



## The role of $\beta_2$ adrenergic receptor on infection development after ischaemic stroke



Raymond Shim<sup>a</sup>, Jenny L. Wilson<sup>a</sup>, Sarah E. Phillips<sup>b,c</sup>, Gavin W. Lambert<sup>b,c</sup>, Shu Wen Wen<sup>a,\*</sup>, Connie H.Y. Wong<sup>a,\*\*</sup>

<sup>a</sup> Centre for Inflammatory Diseases, Department of Medicine, School of Clinical Sciences at Monash Health, Monash Medical Centre, Monash University, Clayton, Victoria, Australia

<sup>b</sup> Inversion Health Innovation Research Institute and School of Health Sciences, Swinburne University of Technology, Victoria, Australia

<sup>c</sup> Human Neurotransmitters Laboratory, Baker Heart and Diabetes Institute, Victoria, Australia

### ARTICLE INFO

**Keywords:**  
Stroke  
Infection  
Adrenergic

### ABSTRACT

Mechanisms underlying post-stroke immune impairments and subsequent development of fatal lung infection have been suggested to involve multiple pathways, including hyperactivation of the sympathetic nervous system (SNS), which results in the excessive release of catecholamines and activation of  $\beta$ -adrenergic receptors ( $\beta$ ARs). Indeed, previous reports from experimental studies demonstrated that post-stroke infection can be inhibited with treatment of  $\beta$ -blockers. However, the effectiveness of  $\beta$ -blockers in reducing post-stroke infection has yielded mixed results in retrospective clinical trials and its use remain controversial. In this study, we performed mid-cerebral artery occlusion in mice either genetically deficient in  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) or treated with non-selective and selective  $\beta$ AR antagonists to explore the contributions of the SNS in the development of post-stroke lung infection. Stroke induced a systemic activation of the SNS as indicated by elevated levels of plasma catecholamines and UCP-1 activity. However,  $\beta_2$ AR deficient mice showed similar degrees of post-stroke immune impairment and infection rate compared to wildtype counterparts, potentially due to compensatory mechanisms common in transgenic animals. To overcome this, we treated post-stroke wildtype mice with pharmacological inhibitors of the  $\beta$ ARs, including the non-selective antagonist propranolol (PPL) and selective  $\beta_2$ AR antagonist ICI-118551. Both pharmacological strategies to block the action of SNS signalling were unable to reduce infection in mice that underwent ischaemic stroke. Overall, our data suggests that other mechanisms independent or in combination with  $\beta_2$ AR activation contribute to the development of post-stroke infection.

### 1. Introduction

Bacterial infection is a leading complication in patients with stroke, and its incidence peaks in the first 3 days after cerebral infarction (Lan-ghorne et al., 2000). In fact, bacterial pneumonia is independently associated with poor patient outcome and contributes up to 30% of mortality in patients with stroke (Heuschmann et al., 2004; Kwan and Hand, 2007; Vermeij et al., 2009). The high prevalence of post-stroke infection is strongly suggestive of impaired antibacterial immunity following this brain injury. Indeed, we and others have previously found indicators of post-stroke immune impairment, including the loss and functional impairment of circulating and tissue-resident leukocytes, as well as reduced spleen size, shortly after experimental and clinical stroke

onset (Offner et al., 2006; Prass et al., 2003; Shim et al., 2019). However, the mechanisms underlying these observations remain poorly understood.

A proposed mechanism of post-stroke immune impairment involves the activation of sympathetic nervous system (SNS). After stroke, symptoms of impaired cardiac function, heart rate variability, baroreflex sensitivity, and blood pressure changes provide strong evidence of altered SNS activity (Bassi et al., 2007; Xiong et al., 2018). Indeed, elevated catecholamines in the form of noradrenaline (NA) has been reported in the plasma of patients within the first 7 days following stroke onset, further suggestive of SNS hyperactivity (Eşref et al., 2015; McCulloch et al., 2019; Myers et al., 1981; Naredi et al., 2000, 2006). Of the adrenergic receptors,  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) is most widely

\* Corresponding author.

\*\* Corresponding author.

E-mail addresses: [shu.wen@monash.edu](mailto:shu.wen@monash.edu) (S.W. Wen), [connie.wong@monash.edu](mailto:connie.wong@monash.edu) (C.H.Y. Wong).

<https://doi.org/10.1016/j.bbih.2021.100393>

Received 12 August 2021; Received in revised form 31 October 2021; Accepted 21 November 2021

Available online 23 November 2021

2666-3546/© 2021 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

expressed on immune cells (Scanzano and Cosentino, 2015), and findings from various preclinical studies suggest that activation of the  $\beta_2$ AR on immune cells significantly impact immune functions. Specifically,  $\beta_2$ AR activation on immune cells has been shown to inhibit respiratory burst in neutrophils (Hummel et al., 2013), reduce interleukin (IL)-8 production by monocytes (Mizuno et al., 2005), modulate regulatory T cell apoptosis (Wirth et al., 2014), promote lymphocyte apoptosis (Prass et al., 2003), induce alternatively activated macrophages, and down-regulate a number of pro-inflammatory cytokines (Graier et al., 2014). Contrastingly, sympathetic denervation in the kidney of patients with hypertension was shown to induce a systemic anti-inflammatory response in monocytes (Zaldivia et al., 2017). These findings support the concept that catecholamines can directly modulate the immune system and thus regulate the host antibacterial immune response following stroke via  $\beta$ -adrenoceptor activation.

Accordingly, inhibiting the SNS with  $\beta$ -blockers showed the beneficial effects of reducing immune suppression and limiting the development of lung infection after experimental stroke models (Liu et al., 2017; McCulloch et al., 2017; Offner et al., 2009; Prass et al., 2003; Wong et al., 2011). Despite these promising preclinical findings, the clinical translation of  $\beta$ -blockers in preventing infection in patients with stroke yielded mixed findings. Some retrospective studies found that  $\beta$ -blocker use was associated with reduced rates of infection and improved mortality rates (Dziedzic et al., 2007; Sykora et al., 2015), whilst others showed that it did not lower the incidence of post-stroke infection or improve patient outcome (Barer et al., 1988; Maier et al., 2015). In fact, some studies report a positive association between the use of  $\beta$ -blockers with incidence of infection and mortality in patients with stroke (Westendorp et al., 2016). This discrepancy between clinical studies may be due to the ambiguity of which selective or non-selective  $\beta$ -blockers were tested as a recent study reported that non-selective  $\beta$ -blocker use was associated with increased infection, while selective  $\beta$ -blocker use had no effect (Shim et al., 2019). Furthermore, the severity of stroke largely dictates the susceptibility to infection (Shim et al., 2019), thus the effectiveness of  $\beta$ -blocker therapy may depend on the extent of cerebral injury (Starr et al., 2017). These knowledge gaps and conflicting clinical reports demonstrates a need to elucidate the relationship between post-stroke SNS activation, systemic release of catecholamines, activation of adrenergic receptor and development of infection. In this study, we assessed systemic SNS activity at various timepoints following a clinically relevant model of ischaemic stroke by two methods: i) quantifying plasma catecholamines and ii) assessing the expression level of SNS-mediated uncoupling protein 1 (UCP-1). Additionally, we examined whether the development of post-stroke immune impairment and lung infection is mediated through  $\beta_2$ AR signalling with the use of transgenic mice that lack the  $\beta_2$ AR or by targeting this receptor pharmacologically with selective or non-selective  $\beta$ -adrenergic receptor antagonists.

## 2. Materials and methods

### 2.1. Mice

Adult male C57Bl/6J mice of 7–12 weeks were obtained from Monash Animal Research Platform (MARF, Clayton, Victoria, Australia) and housed at Monash Medical Centre Animal Facility (MMCAF) in specific pathogen-free (SPF) conditions. Mice were acclimatised for a minimum of 7 days before starting any experimental procedures. For experiments using knockout mice,  $\beta_2$ AR deficient (*Adrb2*<sup>-/-</sup>) mice were bred on a C57Bl/6J background for 10 generations. The genotype of *Adrb2*<sup>-/-</sup> mice was confirmed by the genotyping facilities from Transnetyx. All procedures were approved by the Monash Medical Centre Animal Ethics Committee (MMCB/2016/10 and MMB/2018/02, Monash Medical Centre, Clayton, Victoria, Australia). Animals were randomly assigned to the experimental groups to prevent selection bias. All animals have access to water and food *ad libitum*, maintained in temperature-controlled rooms under a 12 h light-dark cycle.

### 2.2. Middle-cerebral artery occlusion (MCAO)

To induce ischaemic stroke, mice underwent the intraluminal MCAO experimental model of ischaemic stroke as previously described (Connolly et al., 1996). Briefly, mice were anaesthetised via intraperitoneal injection of 150 mg/kg ketamine and 10 mg/kg xylazine. An incision at the neck was made and the right common carotid artery, external carotid artery, and internal carotid artery was isolated away from connective tissue. A 0.21–0.23 mm silicone-coated monofilament (Doccol Corporation) was inserted into the external carotid artery and advanced along the internal carotid artery to occlude blood flow into the middle cerebral artery. Cerebral blood flow to the right-hemisphere of the brain was monitored using a Doppler probe (PeriFlux System 5000, Stroke model kit 407, Perimed, Järfälla-Stockholm, Sweden) where a successful occlusion was confirmed by a ~70% drop in reading. Clinically, fewer than 15% stroke patients are given thrombolytic agents to restore blood supply to the brain, thus many do not necessarily undergo reperfusion. For this reason, the monofilament remained occluding the middle-cerebral artery for the duration of the experimental timepoint, termed permanent MCAO or pMCAO. Following induction of stroke, the external carotid artery was tied off and the incision at the neck was sutured closed. Alongside experimental animals, separate mice underwent a sham-surgery to control for surgical stress whereby similar surgery was performed but without the insertion of the monofilament. Following surgery, mice were allowed to recover overnight on a heat pad and subsequently culled at various time points post-surgery for tissue collection and analysis.

### 2.3. Treatments

For experiments using the non-selective  $\beta$ -blocker, propranolol (PPL) (Sigma-Aldrich), mice were treated with 30 mg/kg at 0 h, 4 h and 8 h after surgery via intraperitoneal injection (Prass et al., 2003; Wong et al., 2011). For experiments using the  $\beta_2$ AR inhibitor, ICI-118551 (Sigma-Aldrich), mice were treated with 1 mg/kg at 0 h, 4 h and 8 h after surgery via intraperitoneal injection (Mracsko et al., 2014). Saline injections were used as treatment control.

### 2.4. Plasma collection and circulating catecholamine analysis

In naive mice or at 15 min, 30 min, 60 min and 3 h after stroke or sham surgery, whole blood was taken from mice via cardiac puncture and immediately transferred into tubes containing 20  $\mu$ l of 250 mM egtazic acid (EGTA) and 100 mM glutathione. Samples were centrifuged for 10 min at 220 $\times$ g at 4 °C for plasma collection and stored at -80 °C until catecholamine quantification. Plasma catecholamines were extracted and quantified as previously described (Lambert and Jonsdottir, 1998; Smolich et al., 2017). Briefly, plasma was placed in 0.5 ml of 0.4 M perchloric acid containing 0.01% EDTA with 3,4-dihydroxybenzylamine. Samples were centrifuged and supernatant was collected. Catecholamines were extracted from the supernatant using alumina adsorption, separated by high performance liquid chromatography, and quantified by colorimetric detection.

### 2.5. Collection of interscapular brown adipose tissue (iBAT) to qualify sympathetic activity

At 3 and 24 h after stroke or sham surgery, mice were culled and the interscapular brown adipose tissue (iBAT) was dissected carefully and collected for assessment of UCP-1 protein expression. iBAT was homogenised in a modified ice-cold radioimmunoprecipitation assay lysis buffer (10 mM Tris, pH 7.4, 0.1% SDS, NaCl 150 mM, 0.5% deoxycholic acid, 1% Triton X-100) containing Protease Inhibitor cocktail (Roche). The homogenates were centrifuged at 9600 $\times$ g for 5 min at 4 °C and the protein concentration of the supernatant was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Laemmli

sample buffer and NuPAGE Sample reducing agent (Invitrogen) was then added to each sample. Samples were heated at 95 °C for 10 min before loading them (20 µg of protein/sample) onto 4–12% NuPAGE Bis-Tris gels (Invitrogen) with MOPS SDS Running Buffer (Invitrogen) and SeeBlue Plus2 protein standard (Invitrogen). After electrophoresis at 110 V for 2 h, the proteins were transferred at 20 V for 1.5 h at 4 °C onto Immobilon-P polyvinylidene difluoride membranes (Merck Millipore) in NuPAGE transfer buffer (Life Technologies) containing 20% methanol. The membranes were blocked in 5% non-fat milk for 1 h, and then incubated overnight at 4 °C with a rabbit polyclonal antibody raised against UCP-1 (Thermo Fisher PA5-29575, 1:1000 dilution), followed by incubation with a goat anti-rabbit HRP antibody (1 h at room temperature, 1:1000 dilution; Enzo, ADI-SAB-300J). The membranes were developed by Amersham ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences). The membrane was then washed in Tris-buffered saline-Tween 20 and bound antibodies removed with stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween-20, pH 2.2) before blocking in 5% non-fat milk for 1 h. The membranes were then incubated overnight at 4 °C with a mouse monoclonal antibody against β-actin (1:10,000 dilution, Sigma, A5441), followed by a goat anti-mouse HRP antibody (1 h at room temperature, 1:10,000; Cayman Chemical, 10004302). The membranes were imaged using a GE ImageQuant LAS 4000 and ImageQuant software (GE Healthcare Life Sciences). Subsequent densitometric quantitative analysis was performed using Image Studio Lite. Densitometry values for UCP-1 and β-actin were assessed, and the ratio of the densitometry value of UCP-1 to β-actin was calculated.

## 2.6. Infarct quantification

At experimental endpoints, brains dissected from culled animals were slowly frozen over liquid nitrogen. Coronal sections at a thickness of 30 µm were collected at every 420 µm intervals. For infarct staining, sections were submerged in 0.1% thionin (Sigma) for 2 min, 70% ethanol for 2 min, and 100% ethanol for 2 min, with washing performed with deionised water between each step. Sections were dried and submerged in HistoSol before mounting coverslips using DPX Mountant (Thermo Fisher). Images were captured using a Canon PowerShot SX730 HS mounted above a light box. Infarct volume was measured using ImageJ (NIH, Bethesda, MD, USA) and corrected for brain oedema using the formula: corrected infarct volume = [left hemisphere area – (right hemisphere area – right hemisphere infarct area) × distance of sections between each slice].

## 2.7. Examining for signs of immune suppression

At experimental endpoints, mice were anaesthetised via inhalation of isoflurane and a cardiac puncture was performed to collect blood using a 26G needle. Blood was dispensed into a heparin-containing tube and a haemocytometer was used to count the number of circulating white blood cells. Subsequently, mice were culled by cervical dislocation and the spleen was dissected and weighed.

## 2.8. Bacteriological analysis

At experimental endpoints, mice were humanely culled and sterilised with 80% ethanol. The whole lung was dissected, weighed and placed in sterile PBS. Lungs were homogenised under sterile conditions. To determine bacterial load, lung homogenate was serially diluted and plated in triplicates onto brain heart infusion agar plates, supplemented with 5% sheep blood as supplied by the Department of Microbiology at Monash University (Clayton, Victoria, Australia). Plates were incubated at 37 °C in aerobic conditions and bacterial colonies quantified 24 h later.

## 2.9. Statistical analysis

Data were analysed in GraphPad Prism 9. To compare data between two groups, an unpaired *t*-test or Mann-Whitney *u*-test was performed depending on the normality of the data. For comparisons between more than two groups, a Two-way ANOVA with Tukey's multiple comparisons test was used for normally distributed data. All graphs are presented as mean ± standard error of the mean (SEM). Comparisons were considered statistically significant when  $p < 0.05$ .

## 3. Results

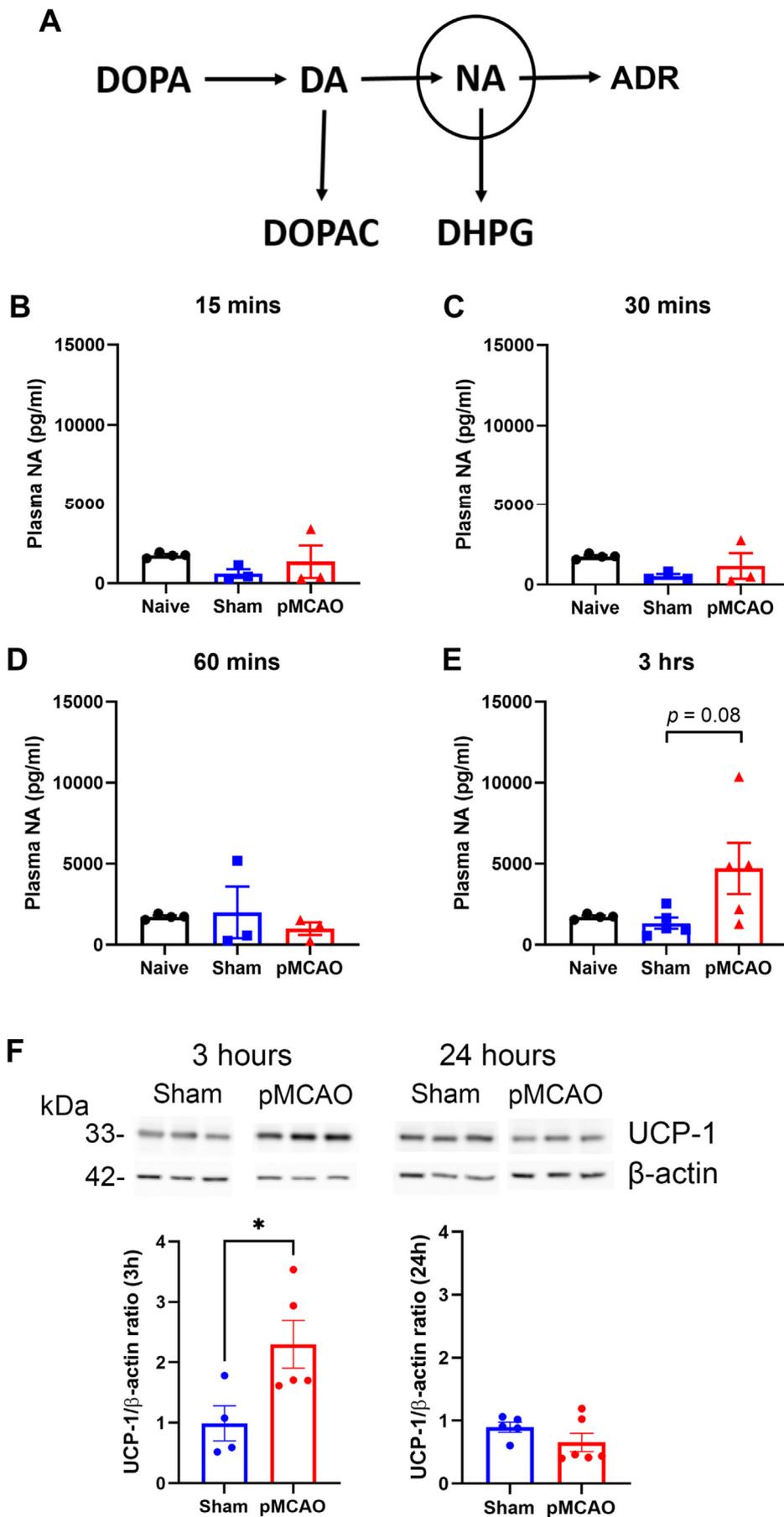
### 3.1. Systemic noradrenaline and dopamine are elevated after stroke

To investigate the effect of stroke on the SNS, we assessed circulating levels of NA, its precursors and metabolites at 15 min, 30 min, 60 min and 3 h following induction of experimental stroke and compared it to naïve and sham-operated animals. It is of note that the sham-operated group is a key surgical control for experiments with stroke induction. Following conversion by dopamine beta-hydroxylase (DBH), NA was shown to be slightly elevated at 3 h following stroke (4713 pg/ml ± 1583 vs sham 1337 pg/ml ± 348.5,  $p = 0.08$ ), but not at earlier timepoints (Fig. 1a–e). However, circulating levels of NA only represents approximately 5–10% of total NA released from nerve endings (Eisenhofer et al., 1990), and the peak of NA release systemically may occur after 3 h post-stroke. Therefore, to demonstrate stroke induces systemic sympathetic activation, we examined the expression of UCP-1 in the iBAT of sham-operated and post-stroke mice at 3 and 24 h. UCP-1 is a mitochondrial protein expressed in the densely SNS-innervated iBAT, the activity in this setting is indicative of global SNS activation after stroke (Richard, 2007). We found the expression of UCP-1 in iBAT was transiently elevated at 3 h following stroke onset (2.30 ± 0.39 vs sham 0.99 ± 0.29,  $p = 0.039$ ), with levels of expression returning to sham-operated levels at 24 h (0.65 ± 0.15 vs sham 0.89 ± 0.08,  $p = 0.21$ ; Fig. 1f).

Amongst the other precursors and metabolites, we did not detect any changes in the circulating levels of 3, 4-dihydroxyphenylalanine (DOPA), dihydroxyphenylacetic acid (DOPAC), adrenaline (ADR), or dihydroxyphenylglycol (DHPG) within the first 3 h following induction of ischaemia compared to sham-operated or naïve animals (summarised in Table 1). We also examined the level of circulating dopamine (DA) which is the primary precursor for the production of NA (Fig. 2a). While there were no changes to circulating DA levels at 15 min (376.4 pg/ml ± 246.3; Figs. 2b), 30 min (329.4 pg/ml ± 181.7; Fig. 2c), or 60 min (239.5 pg/ml ± 81.95; Fig. 2d) following stroke onset compared to sham-operated or naïve mice, DA was significantly elevated by 3 h in post-stroke mice (1133 pg/ml ± 354.8) compared to both naïve (192.5 pg/ml ± 29.46,  $p = 0.049$ ) or sham-operated mice (253.1 pg/ml ± 50.1,  $p = 0.040$ ; Fig. 2e). Taken together, these findings suggest that stroke induces the systemic activation of the sympathetic nervous system within hours of the brain injury, as evidenced by an elevation of UCP-1 activity in iBAT, and circulating DA which is primarily metabolised into NA at 3 h following experimental stroke.

### 3.2. Deficiency in the β<sub>2</sub>AR does not alter post-stroke lung infection

To test the hypothesis that elevated SNS activity after stroke induces post-stroke immunosuppression and susceptibility to infection via activation of the β<sub>2</sub>AR (the most predominant β<sub>2</sub>AR expressed on immune cells), we examined the effect of stroke on mice deficient of β<sub>2</sub>AR. At 24 h after pMCAO, the infarct volume was comparable between post-stroke WT (42.40 mm<sup>3</sup> ± 6.56) and β<sub>2</sub>AR deficient mice (50.48 mm<sup>3</sup> ± 6.31,  $p = 0.67$ ; Fig. 3a), suggesting genetic deficiency of β<sub>2</sub>AR does not impact on brain injury development in this model. Consistent with previous findings, spontaneous lung infection was evident in post-stroke WT mice at 24 h (395,126 CFU/g ± 371,281) compared to those that underwent sham surgery ( $p = 0.007$ ; Fig. 3b), and this was accompanied by the

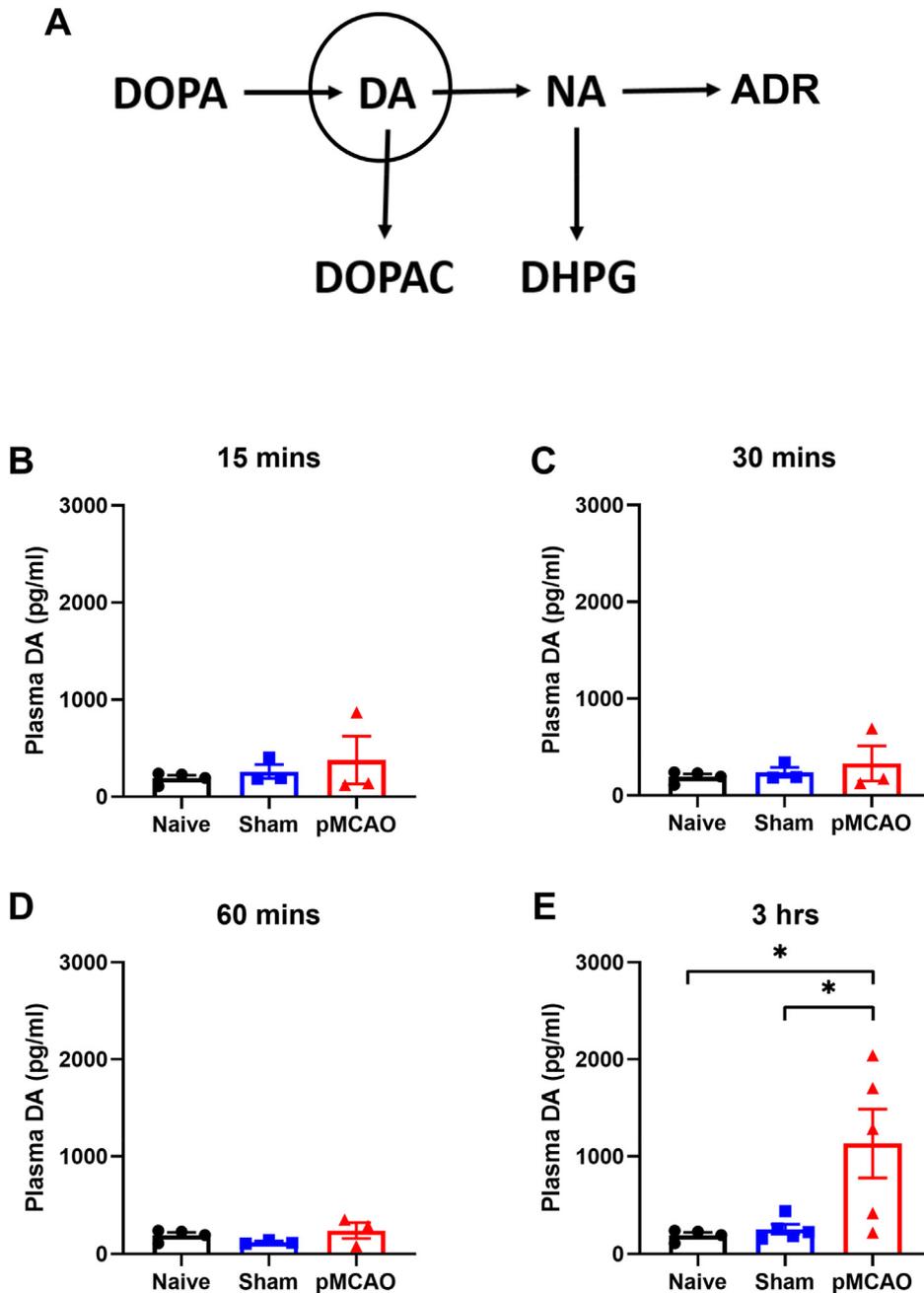


**Fig. 1.** Evidence for systemic activation of SNS at 3 h following stroke. Noradrenaline (NA) is synthesized from dopamine by the dopamine  $\beta$ -hydroxylase (DBH) enzyme (A). Naïve mice and mice that underwent pMCAO or sham surgery and were culled and plasma was collected via cardiac puncture at 15 min (B), 30 min (C), 60 min (D) and 3 h (E) post-surgery to assess the levels of plasma NA. Data are represented as mean  $\pm$  SEM,  $n = 3$  mice per group in 15 min, 30 min and 60 min time-points, and  $n = 5$  for 3 h timepoints,  $p = 0.08$ ,  $t$ -tests. (F) The protein levels of UCP-1 in the iBAT of sham-operated and post-stroke mice were assessed via Western blots at 3 h and 24 h. Data are represented as mean  $\pm$  SEM,  $n \geq 4$  mice per group. \* $p < 0.05$ ,  $t$ -tests.

**Table 1**

Plasma catecholamine levels at 15 min, 30 min, 60 min and 180 min after pMCAO. Mean concentrations in pg/ml with  $\pm$ SEM. Abbreviations: ADR – adrenaline; DOPA – 3,4-dihydroxyphenylalanine; DOPAC – 3,4-dihydroxyphenylacetic acid; DHPG – 3,4-dihydroxyphenylglycol

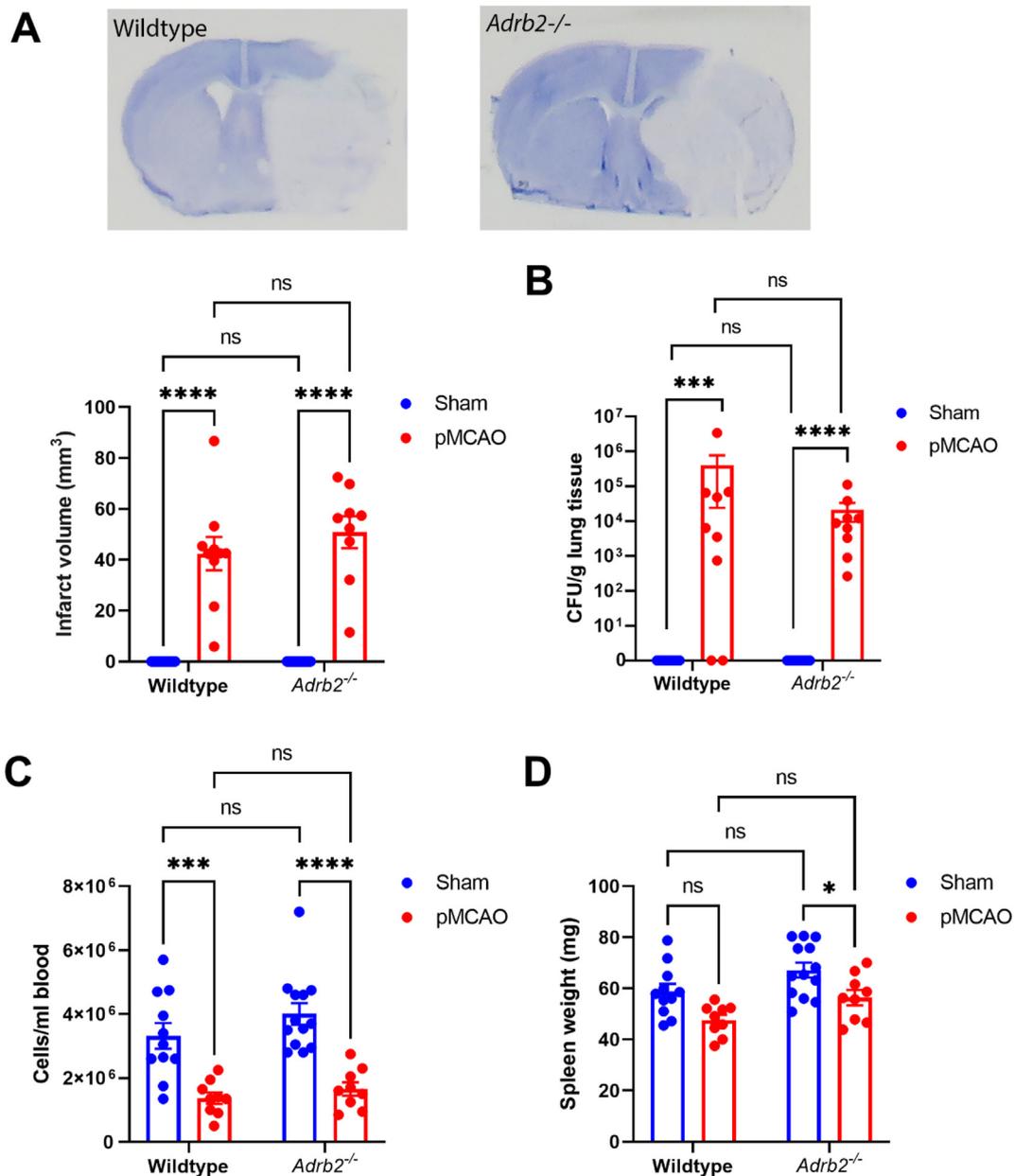
	Naive	Sham	15 min post-pMCAO	30 min post-pMCAO	60 min post-pMCAO	180 min post-pMCAO
DOPA	977.9 ( $\pm$ 64.99)	1700 ( $\pm$ 115.3)	1335 ( $\pm$ 131.0)	1338 ( $\pm$ 94.24)	1293 ( $\pm$ 82.14)	841.0 ( $\pm$ 111.3)
ADR	586.1 ( $\pm$ 109.6)	256.0 ( $\pm$ 45.07)	269.1 ( $\pm$ 70.3)	460.2 ( $\pm$ 175.3)	718.4 ( $\pm$ 271.4)	401.1 ( $\pm$ 73.1)
DOPAC	2275 ( $\pm$ 346.1)	4675 ( $\pm$ 1490)	4347 ( $\pm$ 1004)	3916 ( $\pm$ 399.0)	4086 ( $\pm$ 720.3)	1704.0 ( $\pm$ 295.2)
DHPG	5920 ( $\pm$ 100.3)	2099 ( $\pm$ 280.3)	2030 ( $\pm$ 212.0)	1612 ( $\pm$ 120.5)	1844 ( $\pm$ 154.8)	3533.0 ( $\pm$ 578.8)



**Fig. 2.** Plasma DA is elevated by 3 h following experimental stroke. The conversion of DOPA to dopamine (DA) is a one-step enzymatic reaction catalysed by aromatic L-amino acid decarboxylase (AADC; A). Naive mice and mice that underwent pMCAO or sham surgery were culled and plasma was collected via cardiac puncture at 15 min (B), 30 min (C), 60 min (D) and 3 h (E) post-surgery to assess the levels of plasma DA. Data are represented as mean  $\pm$  SEM, n = 3 mice per group in 15 min, 30 min and 60 min timepoints, and n = 5 for 3 h timepoints, \* $p$  < 0.05,  $t$ -tests.

characteristic signs of post-stroke immunosuppression such as marked decrease of circulating leukocyte numbers (1,372,222 cells/ml  $\pm$  178,946 vs sham 3,318,182 cells/ml  $\pm$  403,635,  $p$  = 0.001; Fig. 3c), though spleen weight remain unchanged (47.64 mg  $\pm$  2.04 vs sham 58.81 mg  $\pm$  3.02,  $p$  = 0.063; Fig. 3d). Similarly, post-stroke *Adrb2*<sup>-/-</sup> mice had comparable levels of bacterial load in the lung following

pMCAO when compared to their post-stroke WT counterpart (21,285 CFU/g  $\pm$  11,792,  $p$  = 0.49; Fig. 3b), as well as similar stroke-induced reduction of circulating leukocyte count (1,661,111 CFU/g  $\pm$  207,796,  $p$  = 0.99; Fig. 3c) and spleen weight (56.39 mg  $\pm$  3.01,  $p$  = 0.28; Fig. 3d). Overall, these findings suggest that the lack of *Adrb2* in mice does not alter brain infarct development after the pMCAO model of stroke nor the



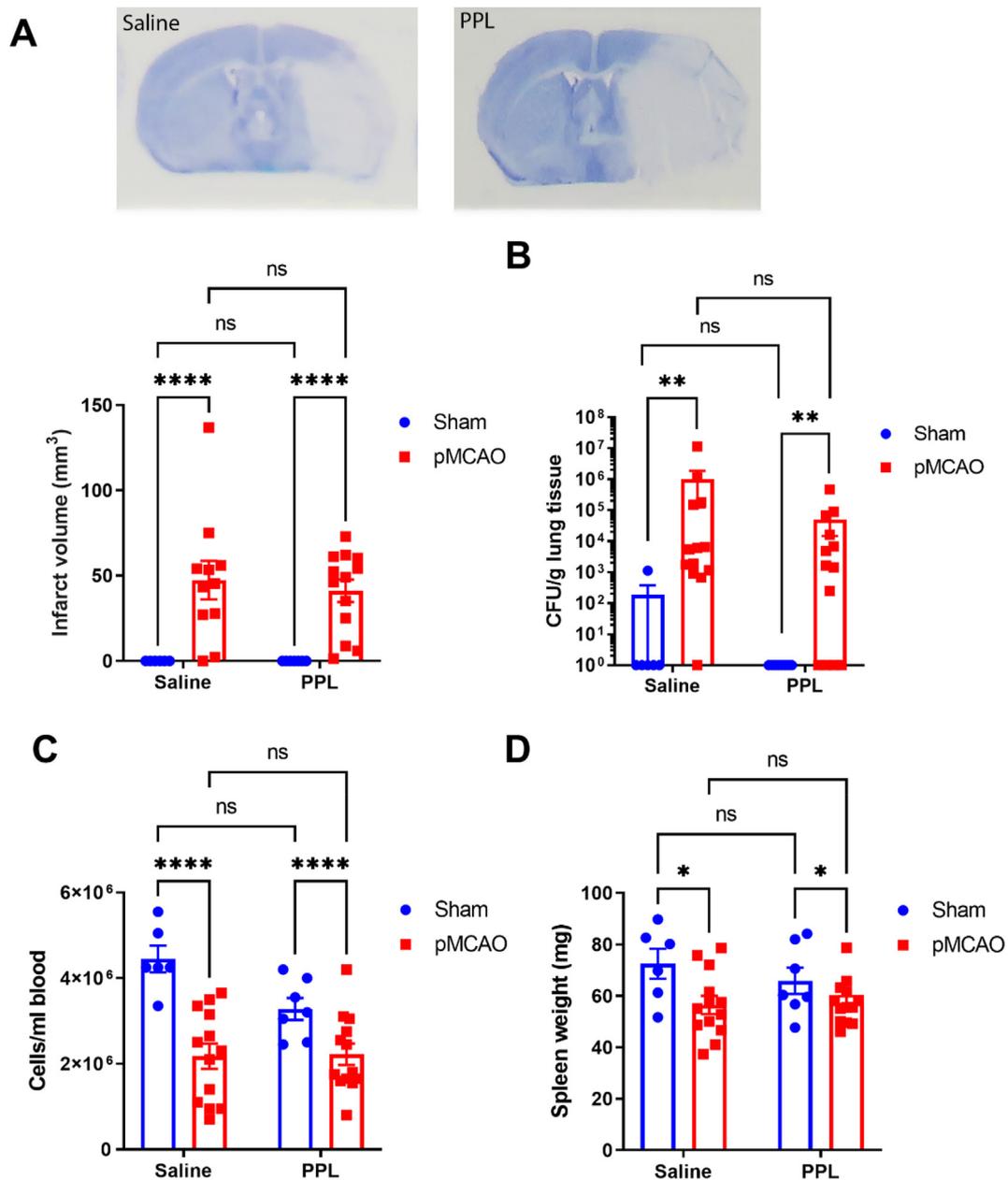
**Fig. 3.** Deficiency of the  $\beta_2$ AR does not protect from post-stroke infection. Wildtype (WT) or  $\beta_2$ AR deficient (*Adrb2*<sup>-/-</sup>) mice underwent pMCAO or sham surgery. At 24 h following surgery, mice were culled, where brains were collected, frozen and sliced to quantify infarct volume (A, unstained area signify infarct region), lungs were collected for bacteriological analysis (B), circulating leukocytes enumerated (C), and spleen weights assessed (D). Data are presented as mean  $\pm$  SEM,  $n \geq 9$  mice per group, ns denotes no statistically significant difference, \* $p > 0.05$ , \*\*\* $p > 0.001$ , \*\*\*\* $p > 0.0001$ , two-way ANOVA for normally distributed data or Mann-Whitney *u*-test for bacteriological analysis.

susceptibility to post-stroke infection.

### 3.3. Non-selective $\beta$ -blocker treatment and stroke

Previous studies have suggested that mice which are genetically deficient in  $\beta_2$ AR may develop compensatory mechanisms involving the overexpression of the  $\beta_1$ - and  $\beta_3$ -adrenergic receptors (Shim et al., 2019). As such, the lack of difference in post-stroke infection and immune suppression between WT and *Adrb2*<sup>-/-</sup> mice may be due to such compensatory mechanisms. Additionally, multiple reports have previously shown post-stroke administration of  $\beta$ -blocker is effective in reducing lung infection following cerebral ischaemia-reperfusion injury (Prass et al., 2003; Wong et al., 2011), however a majority ischaemic strokes in the clinics do not have a reperfusion component (Kruyt et al., 2013). Therefore, we next examined if the reduced post-stroke infection

following treatment with  $\beta$ -blocker is also seen after a non-reperused pMCAO stroke model. Administering PPL in post-stroke mice did not affect infarct size ( $41.12 \text{ mm}^3 \pm 6.58$  vs saline  $47.40 \text{ mm}^3 \pm 11.28$ ,  $p = 0.99$ ; Fig. 4a) nor reduce bacterial load in the lung ( $50,221 \text{ CFU/g} \pm 35,495$  compared to saline-treated post-stroke mice  $990,191 \text{ CFU/g} \pm 869,587$ ,  $p = 0.31$ ; Fig. 4b). Hallmarks of immune suppression were evident following pMCAO in saline-treated mice as there was a clear decrease in circulating leukocytes ( $2,176,923 \text{ cells/ml} \pm 294,698$  vs sham  $4,450,000 \text{ cells/ml} \pm 310,913$ ,  $p = 0.0001$ ; Fig. 4c) and spleen weight ( $56.53 \text{ mg} \pm 3.53$  vs sham  $72.57 \text{ mg} \pm 5.85$ ,  $p = 0.047$ ; Fig. 4d). In comparison to saline-treated mice that underwent pMCAO, PPL treatment post-stroke resulted in similar levels of circulating leukocytes ( $2,223,077 \text{ cells/ml} \pm 249,655$ ; Fig. 4c) and spleen weight ( $57.48 \text{ mg} \pm 2.40$ ; Fig. 4d). Therefore, our data suggests that the PPL treatment was ineffective in reducing post-stroke brain injury, and it did not reverse characteristics of immune



**Fig. 4.** Non-selective inhibition of  $\beta_2$ ARs with PPL does not reduce infection following experimental stroke. Mice that underwent pMCAO or sham surgery were treated with saline or PPL (30 mg/kg) at 0 h, 4 h and 8 h following surgery. At 24 h after surgery, mice were culled, where brains were collected, frozen and sliced to quantify infarct volume (A, unstained area signify infarct region), lungs were collected for bacteriological analysis (B), circulating leukocytes enumerated (C), and spleen weights recorded (D). Data are presented as mean  $\pm$  SEM,  $n \geq 6$  mice per sham group and  $n = 13$  mice per pMCAO group, ns denotes no statistically significant difference, \* $p > 0.05$ , \*\* $p > 0.01$ , \*\*\*\* $p > 0.0001$ , two-way ANOVA for normally distributed data or Mann-Whitney  $u$ -test for bacteriological analysis.

suppression nor infection rate in the lung following this model of non-reperfused stroke.

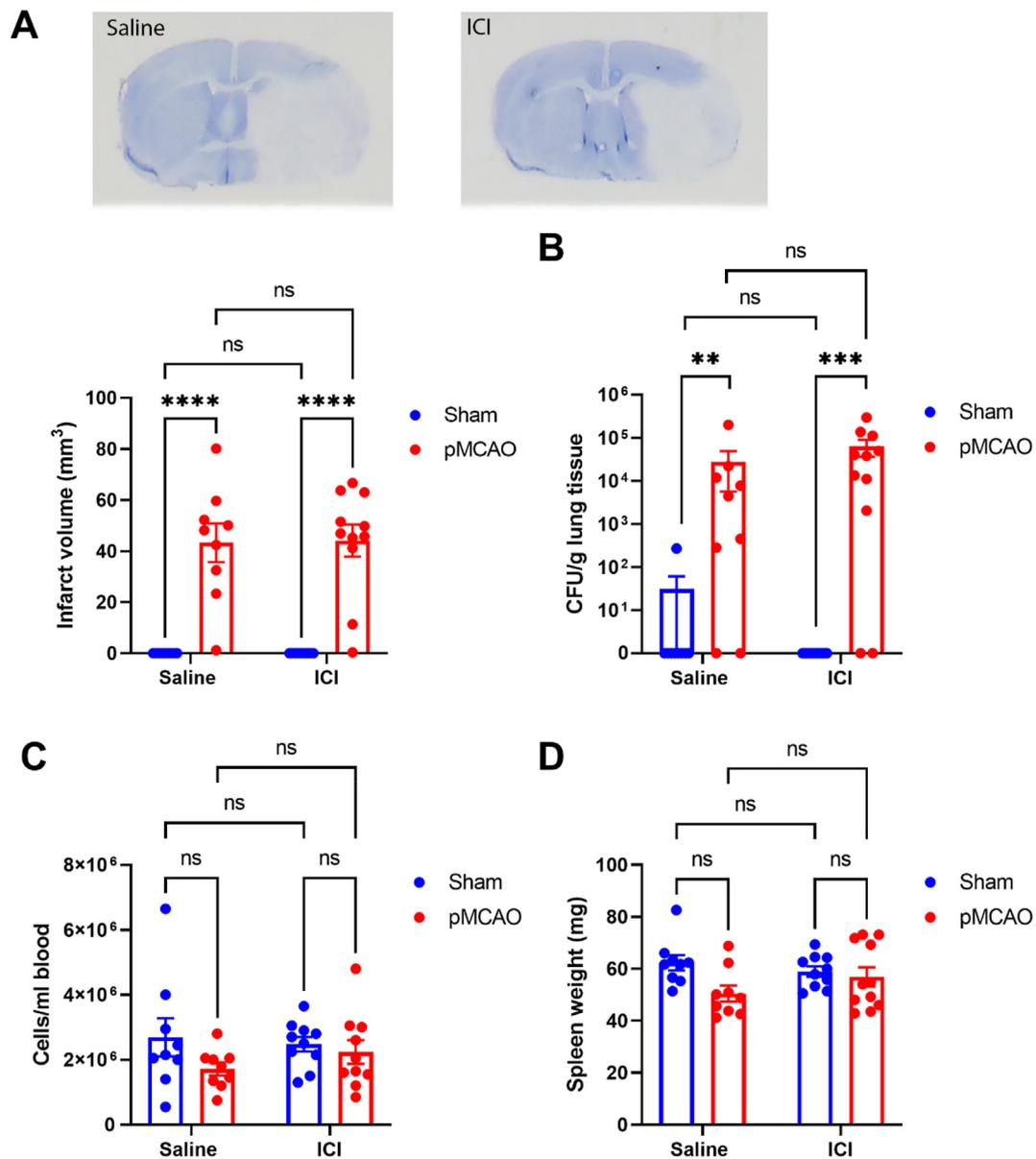
### 3.4. Selective $\beta_2$ AR inhibition and post-stroke outcomes

With the activation of the  $\beta_2$ AR on immune cells being a leading hypothesis by which post-stroke immune suppression occurs, we next tested the effect of selective  $\beta_2$ AR antagonist, ICI-118551 on post-stroke outcomes. We found that treatment with ICI-118551 did not impact on the development of infarction after stroke ( $44.16 \text{ mm}^3 \pm 6.29$  vs saline  $43.34 \text{ mm}^3 \pm 7.52$ ,  $p = 0.99$ ; Fig. 5a). Furthermore, post-stroke administration of ICI-118551 was not effective in reducing bacterial load in mice that underwent pMCAO ( $63,527 \text{ CFU/g} \pm 26,997$ ) when compared to their respective saline-treated counterparts ( $27,522 \text{ CFU/g} \pm 21,776$ ,

$p = 0.17$ ; Fig. 5b). Similar to PPL treatment, ICI-118551 treatment could not rescue the loss of circulating leukocytes ( $2,235,000 \text{ cells/ml} \pm 368,182$  vs saline  $1,716,667 \text{ cells/ml} \pm 200,347$ ,  $p = 0.76$ ; Fig. 5c) nor splenic atrophy ( $56.87 \text{ mg} \pm 3.75$  vs saline  $50.44 \text{ mg} \pm 3.11$ ,  $p = 0.45$ ; Fig. 5d) following stroke compared to their saline-treated counterparts.

## 4. Discussion

The occurrence of infection following stroke has traditionally been associated with aspiration, indwelling catheters, immobility, and other health comorbidities. Although these factors may play a role, recent evidence suggests that the high incidence of infection is attributed to stroke-induced impairment of the host defence. In light of new evidence showing a role for the SNS on immune regulation, we explored the effect



**Fig. 5.** Specific  $\beta_2$ AR antagonism does not alter post-stroke infection. Mice underwent pMCAO or sham surgery and were treated with saline or 1 mg/kg of ICI-118551 (ICI) at 0 h, 4 h and 8 h after surgery. At 24 h after surgery, mice were culled, where brains were collected, frozen and sliced to quantify infarct volume (A, unstained area signify infarct region), lungs were collected for bacteriological analysis (B), circulating leukocytes enumerated (C), and spleen weights recorded (D). Data are presented as mean  $\pm$  SEM,  $n \geq 9$  mice per group, ns denotes no statistically significant difference,  $**p > 0.01$ ,  $***p > 0.001$ ,  $****p > 0.0001$ , two-way ANOVA for normally distributed data or Mann-Whitney  $u$ -test for bacteriological analysis.

of inhibiting  $\beta$ -adrenergic receptor on post-stroke outcomes. Indeed, plasma NA and other catecholamines are elevated in patients with stroke (Akil et al., 2015; McCulloch et al., 2017; Myers et al., 1981), thus the notion of NA-mediated post-stroke immune suppression is a possibility. In this study, we revealed stroke induced a systemic activation of the SNS as indicated by elevated levels of plasma catecholamines and UCP-1 activity. Using a clinically relevant model of stroke, we found  $\beta_2$ AR deficient mice showed similar degrees of post-stroke immune impairment and infection rate compared to wildtype counterparts. Similarly, post-stroke wildtype mice treated with pharmacological inhibitors of the  $\beta$ ARs, including the non-selective antagonist PPL and selective  $\beta_2$ AR antagonist ICI-118551, were unable to reduce infection in mice that underwent ischaemic stroke. As such, our data suggests that other mechanisms independent or in combination with  $\beta_2$ AR activation contribute to the risk of developing post-stroke infection.

Studies that assess the systemic sympathetic activity by measuring

catecholamines following the time course of stroke onset have been scarce for a number of reasons. The half-life of NA in plasma is short (approximately 2 min) and the sensitivity of commercial NA detection assays is relatively low (De Raedt et al., 2015; Grassi et al., 1997; Holmes et al., 1994). Furthermore, the timepoint of 3 h post-stroke may be too acute for SNS dysregulation to be detected in the circulation. Indeed, a previous study showed plasma NA was elevated at 5 h following experimental stroke (Smith et al., 1986), while others show an elevation after the first day in stroke patients (Akil et al., 2015; McCulloch et al., 2019). Additionally, the concentration of NA in the plasma is dependent on the rate of release and the rate of removal from the plasma whereby reduced removal of NA can increase detection of plasma NA without a change in SNS output (Goldstein et al., 2003). The contrary may also occur, where despite an increase in SNS activity in certain organs, an increase in NA uptake or plasma clearance by various cells in respective organs would lead to no change in plasma NA. While there is some evidence that NA

plasma clearance remains unchanged following haemorrhagic stroke (Naredi et al., 2000), whether the same occurs in response to ischaemic stroke remains unknown. Although plasma NA represents up to 10% released by sympathetic nerve endings (De Raedt et al., 2015; Eisenhofer et al., 1990; Goldstein et al., 2003), previous studies have provided evidence of elevated plasma NA in the acute phase in patients following a stroke (Myers et al., 1981), and elevated muscle sympathetic nerve activity in the 3 months following stroke (Mizushima et al., 1998). Given the uncertainty around interpretation of plasma NA levels, some caution is required for the interpretation of the present data and future investigations could include measures of NA spill-over using isotope dilution (Jansson and Lambert, 1999) or direct nerve recording (Moretti et al., 2012). Additionally, in the present report we further assessed SNS activity by examining the expression of SNS-mediated UCP-1 in iBAT. Indeed, we provide evidence that experimental stroke elevated the global activation of SNS at 3 h post injury, shown via both quantifying NA metabolites and UCP-1 expression in iBAT, while it returns to baseline levels at 24 h.

To explore the hypothesis that NA binds to  $\beta$ AR, or  $\beta_2$ AR specifically, to induce post-stroke immune suppression and lung infection, we examined the effect of inhibiting this interaction genetically with *Adrb2*<sup>-/-</sup> mice or pharmacologically with non-selective (PPL) or selective  $\beta_2$ -adrenergic receptor antagonists (ICI-118551) on post-stroke outcomes. The use of PPL in experimental stroke has previously proven to reduce post-stroke infection following a mild to moderate severity reperfused model of stroke (Prass et al., 2003; Wong et al., 2011). However, given that less than 15% of patients with stroke are eligible for thrombolytic therapy or clot retrieval interventions (Kruyt et al., 2013), it is important to additionally test the role of PPL on non-reperfused stroke models (pMCAO) as that is more reflective of clinical settings. Here, we showed using a non-reperfused stroke model that post-stroke *Adrb2*<sup>-/-</sup> mice, and wildtype mice treated with PPL or ICI-118551 demonstrated similar levels of spontaneous lung infections and characteristics of immune suppression compared to their wildtype or vehicle-treated counterparts. These findings suggest the  $\beta_2$ AR signalling pathway is not single-handedly involved in post-stroke immune impairment and infection sequelae.

In a prospective clinical study, use of non-selective  $\beta$ -blockers within the first 3 days following stroke was associated with increased frequency of infection compared to patients that did not receive  $\beta$ -blockers (Starr et al., 2017; Westendorp et al., 2016). However, in the same study, patients that received selective  $\beta_1$ AR antagonists were not at greater risk to infection, thus implicating a differential role for each  $\beta$ AR in post-stroke infection (Starr et al., 2017). Our study contributes to the literature as we found that specific inhibition of the  $\beta_2$ AR using ICI-118551 did not alter the frequency of infection following experimental stroke. In fact, our previous study indicates that the severity of stroke is a greater contributor to the frequency of post-stroke infection (Shim et al., 2019), suggesting that  $\beta$ AR activation is not the sole driver of post-stroke immune suppression and that alternative or additional mechanisms are at play, especially when stroke severity increases. One such alternative mechanisms that were not assessed in this study include hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis, often determined by elevations in serum cortisol. Increased serum cortisol has been detected in patients during the acute phase following stroke and has also been associated with greater stroke severity and larger infarction (Christensen et al., 2004). Regarding the role of cortisol on post-stroke immunosuppression, elevated serum cortisol is also correlated with lymphopenia in a small cohort of patients (Zierath et al., 2018). Similar findings were echoed in experimental stroke where glucocorticoid receptor blockade prevented the loss of circulating lymphocytes (Mracsko et al., 2014; Prass et al., 2003). Importantly, elevation in serum cortisol was only detected following extensive infarction (Mracsko et al., 2014), and therefore supports the notion that other mechanisms contribute to the susceptibility to infection following severe stroke. Additionally, it is likely that the HPA axis and SNS work in tandem to contribute to post-stroke

infection where glucocorticoid receptor activation induce immune cell death and splenic atrophy, whilst  $\beta$ AR activation promotes immune cell dysfunction.

Considering the intricacy of SNS activation and difficulty in pinpointing the source of catecholamine release after stroke, it is unsurprising that clinical trials investigating the use of  $\beta$ -blockers as treatment options for stroke-associated infection have been inconclusive. Further complication to the concept of SNS inhibition with  $\beta$ -blockers to limit fatal lung infection after stroke is the influence of patient genetic variation. Seminal works have demonstrated that polymorphisms in the  $\beta_2$ -adrenergic receptor genes alter receptor physiology (Dishy et al., 2001; Drysdale et al., 2000), and subsequently therapeutic response (Kaye et al., 2003). Thus, in addition to the failure of preventive antibiotic therapy, this clearly identifies the need for treatments to reduce post-stroke infection. Future studies will need to account for the multiple mechanisms to post-stroke infection for the development of therapeutics, as well as consider the implications of stroke severity on the effectiveness of such treatments.

### Declaration of interest

All authors have no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. G.W.L. reports receiving travel and consultancy support from Medtronic.

### Author contributions

R.S., S.W.W. and C.H.Y.W. conceived and designed the experiments. R.S. wrote the manuscript, carried out all animal experiments and data analysis with assistance from: S.W.W, J.L.W. and C.H.Y.W. (animal surgeries), S.E.P. and G.W.L. (catecholamines quantification), J.L.W. (UCP-1 Western blots). All authors carried out manuscript revision.

### Acknowledgments

This work is supported by the National Heart Foundation, CSL Centenary Fellowship and the National Health and Medical Research Council (NHMRC, Australia: APP1104036). The financial supports have no role in conducting the research and/or preparation of the article.

### References

- Akil, E., Tamam, Y., Akil, M.A., Kaplan, I., Bilik, M.Z., Acar, A., Tamam, B., 2015. Identifying autonomic nervous system dysfunction in acute cerebrovascular attack by assessments of heart rate variability and catecholamine levels. *J. Neurosci. Rural Pract.* 6, 145.
- Barer, D., Cruickshank, J., Ebrahim, S., Mitchell, J., 1988. Low dose  $\beta$  blockade in acute stroke ("BEST" trial): an evaluation. *Br. Med. J.* 296, 737.
- Bassi, A., Colivicchi, F., Santini, M., Caltagirone, C., 2007. Cardiac autonomic dysfunction and functional outcome after ischaemic stroke. *Eur. J. Neurol.* 14, 917–922.
- Christensen, H., Boysen, G., Johannesen, H.H., 2004. Serum-cortisol reflects severity and mortality in acute stroke. *J. Neurol. Sci.* 217, 175–180.
- Connolly, E.S., Winfree, C.J., Stern, D.M., Stern, D.M., Solomon, R.A., Pinsky, D.J., 1996. Procedural and strain-related variables significantly affect outcome in a murine model of focal cerebral ischemia. *Neurosurgery* 38, 523–532.
- De Raedt, S., De Vos, A., De Keyser, J., 2015. Autonomic dysfunction in acute ischemic stroke: an underexplored therapeutic area? *J. Neurol. Sci.* 348, 24–34.
- Dishy, V., Sofowora, G.G., Xie, H.G., Kim, R.B., Byrne, D.W., Stein, C.M., Wood, A.J., 2001. The effect of common polymorphisms of the beta2-adrenergic receptor on agonist-mediated vascular desensitization. *N. Engl. J. Med.* 345, 1030–1035.
- Drysdale, C.M., McGraw, D.W., Stack, C.B., Stephens, J.C., Judson, R.S., Nandabalan, K., Arnold, K., Ruano, G., Liggett, S.B., 2000. Complex promoter and coding region beta 2-adrenergic receptor haplotypes alter receptor expression and predict in vivo responsiveness. *Proc. Natl. Acad. Sci. U. S. A.* 97, 10483–10488.
- Dziedzic, T., Slowik, A., Pera, J., Szczudlik, A., 2007. Beta-blockers reduce the risk of early death in ischemic stroke. *J. Neurol. Sci.* 252, 53–56.
- Eisenhofer, G., Esler, M.D., Cox, H.S., Meredith, I.T., Jennings, G.L., Brush Jr., J.E., Goldstein, D.S., 1990. Differences in the neuronal removal of circulating epinephrine and norepinephrine. *J. Clin. Endocrinol. Metab.* 70, 1710–1720.
- Eşref, A., Tamam, Y., Akil, M.A., Kaplan, I., Bilik, M.Z., Acar, A., Tamam, B., 2015. Identifying autonomic nervous system dysfunction in acute cerebrovascular attack by

- assessments of heart rate variability and catecholamine levels. *J. Neurosci. Rural Pract.* 6, 145–150.
- Goldstein, D.S., Eisenhofer, G., Kopin, I.J., 2003. Sources and significance of plasma levels of catechols and their metabolites in humans. *J. Pharmacol. Exp. Therapeut.* 305, 800–811.
- Grailer, J.J., Haggadone, M.D., Sarma, J.V., Zetoune, F.S., Ward, P.A., 2014. Induction of M2 regulatory macrophages through the  $\beta$ 2-adrenergic receptor with protection during endotoxemia and acute lung injury. *Journal of innate immunity* 6, 607–618.
- Grassi, G., Bolla, G., Seravalle, G., Turri, C., Lanfranchi, A., Mancina, G., 1997. Comparison between reproducibility and sensitivity of muscle sympathetic nerve traffic and plasma noradrenaline in man. *Clin. Sci.* 92, 285–289.
- Heuschmann, P.U., Kolominsky-Rabas, P.L., Misselwitz, B., Hermanek, P., Leffmann, C., Janzen, R., Rother, J., Buecker-Nott, H.-J., Berger, K., 2004. Predictors of in-hospital mortality and attributable risks of death after ischemic stroke: the German Stroke Registers Study Group. *Arch. Intern. Med.* 164, 1761–1768.
- Holmes, C., Eisenhofer, G., Goldstein, D.S., 1994. Improved assay for plasma dihydroxyphenylacetic acid and other catechols using high-performance liquid chromatography with electrochemical detection. *J. Chromatogr. B Biomed. Sci. Appl.* 653, 131–138.
- Hummel, I.B., Reinartz, M.T., Kälble, S., Burhenne, H., Schwede, F., Buschauer, A., Seifert, R., 2013. Dissociations in the effects of  $\beta$ 2-adrenergic receptor agonists on cAMP formation and superoxide production in human neutrophils: support for the concept of functional selectivity. *PLoS One* 8.
- Jansson, T., Lambert, G.W., 1999. Effect of intrauterine growth restriction on blood pressure, glucose tolerance and sympathetic nervous system activity in the rat at 3-4 months of age. *J. Hypertens.* 17, 1239–1248.
- Kaye, D.M., Smirk, B., Williams, C., Jennings, G., Esler, M., Holst, D., 2003. Beta-adrenoceptor genotype influences the response to carvedilol in patients with congestive heart failure. *Pharmacogenetics* 13, 379–382.
- Kruyt, N.D., Nederkoorn, P.J., Dennis, M., Leys, D., Ringleb, P.A., Rudd, A.G., Vermeulen, M., Stam, J., Hacke, W., Roos, Y.B., 2013. Door-to-needle time and the proportion of patients receiving intravenous thrombolysis in acute ischemic stroke: uniform interpretation and reporting. *Stroke* 44, 3249–3253.
- Kwan, J., Hand, P., 2007. Infection after acute stroke is associated with poor short-term outcome. *Acta Neurol. Scand.* 115, 331–338.
- Lambert, G.W., Jonsdottir, I.H., 1998. Influence of voluntary exercise on hypothalamic norepinephrine. *J. Appl. Physiol.* 85, 962–966.
- Langhorne, P., Stott, D., Robertson, L., MacDonald, J., Jones, L., McAlpine, C., Dick, F., Taylor, G., Murray, G., 2000. Medical complications after stroke a multicenter study. *Stroke* 31, 1223–1229.
- Liu, Q., Jin, W.-N., Liu, Y., Shi, K., Sun, H., Zhang, F., Zhang, C., Gonzales, R.J., Sheth, K.N., La Cava, A., 2017. Brain ischemia suppresses immunity in the periphery and brain via different neurogenic innervations. *Immunity* 46, 474–487.
- Maier, I.L., Karch, A., Mikolajczyk, R., Bähr, M., Liman, J., 2015. Effect of Beta-blocker therapy on the risk of infections and death after acute stroke—a historical cohort study. *PLoS One* 10, e0116836.
- McCulloch, L., Allan, S.M., Emsley, H.C., Smith, C.J., McColl, B.W., 2019. Interleukin-1 receptor antagonist treatment in acute ischaemic stroke does not alter systemic markers of anti-microbial defence. *F1000Research* 8.
- McCulloch, L., Smith, C.J., McColl, B.W., 2017. Adrenergic-mediated loss of splenic marginal zone B cells contributes to infection susceptibility after stroke. *Nat. Commun.* 8.
- Mizuno, K., Takahashi, H.K., Iwagaki, H., Katsuno, G., Kamurul, H.A., Ohtani, S., Mori, S., Yoshino, T., Nishibori, M., Tanaka, N., 2005.  $\beta$ 2-Adrenergic receptor stimulation inhibits LPS-induced IL-18 and IL-12 production in monocytes. *Immunol. Lett.* 101, 168–172.
- Mizushima, T., Tajima, F., Nakamura, T., Yamamoto, M., Lee, K.H., Ogata, H., 1998. Muscle sympathetic nerve activity during cold pressor test in patients with cerebrovascular accidents. *Stroke* 29, 607–612.
- Moretti, J.L., Burke, S.L., Davern, P.J., Evans, R.G., Lambert, G.W., Head, G.A., 2012. Renal sympathetic activation from long-term low-dose angiotensin II infusion in rabbits. *J. Hypertens.* 30, 551–560.
- Mracsko, E., Liesz, A., Karcher, S., Zorn, M., Bari, F., Veltkamp, R., 2014. Differential effects of sympathetic nervous system and hypothalamic–pituitary–adrenal axis on systemic immune cells after severe experimental stroke. *Brain Behav. Immun.* 41, 200–209.
- Myers, M.G., Norris, J.W., Hachinski, V., Sole, M.J., 1981. Plasma norepinephrine in stroke. *Stroke* 12, 200–204.
- Naredi, S., Lambert, G., Eden, E., Zall, S., Runnerstam, M., Rydenhag, B., Friberg, P., 2000. Increased sympathetic nervous activity in patients with nontraumatic subarachnoid hemorrhage. *Stroke* 31, 901–906.
- Naredi, S., Lambert, G., Friberg, P., Zall, S., Eden, E., Rydenhag, B., Tylman, M., Bengtsson, A., 2006. Sympathetic activation and inflammatory response in patients with subarachnoid haemorrhage. *Intensive Care Med.* 32, 1955–1961.
- Offner, H., Subramanian, S., Parker, S.M., Wang, C., Afentoulis, M.E., Lewis, A., Vandenbark, A.A., Hurn, P.D., 2006. Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. *J. Immunol.* 176, 6523–6531.
- Offner, H., Vandenbark, A., Hurn, P., 2009. Effect of experimental stroke on peripheral immunity: CNS ischemia induces profound immunosuppression. *Neuroscience* 158, 1098–1111.
- Prass, K., Meisel, C., Höflich, C., Braun, J., Halle, E., Wolf, T., Ruscher, K., Victorov, I.V., Priller, J., Dirnagl, U., 2003. Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J. Exp. Med.* 198, 725–736.
- Richard, D., 2007. The control of the sympathetic nervous system (SNS)-mediated uncoupling 1 (UCP1) activity. *Appetite* 49, 323.
- Scanzano, A., Cosentino, M., 2015. Adrenergic regulation of innate immunity: a review. *Front. Pharmacol.* 6, 171.
- Shim, R., Wen, S.W., Wanrooy, B.J., Rank, M., Thirugnanachandran, T., Ho, L., Sephezadeh, T., de Veer, M., Srikanth, V.K., Ma, H., 2019. Stroke severity, and not cerebral infarct location, increases the risk of infection. *Translational stroke research* 1–15.
- Smith, K.E., Hachinski, V.C., Gibson, C.J., Ciriello, J., 1986. Changes in plasma catecholamine levels after insula damage in experimental stroke. *Brain Res.* 375, 182–185.
- Smolich, J.J., Kenna, K.R., Esler, M.D., Phillips, S.E., Lambert, G.W., 2017. Greater sympathoadrenal activation with longer prevention intervals after immediate cord clamping increases hemodynamic lability at birth in preterm lambs. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 312, R903–R911.
- Starr, J.B., Tirschwell, D.L., Becker, K.J., 2017. Increased infections with  $\beta$ -blocker use in ischemic stroke, a  $\beta$ 2-receptor mediated process? *Neurol. Sci.* 38, 967–974.
- Sykora, M., Siarnik, P., Diedler, J., Lees, K., Alexandrov, A., Bath, P., Bluhmki, E., Bornstein, N., Claesson, L., Davis, S., 2015.  $\beta$ -Blockers, pneumonia, and outcome after ischemic stroke: evidence from virtual international stroke trials archive. *Stroke* 46, 1269–1274.
- Vermeij, F.H., op Reimer, W.J.S., De Man, P., Van Oostenbrugge, R.J., Franke, C.L., De Jong, G., De Kort, P.L., Dippel, D.W., 2009. Stroke-associated infection is an independent risk factor for poor outcome after acute ischemic stroke: data from The Netherlands Stroke Survey. *Cerebrovasc. Dis.* 27, 465–471.
- Westendorp, W.F., Vermeij, J.-D., Brouwer, M.C., Roos, Y., Nederkoorn, P.J., van de Beek, D., Investigators, P., 2016. Pre-stroke use of beta-blockers does not lower post-stroke infection rate: an exploratory analysis of the preventive antibiotics in stroke study. *Cerebrovasc. Dis.* 42, 506–511.
- Wirth, T., Westendorf, A.M., Bloemker, D., Wildmann, J., Engler, H., Moller, S., Wadwa, M., Schäfer, M.K.-H., Schedlowski, M., del Rey, A., 2014. The sympathetic nervous system modulates CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells via noradrenaline-dependent apoptosis in a murine model of lymphoproliferative disease. *Brain Behav. Immun.* 38, 100–110.
- Wong, C.H., Jenne, C.N., Lee, W.-Y., Léger, C., Kubes, P., 2011. Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. *Science* 334, 101–105.
- Xiong, L., Tian, G., Leung, H., Soo, Y.O., Chen, X., Ip, V.H., Mok, V.C., Chu, W.C., Wong, K.S., Leung, T.W., 2018. Autonomic dysfunction predicts clinical outcomes after acute ischemic stroke: a prospective observational study. *Stroke* 49, 215–218.
- Zaldivia, M.T., Rivera, J., Hering, D., Marusic, P., Sata, Y., Lim, B., Eikelis, N., Lee, R., Lambert, G.W., Esler, M.D., Htun, N.M., Duval, J., Hammond, L., Eisenhardt, S.U., Flierl, U., Schlaich, M.P., Peter, K., 2017. Renal denervation reduces monocyte activation and monocyte-platelet aggregate formation: an anti-inflammatory effect relevant for cardiovascular risk. *Hypertension* 69, 323–331.
- Zierath, D., Tanzi, P., Shibata, D., Becker, K.J., 2018. Cortisol is more important than metanephrines in driving changes in leukocyte counts after stroke. *J. Stroke Cerebrovasc. Dis.* 27, 555–562.