCAGCCCA	GGCGGGTCGATGGAGTCACA
TNFα	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
IL-1β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC

knockout mice have attenuated seizure severity in kainate-induced epilepsy (Eyo et al., 2014). CX3CR1 is the only receptor for the chemokine CX3CL1, also known as fractalkine (Jung et al., 2000). CX3CR1 is primarily expressed by microglia, and CX3CL1 is usually found in its membrane-bound form on neurons (Maciejewski-Lenoir et al., 1999). Although CX3CR1-CX3CL1 binding can have beneficial or detrimental effects depending on the time-course and disease model (Febinger et al., 2015), it appears to be protective against excitotoxicity (Limatola et al., 2005) and can reduce blood pressure at the level of the paraventricular nucleus of the hypothalamus (Ruchaya et al., 2014). Activation of microglial P2Y12R or CX3CR1 triggers intracellular calcium increases, cytoskeleton rearrangement, and migration towards the agonist (Eyo et al., 2014; Maciejewski-Lenoir et al., 1999). Recent evidence shows that microglial phenotype and activity is heterogeneous between distinct functional brain regions (De Biase et al., 2017).

We investigated the expression of P2Y12R and CX3CR1 in the RVLM of spontaneously hypertensive rats, along with the morphological state of microglia in that area, to determine if genetic hypertension is associated with microglial dysfunction in the RVLM. We also assessed the expression of several genes related to microglial activity and inflammation in the RVLM, including CD32 and CD206 (commonly used as markers of fully polarised microglia in a pro-inflammatory or anti-inflammatory state, respectively), CX3CL1 (the only ligand of CX3CR1), IL-6, iNOS, TNFα, and IL-1β (signalling molecules indicating high levels of inflammation). In a normal state, microglia have a small cell body and many highly branched or ramified processes (Tremblay et al., 2010). These are used to survey the local microenvironment (Nimmerjahn et al., 2005), contacting and sometimes phagocytosing synaptic elements (Wake et al., 2009), and enwrapping the axons of excitotoxic neurons to reduce their electrical activity (Kato et al., 2016). In a pro-inflammatory state, microglial processes retract, reducing the branching complexity and branch length. This corresponds to a loss of P2Y12R immunoreactivity (Haynes et al., 2006) and a functional change in state, where microglial motility and responses to ATP are impaired (Gyoneva et al., 2009). The morphology of a microglial cell changes during inflammation and can be used as an indicator of inflammation (Fernandez-Arjona et al., 2017).

2. Materials and methods

All animal work was approved by the Sydney Local Health District Animal Welfare Committee and performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, using male 15-week-old SHR and WKY (Animal Resource Centre; Perth, Australia) weighing 300–320 g. These guidelines comply with the ARRIVE guidelines and were therefore carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Blood pressure was measured in conscious rats using a non-invasive tail cuff (IITC Life Science) to verify their hypertensive or normotensive phenotype. Rats were euthanised with sodium pentobarbitone (Lethabarb, Virbac, 150 mg IP) and transcardially perfused with ~200 mL of phosphate buffered saline (PBS). Brainstems were extracted and snap frozen for gene expression studies or fixed in 4% paraformaldehyde for immunohistochemistry.

2.1. RNA isolation, cDNA synthesis and quantitative polymerase chain reaction (qPCR)

Frozen brainstems were sectioned using a vibratome (Leica VT 1200S) at 500um. The RVLM was removed bilaterally from 2 to 4 coronal sections using the Palkovits punch technique (Palkovits, 1983), using the nucleus ambiguus as a visual landmark (Fig. 5A). The facial nucleus was also removed bilaterally from 2 to 4 sections (Fig. 5B). RVLM and facial nucleus tissue was stored separately at −80 °C and was not pooled. Tissue was homogenised (Precellys 24, Bertin Technologies) and total RNA was extracted using 1-bromo-3-chloro-propane and Tri Reagent, followed by isopropanol precipitation and ethanol washes (Sigma). 50 ng of total RNA was reverse-transcribed into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) in a Bio-Rad CFX96 C1000 thermal cycler. Primer sequences are listed in Table 1. The Cq values for P2Y12R and CX3CR1 were normalised to HPRT using the $2^{(-\Delta Cq)}$ method.

2.2. Histological analysis

For histological analysis, rats were also perfused with 400 mL of freshly-made paraformaldehyde (PFA, 4% in PBS, Sigma). Brains were extracted and post-fixed in PFA overnight, then sectioned at 40 µm using a vibratome (Leica VT 1200S). Sections were incubated with the microglial marker anti-Iba1 (rabbit polyclonal, Wako Pure Chemical Industries Ltd., 019-19741; 1:2000) for three days, then with donkey anti-rabbit AlexaFluor488 (Jacksons Immunoresearch 711-546-152; 1:500) overnight, before being mounted and cover-slipped with Vectashield (H-1000, Vector Laboratories). Imaging was performed with a Zeiss Axio Imager Z2. The RVLM was defined as a ~1 mm circular area ventral to the nucleus ambiguus, lateral to the inferior olive and medial to the spinal trigeminal tract, between Bregma -12.12 and Bregma -12.36 (Paxinos & Watson, 1986; Nedoboy et al., 2016). *Z*-stack images covering the RVLM were taken at 20× magnification and a maximum intensity projection was used for microglial morphological analysis.

ImageJ software was used for morphological analysis of microglia. A circular area 1 mm in diameter containing the RVLM was selected (Fig. 1a, b, c). Custom macros were used to convert the image to a binary representation to count the number of microglia within this area, then skeletonised to quantify the degree of ramification or branching (Fig. 1d-h). Morphological parameters included the average branch length, number of branches, and number of endpoint voxels (branch

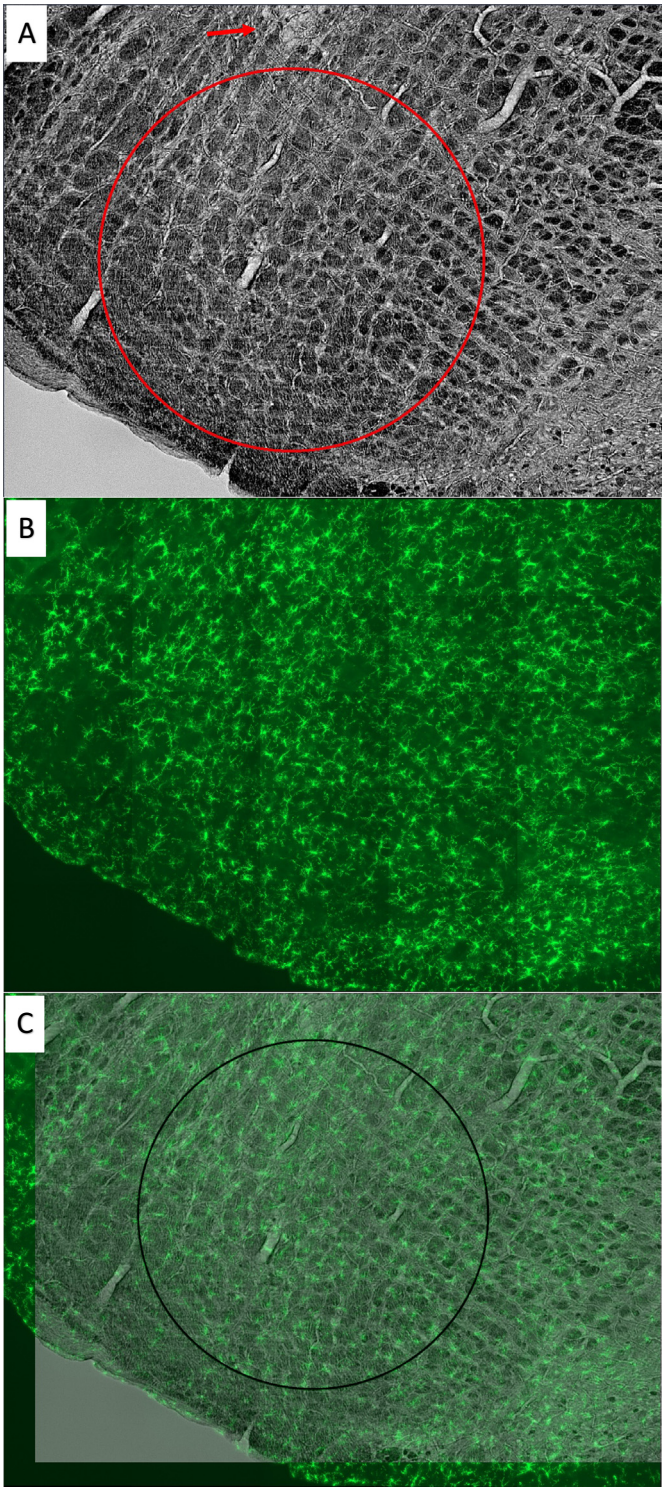

Fig. 1. A) The brainstem was sectioned at 40 μm and the RVLM (circled) was identified based on morphological landmarks in the tissue. The nucleus ambiguus is visible dorsal to the RVLM (red arrow). B) Sections were stained for the microglial marker Iba1. C) Merged brightfield and fluorescent images of the RVLM (circled). D) through H) illustrate the process of binary conversion, skeletonisation, and morphological analysis conducted by the ImageJ plugin (Supplementary Information). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

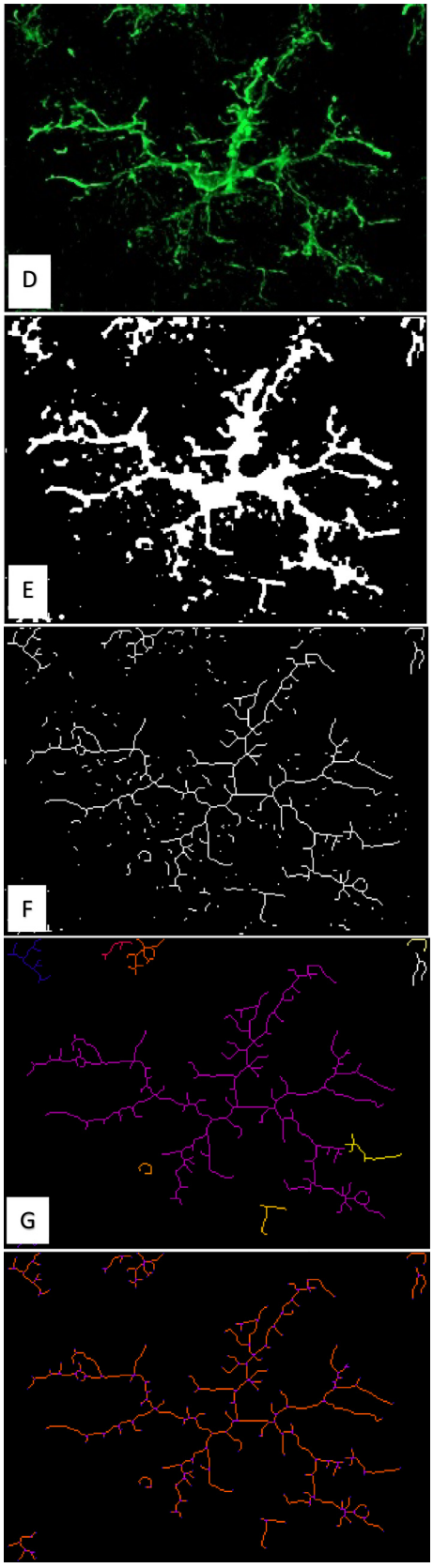

Fig. 1. (*continued*)

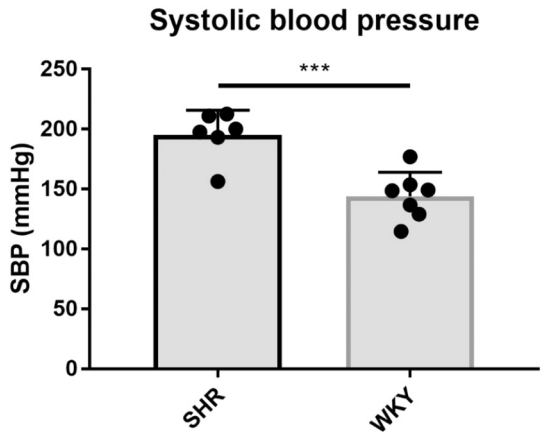

Fig. 2. Average systolic blood pressure for SHR ($n = 6$) and WKY ($n = 7$), $^{***}p < 0.005$ *t*-test.

terminals) per cell. The average of three images was used from each animal.

3. Results

3.1. Phenotype verification

SHRs had a significantly higher systolic blood pressure (SBP) compared to normotensive WKY rats (SHR SBP 195 ± 8 mm Hg, WKY SBP 144 ± 8 mm Hg, Fig. 2).

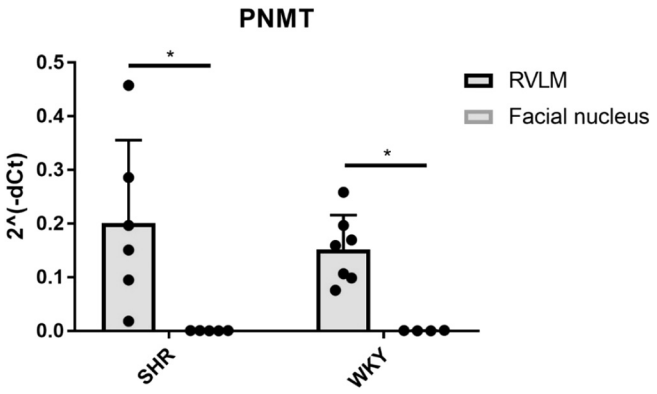

Fig. 4. Gene expression of PNMT relative to HPRT in SHR RVLM vs SHR facial nucleus (0.2008 ± 0.15, $n = 6$ vs 0.0008 ± 0.0002, $n = 5$) and in WKY RVLM vs WKY facial nucleus (0.1524 ± 0.06, $n = 7$, vs 0.0008 ± 0.0004, $n = 4$) * adjusted $p < 0.05$ two-way Anova, Holm-Sidak multiple comparisons test.

3.2. Gene expression

When we examined P2Y12R expression in the RVLM, we found that P2Y12R mRNA expression was 37.3% lower in SHR compared to WKY (Fig. 3A). The facial nucleus showed no difference in P2Y12R

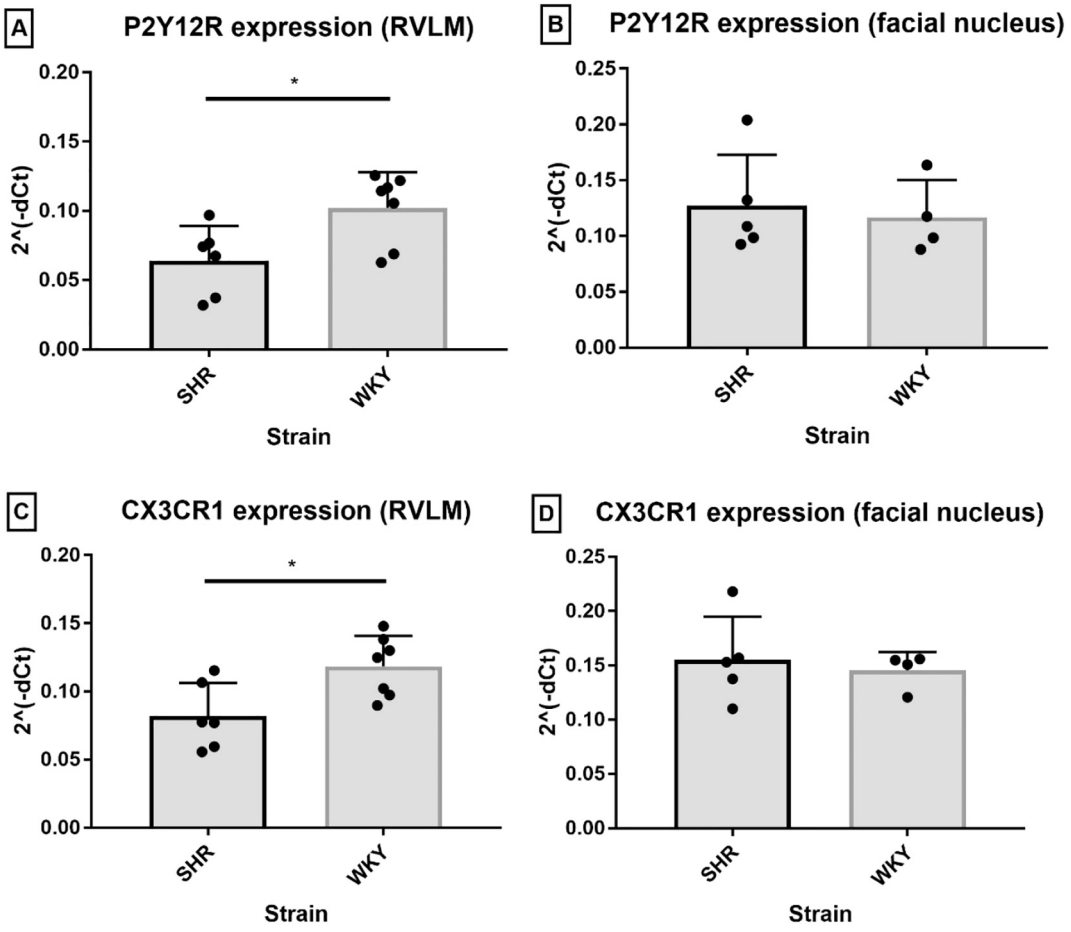

Fig. 3. A & B, gene expression of P2Y12R relative to HPRT in SHR RVLM vs WKY RVLM (0.641 ± 0.025, $n = 6$, vs 0.1023 ± 0.026, $n = 7$) and in SHR facial nucleus vs WKY facial nucleus (0.1272 ± 0.046, $n = 5$, vs 0.1170 ± 0.033, $n = 4$), $^{*}p < 0.05$, *t*-test
C & D, gene expression of CX3CR1 relative to HPRT in SHR RVLM vs WKY RVLM (0.0820 ± 0.024, n = 6, vs 0.1186 ± 0.022, n = 7) and in SHR facial nucleus vs WKY facial nucleus (0.1152 ± 0.040, n = 5, vs 0.1456 ± 0.017, n = 4), * $p < 0.05$, *t*-test.

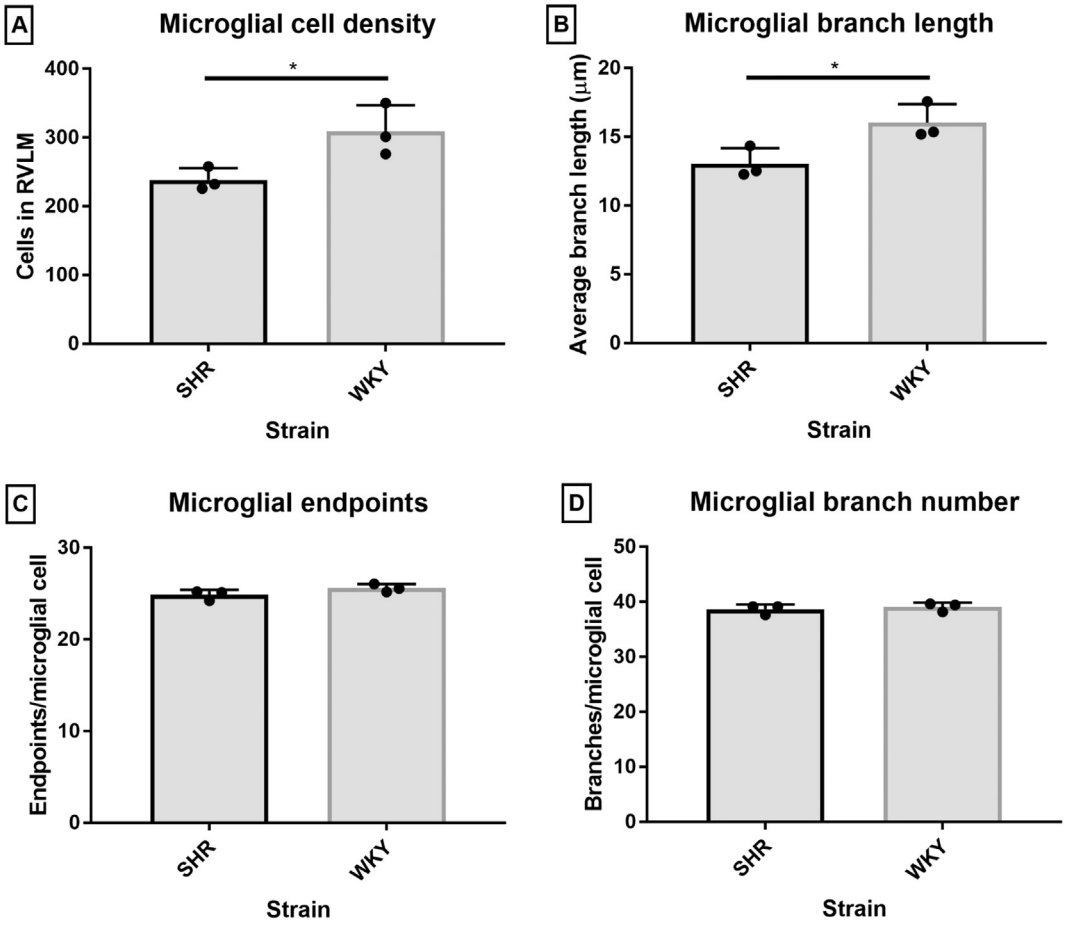

Fig. 5. A, average number of microglial cells per site of 1 mm diameter in SHR RVLM (238.44 ± 16.95, $n = 3$) vs WKY RVLM (309.33 ± 37.64, n = 3), $^*p < 0.05$, *t*-test.
B, average process branch length in SHR RVLM (13.05 ± 1.13, n = 3) vs WKY RVLM (16.04 ± 1.33, n = 3), $^*p < 0.05$, *t*-test.
C, average number of endpoints per microglial cell in SHR RVLM (24.86 ± 0.55, n = 3) vs WKY RVLM (25.61 ± 0.43, n = 3), n.s. *t*-test.
D, average number of branches per microglial cell in SHR RVLM (38.65 ± 0.86, n = 3) vs WKY RVLM (39.09 ± 0.77, n = 3), n.s. *t*-test.

expression between strains (Fig. 3B).

We next examined mRNA expression of CX3CR1, which was 30.9% lower in the RVLM of SHR compared to WKY (Fig. 3C). No difference in CX3CR1 mRNA was observed in the facial nucleus (Fig. 3D).

PNMT mRNA was expressed in the RVLM of both strains, and its expression was negligible in the facial nucleus (Fig. 4), confirming that the correct site was obtained during tissue harvest.

The gene expression of CD32, CD206, CX3CL1, IL-6, iNOS, TNFα, and IL-1β was assessed in the RVLM. No significant differences were found between SHR and WKY (data not shown).

3.3. Microglial morphology

Because we predicted that changes in gene expression would reflect differences in the number of microglia present in the RVLM, we next assessed microglial cell density. Indeed, the number of microglia in the SHR RVLM was significantly lower than WKY, by 22.9% (Fig. 5A). Because microglial morphology can reflect changes in microglial activity, we also examined microglial branch length, number of branch points, and end-points. Microglia in the RVLM of SHR had branch lengths which were 18.7% shorter than WKY (Fig. 5B). The average number of branches, and the average number of endpoints per cell, were not significantly different between strains (Fig. 5C & D).

4. Discussion

Microglia are responsible for the defence and repair of the CNS. The microglia in cardiovascular nuclei can become pro-inflammatory during peripheral events such as myocardial infarction, or during neuronal pathologies such as epilepsy. The relationship between microglia, CNS inflammation, and essential hypertension has not been comprehensively explored. This study focused on identifying microglial changes in the RVLM of hypertensive rats. We found that mRNA expression of P2Y12R and CX3CR1, two microglia-specific receptors, were significantly reduced in the RVLM of SHR compared to WKY. This effect was not seen in the adjacent facial nucleus, suggesting that is specific to blood pressure control. These results may indicate a subtle loss of function, or activation, in the microglia of the RVLM during essential hypertension.

This is supported by the morphological data. Microglia become more ameboid and less ramified as they become more inflamed (Karperien et al., 2013). Microglial 'activation' is a broad term used to describe a functional and visible change in state. This includes a loss of normal motility, a reduction in process length and branch complexity, and changes in microglial responses to endogenous stimuli (De Simone et al., 2010; Henry et al., 2009; Gyoneva & Traynelis, 2013). Here, the microglia in the RVLM of SHR were less dense and had shorter processes compared to WKY (Fig. 6), although the degree of branching and the number of endpoints was the same between strains. The shorter processes indicate a small degree of inflammation in this nucleus. Acute

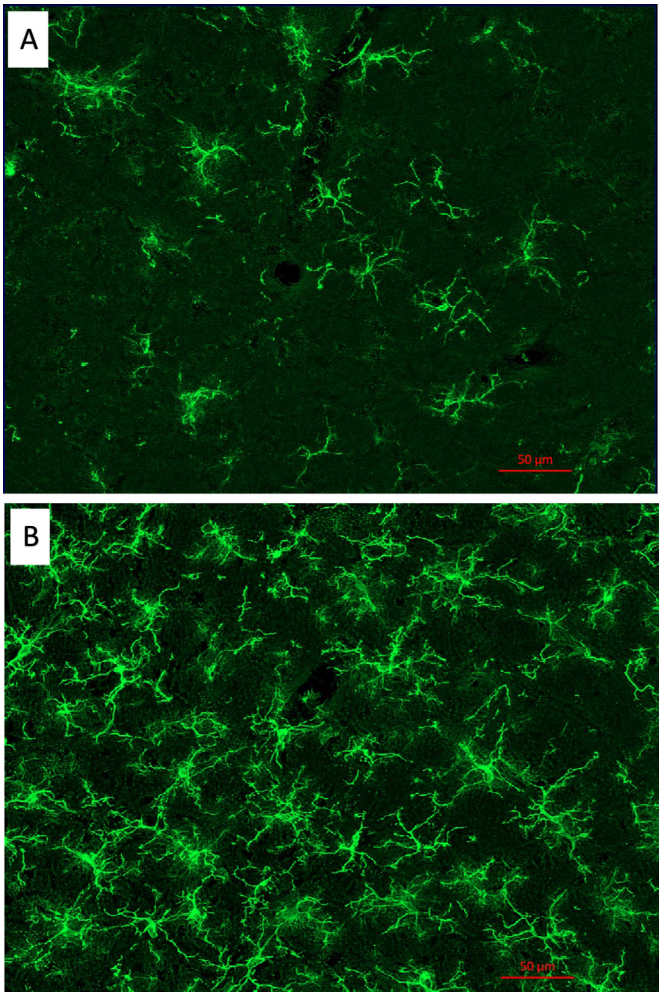

Fig. 6. A, example of microglia in the RVLM of SHR.
B, example of microglia in the RVLM of WKY.

microglial activation often involves migration towards a source of injury, leading to increased cell density along with the transition to a more amoeboid phenotype. The morphological changes in microglia found in the present study were accompanied by a reduction in cell density. This is in direct contrast to the typical behaviour of microglia during acute focal injury, where the microglial processes converge on the injured area, shortly followed by chemotaxis of the cell bodies themselves. However, the lack of sufficient microglia is a possible mechanism by which inflammation occurs in the RVLM of SHR. It is also possible that the decreased abundance is characteristic of a chronic state of inflammation, which may be more similar to neurodegenerative conditions than to acute injury. This is consistent with other reports of decreased microglial density in some neurodegenerative disorders (Ma et al., 2003). On the other hand, Shi et al. (2010) found increased microglial density in the paraventricular nucleus of the hypothalamus after chronic angiotensin II was used to cause hypertension. This study highlighted the relevance of inflammation in hypertension, but it also demonstrates the importance of brain region specificity.

Here, we found a reduction in P2Y12R and CX3CR1, lower microglial density, and a loss of process length, but not a reduction in process numbers in SHR RVLM compared to WKY. These gene expression changes were site-specific. Both P2Y12R and CX3CR1 are required for normal microglial function. CX3CR1 can be protective or detrimental during pathology, indicating a time-course effect, whereby CX3CR1 confers chronic but not acute neuroprotection after inflammation or injury (Febinger et al., 2015; Zanier et al., 2016). Without CX3CR1, microglia become more neurotoxic during inflammation, resulting in greater neuronal loss (Cardona et al., 2006). Although the expression of P2Y12R and CX3CR1 can be affected by inflammation, the mechanisms behind these changes are still under investigation. Although other studies have demonstrated that both receptors can be downregulated on contact with lipopolysaccharide (Haynes et al., 2006; Wynne et al., 2010) – indicating their importance in 'classical' inflammation – this is quite different to the SHR model. It is also possible that the reduced expression of P2Y12R and CX3CR1 is a function of the morphological changes in microglia.

P2Y12R gene and protein expression is inversely correlated with microglial activation state; after striatal injections of LPS, P2Y12R is progressively lost, while microglia transition to a completely polarised morphology (Haynes et al., 2006). Haynes et al. (2006) also reported that P2Y12R expression is inversely correlated with the average number of branches (primary processes) in a microglial cell in hippocampal slices, although branch length was not assessed. Neuroinflammation can in turn result in a loss of P2Y12R immunoreactivity (Mildner et al., 2017), and LPS-induced inflammation reduces microglial migration and P2Y12R expression in culture (De Simone et al., 2010). Other models of chronic neuroinflammation, such as MPTP treatment which simulates Parkinson's disease, show a similar loss of damage-induced microglial process motility (Gyoneva, 2014). A loss of P2Y12R expression and/or function appears to be detrimental in models of chronic neuroinflammation.

PNMT, the enzyme which converts noradrenaline to adrenaline, is only found in specific brain regions (Hokfelt et al., 1974). This makes it a robust specific marker for adrenergic neurons, which are present in the RVLM and not the facial nucleus. In this study, the degree of PNMT expression in the SHR RVLM was variable, which suggests that the RVLM tissue may not have been completely isolated. However, the lowest level of PNMT expression in the RVLM was an order of magnitude greater than the highest level of PNMT expression in the facial nucleus, indicating that the tissue harvest had captured enough of the RVLM to effectively assess gene expression.

Since microglia are immune cells, several other genes related to inflammation were assessed in the RVLM of SHR and WKY, including CD32, CD206, CX3CL1, IL-6, TNFα, and IL-1β. These did not show significant expression changes between sites in either strain, which underscores the subtle nature of the microglial changes observed. In particular, CD32 and CD206 are commonly used as markers of complete microglial polarisation, which occurs in response to injury or overwhelming inflammation.

Microglial morphology and inflammation are known to occur in hypertension. However, any cytokine changes must be assessed in the context of the specific brain region being investigated. For example, Takesue et al. (2017) demonstrated that microglia are de-ramified in the paraventricular nucleus of stroke-prone SHR, but that preventing this morphological change with the anti-inflammatory antibiotic minocycline did not affect blood pressure. Other markers of inflammation were not assessed. On the other hand, Tayebati et al. (2016) showed increased IL-1β and TNFα expression in some areas of the brain which are not related to blood pressure control, but did not analyse blood pressure itself. Lastly, Silva et al. (2018) assessed the microglial response to acute hypoxia in the paraventricular nucleus and the RVLM, and found that minocycline pre-treatment could prevent some of the cytokine upregulation seen in these areas without affecting blood pressure changes. From these reports, and from other studies in this area, it is evident that microglial activity is related to neuronal activity in autonomic areas. However, the direction of this relationship remains unclear. It seems likely that a positive feedback loop exists between blood pressure control and neuroinflammation. In clinical practice, normalising blood pressure may improve inflammation in the brain, and vice versa.

The RVLM is a critical area for blood pressure control, and exhibits chronic over-activation in essential hypertension (Kumagai et al.,

2012), the pathology modelled by the SHR strain. The gene expression and morphological differences shown here imply a functional change in microglial state or activity in the RVLM. This suggests a potential dysfunction in the communication between RVLM neurons and the surrounding microglia during hypertension. What remains to be determined is a) the exact nature of this microglial dysfunction, and b) whether the microglial changes are a cause or consequence of the RVLM over-activation seen in hypertension. Since the animals used here were perfused almost immediately after anaesthetic administration, direct nerve recordings could not be performed; however, earlier studies have shown that SHR have increased sympathetic nerve activity compared to normotensive rats (Judy & Farrell, 1979; Judy et al., 1976). This question may be resolved by using young normotensive or pre-hypertensive SHR to determine if the microglial changes occur prior to the development of hypertension. Future investigations may target RVLM-specific microglial P2Y12R or CX3CR1 function, to assess the impact on long-term blood pressure control. Alternately, other non-pharmacological models of hypertension (such as Goldblatt hypertension) could be used to evaluate whether the receptor downregulation seen here is unique to the SHR strain, or a result of sustained RVLM neuron activation.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.autneu.2018.12.002.

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