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Long-term stimulation of cardiac vagal preganglionic neurons reduces blood pressure in the spontaneously hypertensive rat

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Authors contribution
TSM, VRA and NM designed research; TSM, VRA and BF performed research and analyzed data; TSM, VRA and NM wrote the paper. TSM, VRA, BF and NM preformed critical review of the manuscript. All authors approved the final version.

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Abstract

BACKGROUND: Arterial hypertension is associated with autonomic nervous system dysfunction. Different interventional strategies have been implemented in recent years for the reduction of sympathetic activity in subjects with hypertension. However, the therapeutic benefit of increasing vagal tone in hypertensive subjects remains largely unexplored.

OBJECTIVE: Here we describe the effects of long-term activation of vagal neural pathways on arterial pressure (AP), heart rate (HR), AP variability and spontaneous baroreflex sensitivity in spontaneously hypertensive rats (SHR) and normotensive Wistar rats.

METHODS: Brainstem vagal preganglionic neurons residing in the dorsal vagal motor nucleus (DVMN) were targeted with a lentiviral vector to induce the expression of an artificial G(s) protein-coupled receptor termed Designer Receptors Exclusively Activated by Designer Drugs (DREADD-Gs). The transduced neurons were activated daily by systemic administration of otherwise inert ligand clozapine-n-oxide (CNO). AP measurements were recorded in conscious freely-moving animals after 21 consecutive days of DVMN stimulation.

RESULTS: Resting AP was significantly lower in SHRs expressing DREADD-Gs in the DVMN, compared to control SHRs expressing eGFP. No changes in AP were detected in Wistar rats expressing DREADD-Gs compared to rats expressing eGFP in the DVMN. Pharmacogenetic activation of DREADD-Gs-expressing DVMN neurons in SHRs was accompanied with increased baroreflex sensitivity and a paradoxical decrease in cardio-vagal components of HR and systolic AP variability in SHRs.

CONCLUSION: These results suggest that long-term activation of vagal parasympathetic pathways is beneficial in restoring autonomic balance in an animal model of neurogenic hypertension and might be an effective therapeutic approach for the management of hypertension.

Introduction

Arterial hypertension increases the risk of death from cardiovascular disease in adults aged between 40-69 years (1). Arterial hypertension is believed to affect one in four people and accounts for approximately one billion cases worldwide (2). Increased arterial blood pressure is one of the most crucial cardiovascular risk factors and has a dominant role in the pathogenesis of ischemic heart disease, heart failure, stroke, kidney failure and overall cardiovascular death. Despite important advances in the diagnosis and treatment of arterial hypertension, a significant proportion of patients have insufficient disease management due to either resistance to current drug therapy (3) or poor drug compliance. Thus, there is a significant unmet clinical need to find alternative strategies to conventional pharmacotherapy.

Over the last three decades, a growing body of evidence has unequivocally shown that both the development and maintenance of arterial hypertension are intimately linked to autonomic dysfunction, characterized by increased sympathetic activity (4; 5) and reduced cardio-vagal tone (6). The great majority of pre-clinical and clinical studies have historically concentrated on the detrimental effects of sustained sympathetic nervous system (SNS) overactivity on arterial blood pressure control (7-12). This has generated considerable interest in novel autonomic modulation approaches such as renal nerve ablation (13), deep brain stimulation (14) and carotid sinus stimulation (15) which have been shown to lower SNS activity, reduce arterial blood pressure levels and prevent end-organ damage, particularly in treatment-resistant subjects. However, the pathophysiological role of impaired vagal function in arterial hypertension remains largely unexplored and despite considerable evidence showing the beneficial effects of vagal nerve modulation on cardiovascular function (16), it remains unknown whether direct activation of vagal efferent pathways could attenuate the progression of the disease. Here, we employed a pharmacogenetic approach in the

spontaneously hypertensive rat model to investigate the physiological consequences of long-term activation of vagal preganglionic neurons on the development of arterial hypertension.

Methods

Animals

All animal experimentations were carried out in accordance with guidelines approved by University of São Paulo Institutional Animal Care and Use Committee (CEUA: 118/109-2). Experiments were performed on male adult hypertensive SHRs (8 weeks of age) and age-matched normotensive Wistar rats.

Viral vectors

Vagal preganglionic neurons of the DVMN, which express the transcriptional factor Phox2, were transduced using an artificial Phox2-activated promoter-PRSx8 as described previously (17-20). The DVMN neurons were targeted with a lentiviral vector to express an artificial G protein-coupled receptor (GPCR) termed Designer Receptors Exclusively Activated by Designer Drugs (DREADD). These are mutated muscarinic receptors, which are selectively activated by an otherwise biologically inert ligand clozapine-N-oxide (CNO) (21). A Gs-coupled version of this system (DREADD-Gs) fused with enhanced green fluorescent protein (eGFP) was used to activate neurons in a similar manner to that following activation of other Gs-coupled receptors. A lentiviral construct was used to induce the expression of DREADD-Gs in DVMN neurons under the control of the PRSX8 promoter (PRSx8-DREADD-Gs-eGFP-LV). For control experiments, DVMN neurons were transduced with a lentiviral vector to express eGFP (PRSx8-eGFP-LV). Vectors were generated in our laboratory facilities as described previously using a DREADDs construct kindly provided by J. Wess (22, 23). LVV titers were approximately between 1×10^{12} transducing units ml^{-1}.

Validation of efficacy of CNO-induced activation of Gs signaling in cells expressing DREADD-Gs was described in detail previously (22).

In vivo gene transfer

Rats were anesthetized [ketamine (80 mg kg-1; ip) and xylasine (7 mg kg−1, ip)] and placed in a stereotaxic frame (model 900; David Kopf Instruments). Adequate level of anesthesia was confirmed by the absence of a withdrawal response to a paw pinch. SHRs and Wistar rats were injected in the DVMN with two microinjections per side (0.25 μL each, 0.05 μL min^{-1}) of a viral suspension containing PRSx8-DREADD-Gs-eGFP-LV (DREADD-Gs; n=8 and n=4, respectively) or PRSx8-eGFP-LV (e-GFP, n=7 and n=5, respectively) using the following coordinates from *calamus scriptorius* (i) 0.5 mm rostral, 0.6 mm lateral, 0.8 mm ventral and (ii) 1.0 mm rostral, 0.8 mm lateral, 0.6 mm ventral. Anesthesia was reversed with atipamezole (1 mg kg^{-1}). All animals recovered normally without complications, received daily injections of buprenorphine for post-operative analgesic control (0.05 mg kg^{-1} subcutaneously for 5 days) and gained weight as expected for their age and size.

Application of CNO

Systemic administration of CNO allowed us to activate Gs signaling in DVMN neurons expressing DREADDs-Gs. The dose of CNO (0.3 mg/kg in 0.1 ml, subcutaneously) was based on the lowest concentration used in a previous study that was found to elicit robust activation of targeted neuronal populations and behavioral changes in conscious animals (24, 25). Animals were allowed to recover from viral gene transfer surgery for 8 days before the start of CNO treatment. CNO injections were delivered daily by the same experimenter and at the same time every day for 21 consecutive days.

Arterial blood pressure measurements

After 19 days of CNO treatment (i.e., at 11 weeks of age), rats were implanted with intra-arterial catheters. Animals were anesthetized with halothane (3% in O_2) and a polyethylene tube (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ, USA) filled with sterile saline containing heparin (50 U ml-1) and penicillin G (2000 U ml-1) was inserted into the left femoral artery and its tip was placed in the abdominal aorta caudal to the renal arteries (26). The opposite end was tunneled subcutaneously and exteriorized through the upper back and sealed with a plastic cap. All animals received an intramuscular injection of penicillin G (24,000 IU) and streptomycin (10 mg). Catheters were flushed the following day with the same solution containing heparin and penicillin. Arterial pressure recordings were performed 2 days after arterial catheterization. On the day of the experiments, the catheter was connected to a pressure transducer (MLT844, ADInstruments, Sydney, NSW, Australia) coupled to a preamplifier (Bridge Amp, ML221, ADInstruments, Sydney, NSW, Australia) that was connected to a Powerlab computer data acquisition system (PowerLab 16/30, ML880, ADInstruments). Arterial pressure measurements were taken between 8 am and 5 pm by an experimenter that was blind to the animal condition. Data was acquired in conscious animals for 60 minutes during quiet resting periods.

Power spectral analysis

Spectral analysis of the systolic pressure variability was performed off-line to estimate the relative level of sympathetic and parasympathetic activity in rats at 11 weeks of age. Mean, systolic and diastolic arterial pressures (MAP, SAP, and DAP; mmHg, respectively), pulse interval (PI; ms) and heart rate (HR; bpm) were measured from the AP recording using LabChart 8.0 (model Powerlab 8SP ADInstruments). Data was acquired after a 10 minute period of stabilization. Only data sets containing stable measurements of SAP and PI without artifacts or large sudden blood pressure changes were selected for analysis.

An algorithm was used to detect beat-to-beat inflection points in the PAP signal. SAP and PI variability analysis was carried out in time and frequency domains using custom software CardioSeries V2.4 (http://www.danielpenteado.com/) as described previously (27). Briefly, SAP and PI power spectral density were estimated by Fast Fourier Transform algorithm for time series. Using 10 Hz of interpolation rate, beat-by-beat series were divided in half overlapping sequential sets with 512 points. The spectra of SAP and PI were integrated into a low-frequency band (LF: 0.2-0.75 Hz, indicating mainly sympathetic influences) and a high-frequency band (HF: 0.75-3 Hz, indicative mainly of cardio-vagal tone). The results were expressed in absolute (ms^2) and normalized units (nu) obtained by calculating the percentage of LF and HF power with regard to the total power of the spectrum minus the very low frequency band (VLF: <0.2 Hz) (28). Sympathovagal balance was also calculated by assessing the LF/HF ratio of PI variability (27-30).

Spontaneous baroreflex sensitivity (BRS)

A beat-by-beat time series of SAP and PI was scanned in a 60-min data set acquired during quiet rest, searching for sequences of at least four consecutive beats in which AP increases were followed by PI lengthening (up-sequence) and AP decreases were followed by PI shortening (down-sequence) with a delay of 0, with no threshold in SAP and PIs and a linear correlation of >0.8. The slope of the linear regression lines between systolic SAP and PI was taken as a measure of spontaneous BRS. The baroreflex effectiveness index, which provides information on baroreflex function that is complementary to spontaneous BRS, was also calculated (31). It was defined as the ratio between the number of SAP ramps followed by respective reflex changes in PI and the total number of SAP ramps (independent of whether accompanied by the corresponding reflex PI ramps) observed over the time window studied.

Immunohistochemistry procedures

At the end of the *in vivo* experiments, the rats transduced to express DREADD-Gs or eGFP in the DVMN neurons were deeply anesthetized with sodium pentobarbital (60 mg/kg), injected with heparin (500 units, intracardially) and perfused through the ascending aorta with 0.9% saline solution followed by 4% phosphate-buffered (0.1 M, pH 7.4) paraformaldehyde. After 12 h of post-fixation and subsequent cryoprotection in 30% sucrose, 40-µm-thick coronal sections were collected along the rostro-caudal extent of the medulla oblongata. Sections were processed for the immunohistochemical detection of choline acetyl-transferase (ChAT). Tissue was incubated in goat anti-ChAT (1:500, Chemicon, raised against human placental ChAT) followed by donkey anti-goat Alexa Fluor 594 (Jackson). The specificity of the antibodies has been validated previously (32; 33).

Cell mapping, counting and imaging

A conventional multifunction microscope [brightfield, darkfield and epifluorescence; Zeiss Axioskop 2 microscope (Oberkochen, Germany)] was used for all observations except when indicated. ImageJ software (NIH) was used to count the various types of neuronal profiles within a defined area.

A one-in-six series of 40-µm brain sections was used per rat, for a total distance of 240 µm of separation between slices. The sections were counted bilaterally, and the numbers reported correspond exactly to the counts obtained in one series of sections. Section alignment between brains was completed relative to a reference section. Briefly, to align sections around the nucleus of the solitary tract (NTS) level, the section containing the mid-area postrema was identified in each brain and assigned the level 13.8 mm caudal to Bregma (Bregma = -13.8 mm). Levels rostral or caudal to this reference section were determined by

adding a distance corresponding to the interval between sections multiplied by the number of intervening sections.

Photographs were taken with a 12-bit color CCD camera (CoolSnap, Roper Scientific, Tuscon, AZ; resolution 1392 X 1042 pixels). The files were exported to the Canvas 9 software-drawing program for final modifications. The neuroanatomical nomenclature is based on Paxinos and Watson Atlas of Neuroanatomy (34).

Data analysis

Data normality was assessed using the Shapiro-Wilk test, and all the normally-distributed data were expressed as the mean ± SEM. The data regarding number of neurons ($ChAT^+$, $ChAT^+/eGFP^+$) and cardiovascular variables (i.e SAP, DAP, MAP, HR) were compared between groups and across time points using two-way ANOVA, with repeated measures for only the time factor. When applicable, the Student Newman Keuls post hoc test was used. Sigma Stat version 3.0 package (Jandel Corporation, Point Richmond, CA, USA) was used for all analysis. Significance level used was $p<0.05$.

Results

1) Expression of DREADD-Gs-eGFP in DVMN neurons.

The number and location of neurons expressing DREADD-Gs in the DVMN was mapped in each rat by identifying DREADD-Gs-eGFP fluorescence signal in vagal preganglionic neurons immunoreactive to ChAT (Figs. 1A-C). On average, DREADD-Gs was detected in 220±13 neurons counted in 5 coronal sections (n=7). Of the neurons expressing DREADD-Gs in all counted sections, 94±5% had detectable ChAT-immunoreactivity. Thus, 70±6% of ChAT-immunoreactive neurons in the DVMN were found to express the transgene (Fig. 1D).

2) Effect of long-term activation of DVMN neurons on arterial blood pressure

Figure 2 shows arterial blood pressure measurements obtained in SHRs and Wistar rats expressing DREADD-Gs or control eGFP after administration of CNO for 21 consecutive days. Arterial blood pressure levels were significantly lower in SHRs transduced to express DREADD-Gs in the DVMN compared to SHRs expressing eGFP (Figs. 2A-B). The SAP was 159.8±2 in SHRs expressing DREADD-Gs, vs. 177.6±3 mmHg in SHRs expressing eGFP [$F_{(3,31)}$ = 183,89; $p<0.001$]; DAP was 114.3±1.8 in SHRs expressing DREADD-Gs, vs. 124±2.7 mmHg in SHRs expressing eGFP [$F_{(3,31)}$ = 40,55; $p<0.01$]; MAP was 129.5±1.5 in SHRs expressing DREADD-Gs, vs. 142±1.2 mmHg in SHRs expressing eGFP [$F_{(3,31)}$ = 165,73; $p<0.001$] (Figs. 2A-C). HR was also found to be lower in SHRs expressing DREADD-Gs in the DVMN (302±5 bpm) compared to SHRs expressing eGFP (370±6 bpm, [$F_{(3,31)}$ = 68,08; $p<0.01$]; Fig. 2D). Resting SAP, DAP, MAP and HR were significantly higher in SHRs expressing DREADD-Gs compared to Wistar rats expressing either DREADD-Gs or eGFP ($p = 0.021$) (Figs. 2A-D). Cardiovascular parameters were not different between control Wistar rats expressing DREADD-Gs or eGFP in the DVMN (Figs. 2A-D).

3. Repetitive activation of DVMN neurons increases spontaneous baroreflex sensitivity

Figures 3A and B show the spontaneous baroreflex sensitivity gain and baroreflex effectiveness index in SHRs and Wistar rats transduced to express DREADD-Gs or eGFP in the DVMN. The results showed that rats from both groups expressing DREADD-Gs in the DVMN had higher spontaneous baroreflex sensitivity (7.7±0.7 in SHRs expressing DREADD-Gs, vs. 4.7±0.6 in SHRs expressing eGFP, $p = 0.024$ and 7.3±0.5 in Wistars expressing DREADD-Gs vs. 5.1±0.7 in Wistars expressing eGFP, $p = 0.03$) and baroreflex effectiveness index (0.15±0.025 in SHRs expressing DREADD-Gs vs. 0.073±0.03 in SHRs

expressing eGFP, p = 0.035 and 0.13±0.01 in Wistars expressing DREADD-Gs vs. 0.08±0.02 in Wistars expressing eGFP, p = 0.038) (Figs. 3A-B).

2.3) Heart rate and systolic arterial pressure and variability

The results of SAP and HR (PI) variability in the time and frequency domains from Wistar (controls: DREADD-Gs or eGFP) and SHRs (DREADD-Gs or eGFP) are showed in Figs. 3C-D and 4A-D. SAP variance and SAP LF were found to be lower in SHRs expressing DREADD-Gs as compared to SHRs expressing eGFP (p = 0.043) (Figs. 3C-D). The analysis of systolic AP variability showed no differences in PI variance. However, the SHRs expressing DREADD-Gs exhibited lower total power LF, and HF compared with SHR expressing e-GFP (p = 0.034) (Figs. 4B-C). Moreover, sympathovagal balance assessed by the LF/HF ratio, was shown to be lower in SHRs expressing DREADD-Gs compared to SHRs expressing eGFP (p = 0.032, Fig. 4D), suggesting a dominance of parasympathetic influences.

Discussion

In this study, we have shown that using a pharmacogenetic approach for repetitive stimulation of DVMN neurons for 21 days produced significant cardiovascular benefits in SHRs, including decreased arterial blood pressure and improved spontaneous baroreflex sensitivity. These data are consistent with previous studies showing improved cardiac and vascular function by either direct [electrical vagal stimulation, acetylcholine (Ach) administration and ACh receptor activation] or indirect vagal modulation (adenosine, cholinesterase inhibitors, statins and exercise training) (see 16 for review). In recent years, vagal modulation techniques such as baroreflex activation therapy and direct vagal nerve stimulation have been proposed as adjuvant therapies for the treatment of arterial hypertension (35). However, to our best knowledge this is the first study where a well-

defined population of vagal preganglionic neurons has been selectively targeted for repetitive activation of central parasympathetic pathways in an animal model of arterial hypertension.

Our results showed that arterial blood pressure was significantly reduced in SHRs transduced to express DREADD-Gs in the DVMN compared to SHRs expressing control eGFP, whilst Wistar rats showed no changes in cardiovascular parameters. This suggests that DVMN efferents might play an important role in the control of arterial blood pressure, however, the mechanisms are unclear. Ach is a powerful vasorelaxant that decreases blood vessel resistance by inducing phosphorylation of the rate-limiting enzyme involved in the biosynthesis of nitric oxide (NO), endothelial nitric oxide synthase (eNOS). Endothelium-derived NO-induced synthesis decreases the availability of intracellular calcium, resulting in a decrease in myosin phosphorylation and vasorelaxation that would ultimately lead to decreased arterial blood pressure. However, the arterioles in skeletal muscle that determine peripheral resistance are paradoxically devoid of cholinergic innervation and therefore, changes in afterload are highly unlikely to be produced by direct stimulation of parasympathetic efferent pathways from the DVMN. Thus, the arterial blood pressure-lowering effect of DVMN stimulation might be exerted elsewhere, probably at the level of the heart. Recent studies have shown that DVMN neurons provide a tonic inhibitory muscarinic influence on left ventricular contractility. Pharmacological activation of cardiac preganglionic vagal neurons that reside in the left caudal DVMN in the rat trigger profound reductions in left ventricular dp/dt_{max} (19). This suggests that reductions in arterial blood pressure in rats expressing DREADDS-Gs in the DVMN may have been the consequence of changes in cardiac output at the expense of reduced stroke volume and left ventricular inotropy. Long-term measurements of cardiac inotropy is needed to validate these claims.

Stimulation of DVMN neurons was also associated with significant increases in baroreflex gain and effectiveness, both in SHRs as well as in Wistar rats. This suggests that

DVMN stimulation might have improved baroreflex function, perhaps by inducing protective effects in baroreceptor function at the level of the great vessels. In recent years, several reports indicate that mechanical insults that increase arterial stiffening contribute directly to the development of arterial hypertension. Arterial stiffness is caused by disruption of elastin integrity in the aortic wall in particular with ageing, diabetes and chronic kidney disease. Stiffening at the level of the aortic root, impairs the Windkessel mechanism at this important interface, thereby reducing wave reflection and leading to increased central pulsatile pressure and transmission of excessive pulsatile energy that is known to promote end-organ damage. More importantly, reduced arterial compliance at higher pressures are believed to hamper baroreceptor activation thus causing a dysfunction in baroreflex control of heart rate and peripheral resistance. Studies performed in mouse models of impaired elastin expression have shown that stiffening of the aorta precedes the development of hypertension and subsequent increments in systolic pressure were inversely proportional to elastin content in the aorta (36). Interestingly, bilateral vagotomy in rats has been shown to induce similar structural remodeling changes leading to increased stiffening of the aorta (37). Even though arterial blood pressure was not reported in this study, this evidence suggests that the vagus nerve may prevent age-related structural changes in the great vessels that may contribute to the development of arterial hypertension. Future studies should endeavour to investigate whether long-term stimulation of DVMN efferent pathways induce protective metabolic and structural mechanisms that help to preserve and/or restore arterial wall compliance and thus facilitate arterial baroreceptor signaling.

Our findings further suggest that the development of hypertension might be associated with impaired DVMN functionality in the SHR and conversely, increasing neuronal activity by pharmacogenetic methods might have helped to attenuate disease progression. Histological analysis of ChAT immunofluorescence revealed that SHRs and Wistar rats have

similar neuronal numbers in the DVMN. However, the number of DVMN neurons retrogradely labeled from the heart was previously found to be significantly reduced in adult SHRs compared to young SHRs and adult Wistar Kyoto rats (38). This discrepancy suggests that the number of cardiovagal neurons that project to the heart is probably too small as to make a significant contribution to the overall population of vagal neurons residing in the DVMN and thus, a loss of cardiovagal neurons in adult SHRs may not have a major impact on the total number of DVMN neurons. However, age-related reduction in the population of DVMN neurons in the SHRs might also be accompanied with abnormalities in DVMN neuronal excitability. Previous studies have shown that parasympathetic neuronal dysfunction is a common feature in animal models of cardiovascular disease such as heart failure and chronic intermittent hypoxia in which increased GABAergic/glycinergic neurotransmission and decreased excitatory neurotransmission in the DVMN and nucleus ambiguous result in reduced neuronal excitability and lower parasympathetic tone (39, 40). Similar mechanisms are likely to operate in the SHR and should be further investigated in future studies to help determine whether DVMN synaptic dysfunction, a selective loss of parasympathetic preganglionic neurons supplying the heart or a remodelling of their cardiac projections, might promote abnormal changes in left ventricular inotropy and/or loss of structural integrity in the aortic wall which might contribute to the development of arterial hypertension.

Our data showed that long-term activation of vagal preganglionic neurons within the DVMN resulted in a reduction in heart rate and systolic blood pressure variance exclusively in SHRs. This bradycardic effect in SHRs might have been caused by indirect reductions in sympathetic nerve trafficking in SHRs rather than by increased vagal influence on cardiac chronotopy since cardiac vagal preganglionic neurons that control the activity of the sino-atrial node are generally believed to be located in the ventrolateral aspect of the nucleus ambiguous (NA) (41, 42) whilst DVMN neurons that were targeted in this study have

virtually no influence on the control of heart rate (43). This is supported by PI variability analysis, showing significant reductions in the LF component, consistent with decreased sympathetic tone. Thus, a reduced heart rate in SHRs might have further contributed to lower cardiac output and produce concomitant reductions in arterial blood pressure in the SHRs expressing DREADD-Gs. This effect was only evident in SHRs and not in Wistar rats because SHRs have augmented sympathetic activity and would therefore be more sensitive to decreased sympathetic activity. Reference from Jim?

Our results are consistent with recent studies showing that pharmacological activation of parasympathetic signaling elicits beneficial effects in the circulatory system. Transdermal application of scopolamine has been shown to decrease arterial blood pressure, increase baroreflex sensitivity and accentuate vagal-cardiac modulation of sinus node in patients with mild hypertension (44), supporting the hypothesis that increasing cardiovascular parasympathetic activity might have beneficial effects in subjects with arterial hypertension. More recently, systemic administration of choline was shown to restore the impaired cardiac function in SHRs, as evidenced by decreases in heart rate, systolic blood pressure, left ventricular systolic pressure, dp/dt_{max} and increases in ejection fraction and fractional shortening (45). This effect was accompanied by restored baroreflex sensitivity in SHRs and profound anti-inflammatory effects, including downregulation of interleukin (IL)-6 and tumour necrosis factor-α (TNF-α) and increased expression of IL-10 in the mesenteric arteries of SHRs, thus indicating that choline improved vagal activity (45). Similarly, chronic increase of acetylcholine availability induced by administration of anti-acetylcholinesterase agents with central and/or peripheral action, attenuated the development of hypertension, increased cardiac vagal tone, improved arterial pressure and pulse interval variability and reduced plasma levels of TNF-α, IL- 6, and interferon γ (46). Together, these pharmacological studies suggest that acetylcholine might attenuate the harmful

cardiovascular effects of systemic low-grade inflammation, including endothelial dysfunction, oxidative stress and autonomic dysfunction. However, the effect of DVMN neuronal activation on the expression of pro-inflammatory mediators and signalling mechanisms requires further investigation. Control of low-grade signalling inflammation by stimulation of DVMN circuits may have a significant impact on the control of autonomic balance and cardiovascular homeostasis and ultimately contribute to the reduction in arterial blood pressure levels in the SHR.

Our study has some limitations. We employed adult SHRs with fully-established arterial hypertension and 21 days of DVMN stimulation with CNO was relatively short. Thus, it remains to be determined whether these effects are similar or perhaps more effective when animals receive the treatment at different stages (i.e., prehypertensive stage or further down in the presence of end-organ damage) and whether arterial blood pressure-lowering effects can be sustained for considerably longer periods (months to years). From a technical point of view, one of the major technical limitations was that blood pressure measurements were only acquired until the end of the study and for relatively short periods of time (normally 1 hour). Thus, longer data acquisitions were not possible, especially at night, so we were not able to assess the time course of the blood pressure-lowering effects of DVMN activation and whether blood pressure dipping was restored in SHRs. Finally, we did not determine whether the amount of lentiviral particles delivered achieved a maximum infection rate which could have affected the magnitude of the arterial blood pressure-lowering effect we obtained. However, despite these limitations, our study provides unequivocal evidence that shows that activation of DVMN neurons for 3 weeks is enough to substantially lower arterial blood pressure levels in adult SHRs and improve autonomic dysfunction.

In conclusion, using a chemogenetic approach for highly selective recruitment of vagal efferent projections in the DVMN, we demonstrate that long-term activation of central

parasympathetic pathways exerts arterial blood pressure-lowering effects in SHRs. Our data suggest that cardiac preganglionic neurones in the DVMN are important targets for increasing parasympathetic activity to the heart, and may provide a therapeutic strategy to help control arterial hypertension and prevent its detrimental effects.

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Figure legends

Figure 1: Targeting cardiac preganglionic neurons in the dorsal motor nucleus of the vagus nerve (DVMN).

A) Representative image of choline acetyl transferase (ChAT)-immunoreactive neurons (red) in the DVMN. B) DVMN neurons expressing DREADD-Gs-eGFP. C) merge of A and B in the intermediate aspect of the DVMN (13.8 mm caudal from Bregma). Most of the neurons in this image are double-labelled and appear orange. D) average number of counted neurons per section from 7 rats. Counts were made from a 1 in 6 series of 40 μm coronal sections. Abbreviations: AP, Area Postrema; cc, central canal; XII, hypoglossal motor nucleus. Scale bar in C = 500 μm.

Figure 2: Long-term activation of DREADD-Gs-expressing neurons in the dorsal motor nucleus of the vagus attenuates the hypertension in the spontaneously hypertensive rat (SHR).

Summary data showing that long-term activation of DVMN neurons expressing DREADD-Gs within the DVMN results in a significant reduction of resting A) mean (MAP), B) systolic (SAP), C) diastolic arterial blood pressure (DAP) and D) heart rate (HR) in the SHR. Note that activation of DREADD-Gs-expressing neurons in the DVMN did not affect physiological variables in Wistar rats. Data are presented as mean±SEM. *$p<0.05$ or ***$p<0.001$, significant difference between SHRs expressing EGFP and SHRs expressing DREADD-Gs in the DVMN. N = 4-8/group of rats

Figure 3: Power spectral analysis of the systolic arterial pressure (SAP) and spontaneous baroreflex sensitivity (SBR) after long-term activation of DREADD-Gs-expressing neurons in the dorsal motor nucleus of the vagus in SHR and Wistar rats.

Summary data showing that long-term activation of DVMN neurons expressing DREADD-Gs within the DVMN results in significant changes in A) baroreflex gain (ms/mmHg); B) baroreflex effectiveness index; C) systolic arterial pressure (SAP) variance; D) Low frequency (LF) component of the SAP ($mmHg^2$). Data are presented as mean±SEM. *$p<0.05$, significant difference between SHRs or Wistar rats expressing eGFP and SHRs and Wistar rats expressing DREADD-Gs in the DVMN. N = 4-8/group of rats

Figure 4: Power spectral analysis of the pulse interval (PI) variability and spontaneous baroreflex sensitivity (SBR) after long-term activation of DREADD-Gs-expressing neurons in the dorsal motor nucleus of the vagus in SHR and Wistar rats.

Summary data showing that long-term activation of DVMN neurons expressing DREADD-Gs within the DVMN results in significant changes in A) Pulse interval (PI) variability; B) LF component of PI (%); C) High frequency (HF) component of PI (%) and D) LF/HF ratio in SHR and Wistar rats. Data are presented as mean±SEM. *$p<0.05$, significant difference between SHRs or Wistar rats expressing EGFP and SHRs and Wistar rats expressing DREADD-Gs in the DVMN. N = 4-8/group of rats