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An exploratory study in conscious rats

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Master Degree in Neurosciences

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The voyage of discovery is not in seeking new landscapes
but in having new eyes.

Marcel Proust

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RESUMO

Um dos principais contributos para o aparecimento, desenvolvimento e manutenção da hipertensão arterial é a existência e persistência de uma atividade simpática elevada. Dados de modelos experimentais de hipertensão e doentes hipertensos tornaram evidente que a simpatoexcitação excessiva, característica da hipertensão, é deletéria resultando em danos nos órgãos periféricos e aumento da morbilidade e mortalidade. Assim, a identificação da origem desta simpatoexcitação é crucial por forma a serem desenvolvidas novas intervenções terapêuticas. No entanto, até ao momento, os mecanismos exatos responsáveis pela ativação simpática na hipertensão arterial permanecem por elucidar, devido à sua complexidade e origem multifatorial.

A face rostroventrolateral do bulbo (FRVLB) é uma área bulbar simpatoexcitatória pertencente à rede autonómica central, que desempenha um papel importante na fisiopatologia da hipertensão arterial. Em termos fisiológicos, a FRVLB tem um papel fundamental na geração do tónus simpático periférico. Os seus neurónios projetam-se monosinapticamente para os neurónios simpáticos pré-ganglionares do núcleo de células da coluna intermediolateral da medula espinhal, condicionando o tónus simpático periférico para o coração e vasos. O núcleo parabraquial lateral (NPL) e a substância cinzenta periaqueductal (SCP) são outras áreas da rede autonómica central que podem estar envolvidas na geração da atividade simpática. O NPL tem um papel crítico na transmissão central de sinais do tronco cerebral relacionados com a regulação de fluidos e ingestão de eletrólitos e com a função cardiovascular, em particular aqueles que regulam a pressão arterial em resposta à hemorragia e à hipovolémia. As colunas da SCP são capazes de modular as funções cardíacas

simpáticas através de uma variedade de vias indiretas que envolvem neurónios pré-motores simpáticos encontrados em locais específicos do hipotálamo, mesencéfalo, protuberância e bulbo raquidiano.

No presente trabalho, de natureza exploratória, procurámos estabelecer as consequências funcionais no tónus simpático e pressão arterial decorrentes da diminuição da excitabilidade da FRVLB e NPL e SCP em duas situações, em normotensão e em hipertensão, respetivamente.

Para tal, e em cada área central de estudo, os valores da pressão arterial, *output* autonómico, função baro- e quimiorreceptora foram monitorizados e os parâmetros metabólicos e comportamentais avaliados antes e depois da microinjeção do lentivírus com informação genética que codifica os canais *hKir2*.1.

Os resultados mostraram que a microinjeção de LVV-*hKir2.1* na FRVLB de ratos normotensos provocou uma descida significativa do tónus simpático, embora esta não se tenha refletido na pressão arterial sistólica, diastólica e média. Adicionalmente, não se observaram efeitos sobre os reflexos cardiorrespiratórios, parâmetros metabólicos e atividade locomotora/exploratória. Aos 60 dias após a microinjeção lentiviral no NPL de ratos hipertensos, o tónus simpático diminuiu significativamente refletindo-se nos valores de pressão arterial e frequência cardíaca. Verificou-se também uma tendência para a diminuição da variação do reflexo quimiorreceptor; no entanto, não houve neste caso significância estatística. Além disso, o silenciamento da SCP em ratos hipertensos não teve repercussões na pressão arterial, frequência cardíaca e tónus simpático, mas o ganho do barorreflexo diminuiu. O núcleo *Kolliker-Fuse*, localizado na protuberância e responsável pelo controlo pôntico da respiração foi utilizado como núcleo controlo para as duas últimas áreas.

Assim, com este trabalho mostramos a suposta contribuição das áreas do mesencéfalo e da rede autonómica central pôntica para a etiologia da hipertensão de causa neurogénica e fornecemos

pistas para possíveis futuras intervenções terapêuticas no controlo da simpatoexcitação a nível central.

Palavras-chave: Face Rostroventrolateral do Bulbo, Núcleo Parabraquial Lateral, Substância Cinzenta Periaqueductal, Hipertensão, Simpatoexcitação, Reflexo Barorreceptor, Quimiorreflexo, Vector Lentiviral.

ABSTRACT

Sympathetic nervous system hyperactivity is a major contributor to the onset, development and maintenance of essential arterial hypertension. Taking into account data from experimental models of hypertension and from hypertensive patients, it is evident that the excessive sympathoexcitation that characterizes hypertension is deleterious leading to organ damage and increasing morbidity and mortality. Thus, is essential to identify the origin of the sympathoexcitation in order to develop new therapeutic interventions. However, until the moment, the precise mechanisms responsible for the sympathetic activation in essential hypertension remain to be elucidated, since they are complex and multifactorial.

The rostral ventrolateral medulla (RVLM) is a medullar sympathoexcitatory area of the central autonomic network, which plays a crucial role in essential hypertension pathophysiology. Physiologically, RVLM has a key role in peripheral sympathetic tone generation. Its neurons monosynaptically project to the sympathetic preganglionic ones in the intermediolateral column of the spinal cord, conditioning peripheral sympathetic tone to the heart and vessels. Other areas of the central autonomic network that could also be involved in the generation of sympathetic activity are the lateral parabrachial nucleus (LPBN) and the periaqueductal gray matter (PAG). LPBN plays a critical role in relaying signals related to the regulation of fluid and electrolyte intake and cardiovascular function from the brainstem to the forebrain, in particular those regulating blood pressure in response to hemorrhages and hypovolemia. PAG columns are capable of modulating

cardiac sympathetic functions through a series of indirect pathways involving sympathetic premotor neurons found in specific sites of the hypothalamus, midbrain, pons and medulla oblongata.

In the present work, of exploratory nature, we sought to establish the functional consequences on sympathetic tone and arterial blood pressure of decreasing the excitability of RVLM and LPBN and PAG in normotension and hypertension, respectively.

For that, blood pressure, autonomic output, circadian blood pressure, baro and chemoreceptor function were monitored and metabolic plus behavioral parameters evaluated before and after microinjection in each area of genetic information encoding *hKir2.1* channels through a lentivirus.

Results show that LVV-*hKir2.1* microinjection into the RVLM of normotensive rats promoted a significant decrease in sympathetic tone, however it did not affect systolic, diastolic and mean blood pressure values. Additionally, no deleterious effects on cardiorespiratory reflexes, metabolic parameters and locomotor plus exploratory activity were observed. In hypertensive conditions, 60 days post lentiviral microinjection into the LPBN, sympathetic outflow decreased significantly and reflect itself on blood pressure and heart rate values as well. A tendency towards chemoreflex variation attenuation was also observed, however statistical significance was not attained. In addition, PAG silencing had no repercussions on arterial blood pressure, heart rate and sympathetic tone but decreased cardiac baroreflex gain. Has a control area for these functional modifications on physiological parameters, we also microinjected the lentiviral vector at the Kolliker –Fuse nucleus, a pontine nucleus which controls respiration.

Hence, with this exploratory work we unravelled the putative contribution of areas of the midbrain and pontine central autonomic network for the etiology of neurogenic hypertension and provide clues to possible future therapeutic interventions to control sympathoexcitation.

Keywords: Rostral Ventrolateral Medulla, Lateral Parabrachial Nucleus, Periaqueductal Gray Matter, Hypertension, Sympathoexcitation; Baroreceptor Reflex; Chemoreceptor Reflex, Lentiviral Vector.

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ABBREVIATIONS LIST

ACh	Acetylcholine
AngII	Angiotensin II
ANS	Autonomic nervous system
BP	Blood pressure
BRG	Baroreflex gain
CNS	Central nervous system
dBP	Diastolic blood pressure
EPM	Elevated-plus maze
HF	High frequency band
hKir2.1	Human inwardly rectifying potassium channel
HR	Heart rate
IML	Intermediolateral column of the spinal cord
KF	Kolliker-Fuse nucleus
LF	Low frequency band
LPBN	Lateral parabrachial nucleus
LVV-eGFP	LV-Syn-Eff-GAL4BS-Syn-Tetoff; LV-TREtight-eGFP
LVV-*hKir2.1*	LV-Syn-Eff-GAL4BS-Syn-Tetoff, LV-TREtight-*hKir2.1*-IRES-eGFP
MAP	Mean arterial pressure
mBP	Mean blood pressure
NA	Nucleus ambiguus
NTS	Nucleus tractus solitarii

OFT	Open-field test
PAG	Periaqueductal gray matter
pCO_2	Carbon dioxide partial pressure
PNS	Parasympathetic nervous system
pO_2	Oxygen partial pressure
PVN	Paraventricular nucleus of the hypothalamus
RAAS	Renin-angiotensin-aldosterone system
RSA	Respiratory sinus arrhythmia
RVLM	Rostral ventrolateral medulla
SAD	Sino-aortic denervation
SHR	Spontaneously hypertensive rats
SNS	Sympathetic nervous system
sBP	Systolic blood pressure

1. INTRODUCTION

1.1. Essential hypertension and sympathetic overexcitation

Primary, essential or idiopathic hypertension can be defined as a persistent rise in blood pressure (BP, >140/90 mmHg) of unidentifiable cause, which increases the risk for cardiac, cerebral and renal events. With an over 90% chance of becoming a hypertensive individual in industrialized countries, this condition pathophysiology remains to be elucidated, however a greater sympathetic drive has been established in the early stages of hypertension, suggesting that neurohormonal dysregulation may be key to its etiology. [123; 132] Based on the proportion of untreated patients with essential hypertension who present evident sympathetic overexcitation and on the number of individuals who achieve substantial BP lowering as well as the extent of this lowering with anti-adrenergic drugs, it is estimated that causal neurogenic arterial hypertension is no less than 50% of all cases of essential arterial hypertension. [133]

The contribution of sympathetic nervous system (SNS) overactivity to the onset, development and maintenance of hypertension is currently accepted. [81] Increased sympathetic drive to the heart leads to elevated cardiac output and neurally mediated vasoconstriction, which ultimately results in increased blood pressure values. [154]

High levels of sympathetic activity and plasma noradrenaline observed in borderline hypertensive subjects support the high blood pressure of neurogenic nature theory. [73; 164] Additionally, radical denervation of the sino-aortic pressoreceptor areas has produced chronic neurogenic hypertension in dogs and rabbits. [103]

The paraventricular nucleus of the hypothalamus (PVN), a major sympathoexcitatory area, regulates the cardiovascular portion of the sympathetic outflow and innervates the lower brainstem regions, such as the nucleus tractus solitarii (NTS) and rostral ventrolateral medulla (RVLM), and the spinal cord. [24; 38; 81] PVN activation increases sympathetic output and promotes a pressor effect mediated by direct and indirect projections, via the RVLM, to the spinal cord. [33; 71; 86; 142; 158] Experimental evidence indicates that the PVN tonic effect on the control of sympathetic vasomotor tone is enhanced in spontaneously hypertensive anesthetized rats (SHR). [8]

A decrease in blood pressure associated with reduced sympathetic nerve activity was observed in SHR after electrolytic lesions of the PVN. [173] Accordingly, a long-term reduction of PVN neurons excitability, through the expression of a human inwardly rectifying potassium channel (*hKir2.1*), produced an anti-hypertensive response in conscious spontaneously hypertensive rats. [71]

Increased sympathetic nerve activity in the hypertensive state has been demonstrated both in humans and animals, but it was not detected in secondary hypertension subjects. [73; 74; 81] Raised SNS activity was also observed in normotensive subjects with a family history of arterial hypertension. [187] Moreover, increased sympathetic nerve activity to the skeletal muscle arterioles was demonstrated in white coat and borderline hypertensive patients compared to healthy subjects. [74; 164] Therefore, it has been hypothesized that sympathetic nervous system hyperactivity precedes hypertension onset, suggesting a causal neurogenic component for this pathology. [81] Additionally, studies have shown that SNS activity increases progressively and in parallel with arterial hypertension stages. [164]

Kidneys afferent sensory nerves, which project to the brain, have been indicated as a significant source of sympathetic activation. Patients with resistant hypertension, i.e. inadequately responders to concurrent treatment with multiple anti-hypertensive drug classes, who were submitted to renal sympathetic nerves ablation with an endovascular radiofrequency technique showed a remarkable reduction of BP values. [63; 104] Despite the end-points have not been entirely reached on the last large clinical trial using this technique, it comes out as a general observation that sympathetic activity reduction has been considered as an anti-hypertensive strategy. [21; 52; 76]

Both cardiac and renal sympathetic outflows are activated in normal-weight hypertensive subjects. [61; 62] Contrarily, renal sympathetic activation with minimal involvement of sympathetic outflow to the heart has been shown in obese hypertensive patients' studies and in many of these, reduction of cardiac norepinephrine was observed. [150]

It is known that the interaction of genetic factors with behavioral and lifestyle aspects is important for the sympathetic overactivation in essential arterial hypertension, nevertheless its specific causes remain to be elucidated. There is evidence that 30-40% of essential arterial hypertension cases are heritable, however the genetic influence on the SNS may be polygenetic and thus of difficult identification. [111] Reduction of sympathetic nervous activity and preferentially renal sympathetic outflow due to aerobic exercise training, suggests that physical inactivity is also important in arterial hypertension. [165]

Increased systemic and central angiotensin II signaling is one potential cause for the sympathoexcitation behind essential hypertension. Angiotensin II actions are mostly mediated by the angiotensin II type 1 (AT1) receptor and the central nervous system (CNS) is richly endowed with the latter. [191] Increased plasma angiotensin II is observed in both humans and animals with hypertension. This peptide hormone exerts central sympathoexcitatory effects, promotes norepinephrine release and amplifies the adrenoreceptor response to stimuli. Endothelial AT1

receptors activation reduces transmission between baroreceptor afferents and NTS efferent neurons, through nitric oxide production by the capillary endothelium. [135]

Sympathetic overexcitation may also be due to insulin resistance. Hyperinsulinemia is often associated with hypertension and it has been established that insulin resistance/hyperinsulinemia raises sympathetic traffic and norepinephrine release. Nonetheless, the reciprocal is also true making it difficult to determine what precedes what. Furthermore, evidence suggests that a central neural action mediates, at least in part, insulin sympathoexcitatory effects since insulin has the ability to cross the blood-brain barrier and there are several distinct CNS regions with insulin receptors. [129; 152]

Baroreflex modulation and sympathetic traffic resetting towards high BP values characterizes hypertension. Thus, another hypothesis for the sympathetic hyperactivity is that the latter is associated with baroreflex dysregulation. Consequently and due to inhibition of vessels sympathetic outflow and norepinephrine plus renin release, blood pressure is maintained rather than reduced. [75]

Another hypothesis is that brain centers abnormal increased sympathetic drive, such as the paraventricular nucleus of the hypothalamus and the rostral ventrolateral medulla may produce the sympathetic hyperactivity characteristic of hypertension. [70; 71]

1.2. Autonomic control of arterial blood pressure

The Autonomic Nervous System (ANS) is the part of the nervous system responsible for homeostasis. It operates beyond direct conscious control and regulates the involuntary functioning of most organs, including the heart and vasculature, via autonomic reflexes. The ability to adapt to environmental stressors and other challenges is compromised without the ANS. [16; 156]

The reflex arc is considered the basic anatomo-functional unit of the nervous system. Detainer of a sense organ, an afferent neuron, one or more synapses in a central integrating station or sympathetic ganglion, an efferent neuron and an effector, the basic unit of integrated reflex activity works without the intervention of consciousness. A receptor's activation produces a signal which is relayed to an integrating center and the output of the latter is sent to an effector, which plays the overall system response. Generally speaking, the efferent part of the ANS is divided in Sympathetic (SNS) and Parasympathetic (PNS) branches. [16; 156; 185]

The heart is innervated by vagal and sympathetic fibers. The vagus nerve, container of parasympathetic axons, arises from the nucleus ambiguus and the dorsal motor nuclei in the medulla and comprises up to 75% of total parasympathetic activity (figure 1.1). Parasympathetic stimulation triggers acetylcholine (ACh) release by vagal nerve terminals. Ach, the principal neurotransmitter in all autonomic ganglia, acts on muscarinic receptors to slow the sinoatrial node, atrioventricular node and specialized conducting tissues discharge. [156]

Increased vagal tone causes slowing of conduction velocity and decreases heart rate (HR). Since the ventricles are sparsely innervated by parasympathetic nerve fibers, stimulation of the latter has limited direct effect on cardiac contractility. Furthermore, the PNS only innervates a restricted number of vascular beds, having no effect on total peripheral resistance. [5; 144]

Respiratory sinus arrhythmia has been used as an index of vagal control of the heart, which is extremely sensitive to behavioral and physiological variables. Respiratory sinus arrhythmia is defined as heart rate variations synchronized with respiration and characterized by R-R interval shortening during inspiration and R-R interval lengthening during expiration, respectively. This noninvasive measurement of parasympathetic effects on the heart is of extreme importance considering the growing awareness of the autonomic control complexity. [19; 190]

Sympathetic innervation of the heart arises from preganglionic neurons in the intermediolateral column (IML) of the spinal cord, extending from first to fifth thoracic segments. Postganglionic neurons release norepinephrine, which acts on β_1-adrenergic receptors (figure 1.1). Consequently, positive inotropic and chronotropic effects emerge. Blood vessels receive sympathetic innervation only. Tonic vasoconstriction is exerted by vascular sympathetic nerves via α_1-adrenergic receptors on vascular smooth muscle cells, on the arteries, arterioles and veins. Sympathetic nervous system increased activation causes further vasoconstriction. Both β_1 and β_2-adrenergic receptors are also present on vascular smooth muscle cells and have a vasodilator effect in the skeletal and coronary circulation. [16; 144]

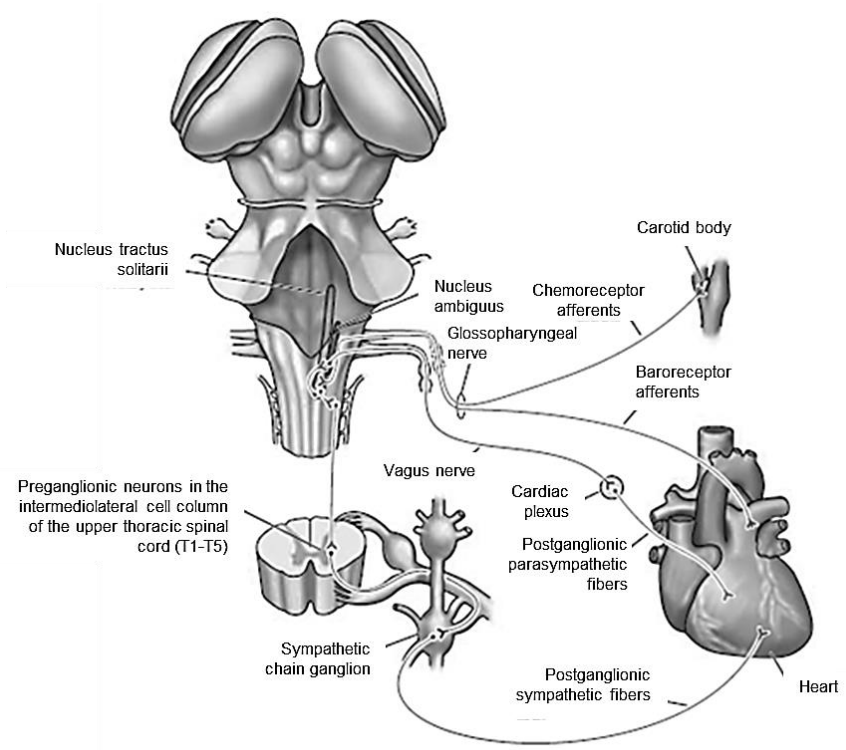

Figure 1.1 – Autonomic control of cardiovascular function. Sympathetic innervation of the heart arises from preganglionic neurons in the intermediolateral cell column of the upper thoracic spinal cord and synapse with postganglionic ones which project to the heart. On the other hand, the vagus nerve supplies the parasympathetic tone to the heart. Baro- and chemoreceptors, structures involved in regulatory mechanisms of the cardiovascular system, relay their information through the glossopharyngeal nerve. Adapted from Purves *et al.*. [1]

In order to have an appropriate oxygenated blood supply, under a wide range of circumstances, the cardiovascular system is dependent on precise reflex regulation. Sensory monitoring for this critical homeostatic process entails primarily, barosensory information about arterial pressure and secondarily, chemosensory information regarding blood oxygen and carbon dioxide levels. [1]

Intrinsic cardiovascular reflexes include the baroreceptor reflex, cardiopulmonary reflexes and chemoreceptor reflex. This works with other local mechanisms like the renin-angiotensin-aldosterone and antidiuretic hormone systems to maintain the mean arterial blood pressure and an adequate cerebral and coronary perfusion. [5]

1.2.1. Baroreceptor reflex

The arterial baroreceptor reflex is a classical negative feedback mechanism which minimizes moment-to-moment fluctuations in mean arterial blood pressure, by reflexively changing heart rate and vascular resistance. However, experimental evidence based both on dogs and rats, indicates that this reflexive mechanism does not provide for the long-term maintenance of blood pressure, since it adapts during a period of 1 to 2 days to the prevailing mean one. [5; 16; 156; 176]

Stretch-sensitive nerve endings, found on the walls of the carotid sinus and aortic arch, are called baroreceptors. These are activated by distension or deformation imposed on the vessel, hence increased transmural pressure in the aorta and carotid sinuses, raises baroreceptors firing rate. Nerve fibers from carotid sinus baroreceptors join the glossopharyngeal nerves, while the ones from the aortic baroreceptors accompany the vagus nerve, traveling both to the nucleus tractus solitarii (NTS). Here are the central terminals of the also called mechanoreceptors. NTS neurons project to the rostral ventrolateral medulla (RVLM) and nucleus ambiguus (NA), where they influence the firing of sympathetic and parasympathetic nerves. [1; 144]

Increased firing rate to the NTS, leads to NA neurons excitation and RVLM neurons neural traffic inhibition. As an end result, there is an increased parasympathetic and decreased sympathetic neural activity to the heart, vessels and veins, causing a drop in cardiac output and systemic vascular resistance. Consequently, mean arterial blood pressure returns to a normal level. Opposite effects occur when arterial blood pressure decreases (figure 1.2). [1; 5; 156]

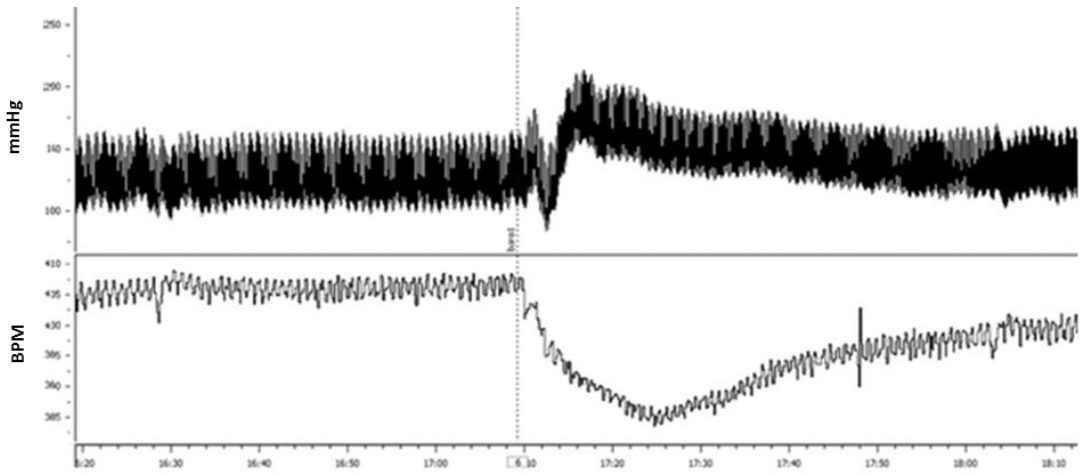

Figure 1.2 – Cardiovascular changes elicited by baroreceptor reflex activation. Blood pressure (top) and heart rate (bottom) evolution are depicted. In this case, baroreceptor reflex was triggered (dotted line) by phenylephrine injection, a selective α_1-adrenergic receptor agonist. (Lab unpublished observations)

In addition to vascular and cardiac muscles, the baroreceptor reflex influences hormone levels, the most important being the renin-angiotensin-aldosterone system (RAAS). Decreased systemic arterial blood pressure results in decreased baroreceptor firing, following increased sympathetic nerve activity to the kidneys and therefore renin release. Renin leads to angiotensin II production, a potent vasoconstrictor that stimulates the release of aldosterone from the adrenal gland, causing the kidneys to reabsorb salt and water. Thus arterial blood pressure rises, due to an increase in vascular resistance and blood volume. [5; 16]

In vivo studies have shown that baroreceptor changes influence breathing and researchers suggest that these are due to alteration of type 1 large A-fibers firing. [55; 78; 96; 113] For instance

baroreceptor stimulation, by rise in carotid sinus pressure, on anesthetized vagotomised dogs decreased respiration rate and heightened end-tidal volume. [25]

1.2.2. Chemoreceptor reflex

Chemoreflexes are important modulators of sympathetic activation and respiratory activity regulators. Chemoreceptors are chemosensitive cells which respond to hypoxia, hypocapnia and acidosis. [99]

Located in the carotid and aortic bodies are the peripheral chemoreceptors. These are activated by a fall in oxygen partial pressure (pO_2) and to a lesser extent by a rise in carbon dioxide partial pressure (pCO_2) or acidity. Via glossopharyngeal and vagus nerves, the chemo-sensors send signals to the medulla and synapse in the NTS. Activation of peripheral chemoreflex elicits hyperventilation (increased air flow volume, respiratory rate and breathing volume) and sympathetic outflow tracts activation (figure 1.3). It also affects parasympathetic efferent tracts of the X cranial nerve. [54; 85; 92]

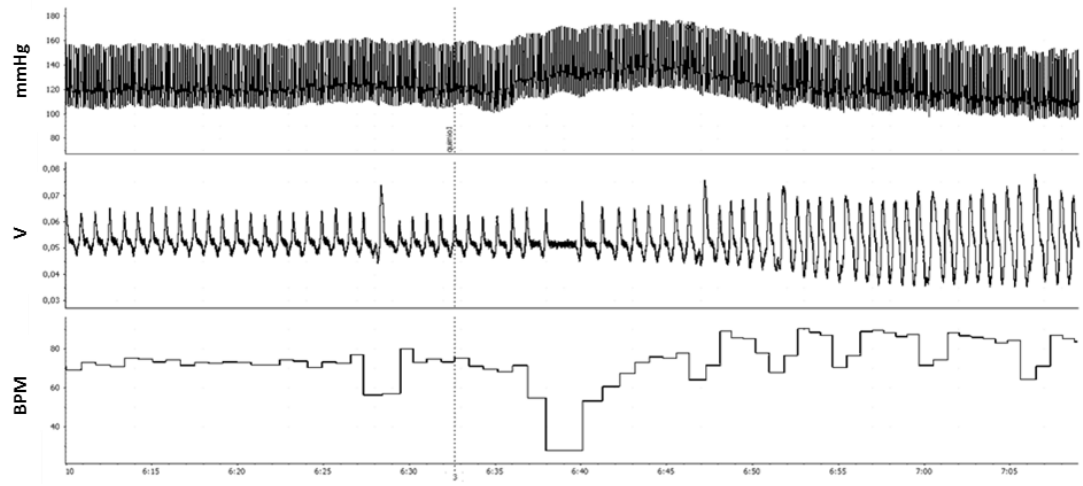

Figure 1.3 – Physiological response of chemoreceptor stimulation. Dotted line denotes chemoreflex activation by lobeline injection. Blood pressure (top), tracheal pressure (middle) and respiratory rate (bottom) are depicted. Lobeline administration elicits a clear hyperventilatory reflex response with an increase on the rate and frequency of respiration. (Lab unpublished observations)

Chemoreceptive areas within the central nervous system, in contrast, respond to changes in pCO_2 and pH. Arterial oxygen saturations less than 50% also activate central chemoreceptors. [23] Initially, central chemoreception was restricted to areas of the ventral medullary surface. Nonetheless, there is substantial evidence that many sites participating in this process are located at a distance from the ventral medulla (figure 1.4). [130]

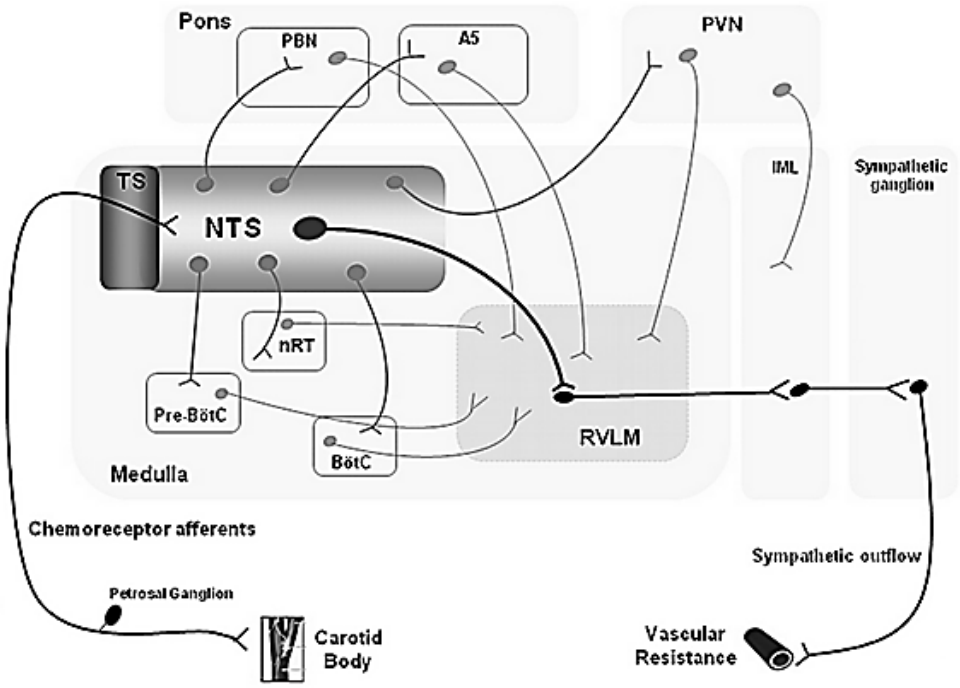

Figure 1.4 – Schematics of chemoreceptor reflex sympathoexcitatory pathways in the brainstem, pons and hypothalamus. NTS, nucleus tractus solitarii; TS, tractus solitarius; PBN, parabrachial nucleus; A5, A5 area; PVN, paraventricular nucleus of the hypothalamus; IML, intermediolateral column of the spinal cord; RVLM, rostral ventrolateral medulla; pre-BötC, pre-Bötzinger nucleus; BötC, Bötzinger nucleus; nRT, retrotrapezoid nucleus. Adapted from Accorsi-Mendonça *et al*. 2004. [7]

Profound peripheral vasoconstriction is the result of both peripheral and central chemoreceptors increased firing rate. Coronary and cerebral perfusions are not subject to the sympathetic

vasoconstrictor effects, exhibiting vasodilation instead, due to the direct effect of the abnormal blood gases and local metabolic effects. [144]

At the central level, chemoreceptors activation raises NTS cells activity and some of those are similar to the ones activated by the baroreceptors. NA and RVLM neurons are simultaneously excited with ensuing increase in sympathetic and parasympathetic tone. Sympathetic nerve activity mediated tachycardia, vasoconstriction and respiration rate are raised in response to chemo-sensors activation. Increase in respiratory activity is due to inspiratory activity of differing NTS neurons. [145]

The chemoreceptor reflex also plays a role in the cardiovascular response to severe hypotension. Chemoreceptor firing rate rises, as BP and as well as blood flow through the carotid and aortic bodies falls. Chemoreceptor activation is owed to changes in local pO2, pCO2 and pH. [144]

Experiments with rats showed that carotid denervation causes a significant decrease in renal sympathetic nerve activity. A transient fall in blood pressure and sympathetic activity was observed in animals exposed to acute hyperoxia, which causes chemoreceptors inactivation. Plus, a decline in chronic BP levels emerged after carotid body artery ligation. [67] Accordingly, peripheral chemoreception triggers the sympathetic nervous system and has a tonic excitatory impact on cardiovascular control, leading to blood pressure upkeep. [67]

1.2.3. Other reflexes

The Bezold-Jarisch and the Bainbridge reflex fall in the category of other reflexes believed to regulate arterial blood pressure. The first is a chemically-sensitive cardiac reflex. As a direct result of receptors found in the ventricles or coronary circulation chemical stimulation, bradycardia and hypotension are evoked. Blood pressure drop is due to both bradycardia and inhibition of sympathetic vasomotor activity which causes vasodilation. Renin release and vasopressin secretion are modulated by the

decrease in BP. Conversely, an increase in sympathetic activity, vascular resistance, plasma renin activity and vasopressin are attained when inhibitory sensory receptors activity falls. [9]

In the Bainbridge reflex, blood pressure is indirectly regulated through heart rate changes. Therefore, right atrial volume increase leads to heart rate rise through sympathetic nerves, by activating low-pressure stretch receptors. The latter respond to low pressures stretch that occur typically in the atria. [26] This reflex efficacy is HR dependent, being more efficient at lower HR values compared to higher ones. The Bainbridge reflex functions opposite to the baroreceptor reflex which triggers a rise in HR in response to stretch decrease, such as in states of hypotension or hypovolemia. [14]

1.3. Central integration of cardiovascular reflexes

Maintenance of normal blood pressure is due to a background level of sympathetic vasoconstriction, cardiac stimulation and adrenal medullary catecholamine secretion. The medulla oblongata is responsible for generating these tonic excitatory signals to spinal sympathetic preganglionic fibers, integrating cardiovascular reflexes and signals from supramedullary neural networks, circulating hormones and drugs. [144]

Placed in the caudal dorsal medulla is an important modulator of autonomic efferent activity to the cardiovascular system, the NTS. [134; 161] The latter is amply innervated by fibers from distinct brain nuclei known to play a key role in cardiovascular control, such as the parabrachial nucleus, medial hypothalamus and amygdala. [37; 42; 110; 127] NTS is considered the primary central station for sensory information reception descendant from peripheral reflexogenic areas. [126; 127]

NTS neurons project to NA and dorsal motor nucleus of the vagus nerve. In addition, NTS also sends projections to caudal ventrolateral medulla inhibitory neurons, which synapse with rostral ventrolateral medulla (RVLM) premotor ones, which then project to preganglionic neurons in the intermediolateral cell column of the spinal cord (figure 1.5). [103; 166]

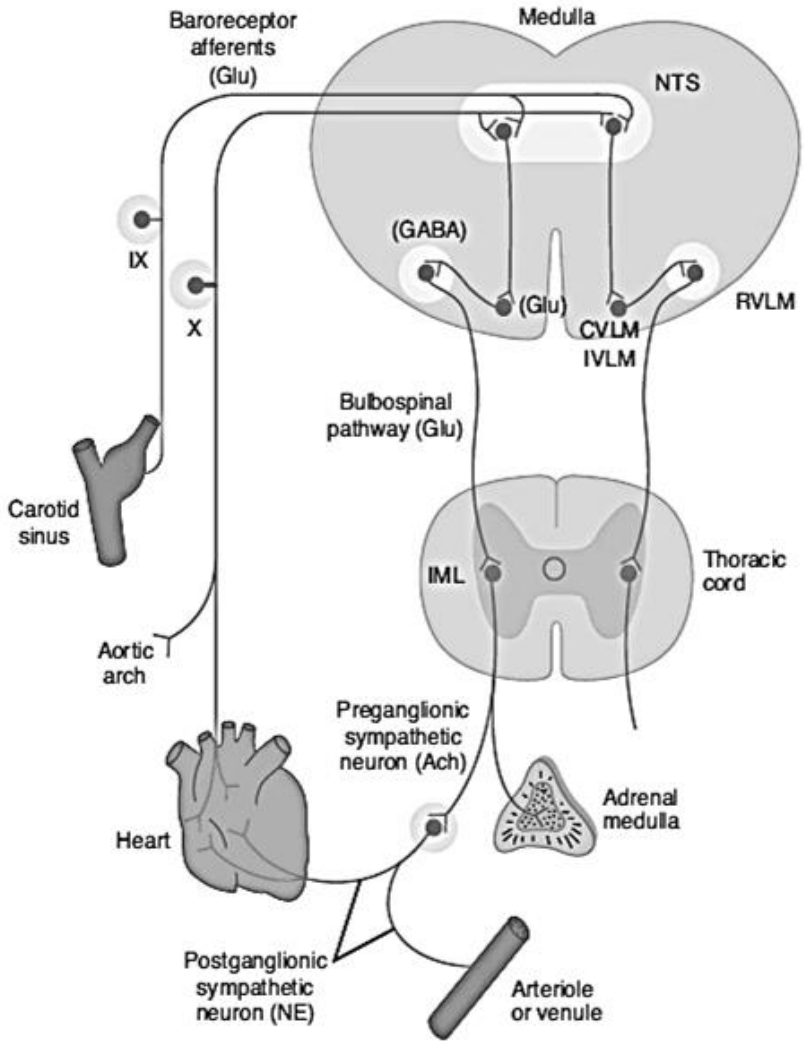

Figure 1.5 – Basic pathways schematics of medullary blood pressure control. Glu, glutamate; NTS, nucleus tractus solitarii; GABA, γ-aminobutyric acid; CVLM, caudal ventrolateral medulla; IVLM, intermediate ventrolateral medulla; RVLM, rostral ventrolateral medulla; IML, intermediolateral column of the spinal cord; Ach, acetylcholine; NE, norepinephrine. Adapted from Barrett *et al.* 2010. [16]

1.3.1. General overview of central autonomic network

Interconnected areas scattered throughout the neuroaxis are involved in the central control of autonomic function. With a key role in moment-to-moment control of visceral function, homeostasis

and adaptation to internal or external challenges, this central autonomic network receives and integrates information from a number of sources [18; 109; 119]:

a) visceral, nociceptive, thermal and muscular sensory information;
b) limbic information via the amygdala central nucleus;
c) direct humoral inputs or through the circumventricular organs;
d) information from central oscillators regulators of pacemaker cells at the suprachiasmatic nucleus;
e) and information originating at pathways regulating the sleep-wake cycle.

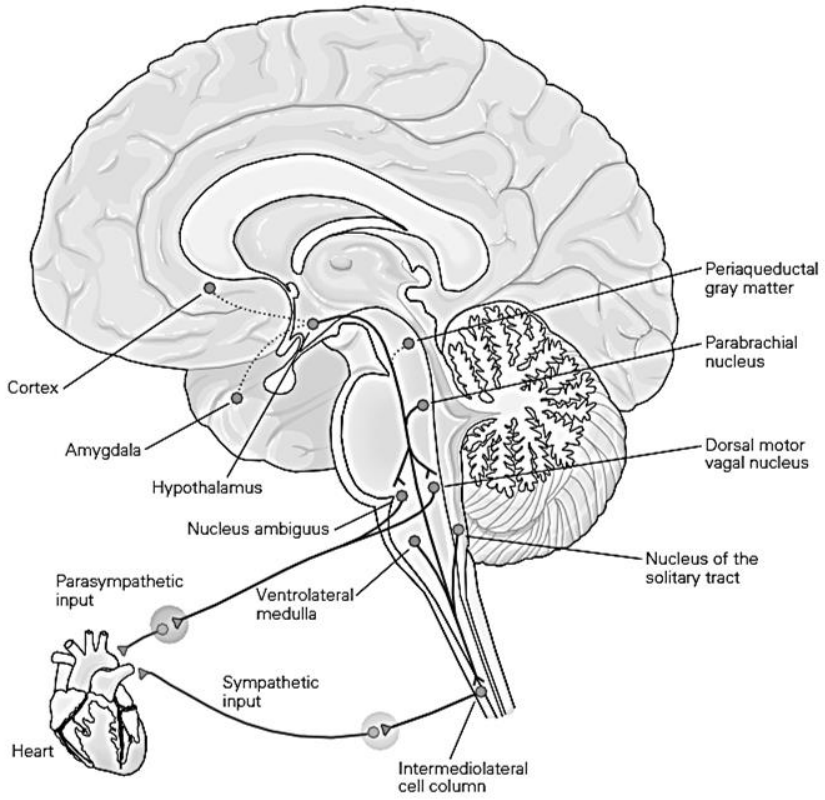

Figure 1.6 – Pathways responsible for autonomic control responses. Direct projections (solid lines) to autonomic preganglionic neurons include the hypothalamic paraventricular nucleus, parabrachial nucleus, nucleus of the solitary tract, ventrolateral medulla and medullary raphe (not depicted). Indirect projections (dashed lines) include the cerebral cortex, amygdala and periaqueductal gray matter. Information originated in the periphery is either processed for reflex responses or for an integrated autonomic, hormonal and behavioral output. Adapetd from Kandel *et al.*[3].

Functionally, the central autonomic network is organized in four, reciprocally interconnected, hierarchical levels: spinal, bulbopontine, pontomesencephalic and forebrain. These areas are convergent inputs receivers of visceral and somatic nature. They also generate stimulus specific profiles of autonomic, endocrine plus motor responses and these are all regulated to the behavioral state. [18; 109; 119]

The spinal level is a mediator of segmental sympathetic or sacral parasympathetic reflexes and participates in stimulus-specific patterned responses under other levels influence.[18; 109; 119]

At the lower brainstem (bulbopontine) level arises reflex control of circulation, respiration, gastrointestinal function and micturition. The NTS is found at this level and considered to be the primary relay station for the reception of peripheral visceral information. Also located in this area is the rostral ventrolateral medulla (RVLM), a container of bulbospinal neurons essential to the vasomotor, cardiac and respiratory functions control and to the coordination of numerous cardiovascular reflexes. RVLM is likewise responsible for hypothalamic function and respiratory rhythmogenesis control. With a more rostral location is the parabrachial nucleus, an important relay center for visceral, nociceptive plus thermoreceptive information convergence and a separate subnuclei container, being the latter linked to taste, salivation, gastrointestinal, cardiovascular and respiratory regulation. [18; 109; 119]

In the pontomesencephalic area is the periaqueductal gray matter (PAG), morphologically divided into columns responsible for cardiorespiratory, urinary, pain, thermoregulation and reproductive function control. PAG also contributes to the autonomic, somatic and anti-nociceptive responses to stressful stimuli integration. [18; 109; 119]

The hypothalamus and anterior limbic circuit constituents, including the insular cortex, anterior cingulated cortex and amygdala, are all comprised in the forebrain level. The autonomic, endocrine

and immune systems communicate in the hypothalamus, having the latter a key role in neuroendocrine integration which is critical for homeostasis and integrative adaptive responses. It is also involved in thermoregulation, food intake, osmolarity, fluid balance and sleep-wake cycle control. Therefore, according to its function, the hypothalamus is divided into three main areas: periventricular, linked to neuroendocrine control; lateral, related to arousal; and medial, involved in behavior control. [18; 109; 119]

The duo hypothalamus-PAG is involved in an acute but active reaction of adaptation to stressful stimuli. This leads to sympathetic activation which generates tachycardia, hypertension, positive inotropism, stroke volume and cardiac output rise, blood flow redistribution, tachypnea, baroreflex inhibition and chemoreceptor reflex facilitation. [159; 160] The PVN also takes part in this state, coordinating the neuroendocrine integration, including sympathoexcitation, vasopressin secretion and adrenomedullary plus adrenocortical systems activation. [18; 109; 119]

In the central nucleus of the amygdala, start the endocrine, autonomic and motor outputs, which are essential for the expression of emotions included in conditioned behaviors. The viscerotopic organized insular cortex is in charge of the instigation of autonomic responses linked to motivation and goal-directed behaviors. [97]

Finally, the anterior cingulate gyrus as well as the ventromedial prefrontal cortex, amygdala, striatum, hypothalamus and PAG constitute a functional unit responsible for the stimuli emotional content assessment. These are too involved in context-dependent autonomic, endocrine and motor outputs, key role players in integrated stress, emotional and motivated behavior responses. [18; 109]

1.3.2. The rostral ventrolateral medulla

Ventral to the rostral part of the nucleus ambiguus (NA), plus the Botzinger complex and caudal to the facial nucleus, is the rostral ventrolateral medulla. Tonic excitatory activity suppliers to the spinal cord and usually active, specific RVLM neurons are crucial in mediating reflex inhibition or

sympathetic activation to the heart and blood vessels. [47; 144; 167] Reciprocal connections between the RVLM and other brain nuclei indicate that the first is also an integrative center managing cardiovascular functions and processing information from peripheral nerves plus other brain nuclei. [27]

Spinal cord sympathetic preganglionic neurons, tonic excitatory drive receivers from supraspinal regions within the brainstem and hypothalamus, produce sympathetic vasomotor and cardiac neural activities. One of these supraspinal regions is the RVLM. [116] Acute bilateral inactivation or ablation of RVLM neurons in anaesthetized animals, led to a fall in BP and sympathetic activity. This decline was similar to the one observed after spinal cord transection or during ganglionic blockade. Therefore, the RVLM significantly contributes to the generation of sympathetic vasomotor tone. [116]

RVLM neurons were first acknowledged, in cats, through glycine and leptazol (a respiratory and circulatory stimulant) topical application on the ventral surface of the brainstem. Glycine administration to RVLM neurons triggered a large fall in arterial pressure, whereas an increase in BP was observed when leptazol was used. Anatomical location of the RVLM followed and cardiovascular neurons were found ventral to the rostral part of the nucleus ambiguus and caudal to the end pole of the facial nucleus. [27; 64; 79]

Sympathoexcitatory RVLM neurons are composed of two groups: a subgroup of the C1 catecholaminergic neurons, containers of adrenaline producing enzymes, and non catecholaminergic neurons, presumably glutamatergic ones. [116] Many of these nerve cells monosynaptically project to the sympathetic preganglionic neurons in the intermediolateral column of the spinal cord and provide the primary tonic excitatory drive to sympathetic vasomotor and cardiac neurons. [47; 83] RVLM C1 neurons also project to many other centers involved in respiratory and autonomic function, including the dorsal vagal motor nucleus. [53; 122] Additionally, the RVLM receives direct

glutamatergic projections from the NTS and the paraventricular nucleus of the hypothalamus (PVN). [98; 102; 140; 149]

The PVN is a command nucleus provider of feed forward excitatory synaptic drive to lower brainstem, coordinator of cardiovascular and respiratory motor activity, and an integrative center for autonomic and neuroendocrine responses. [49] This major sympathoexcitatory area is also involved in the control of blood volume, once it receives afferent information from right atrium and inferior vena cava receptors. These are sensate to volume changes of 8-10%. [112] The PVN projects to a variety of brain structures, including the RVLM. It is due to its connection with the RVLM that the PVN also influences vasomotor sympathetic nerve discharge. [189] It has been shown that in SHR the PVN is in a more active state. [8]

PVN neurons activation prompts, via RVLM excitatory connections, pressor and sympathoexcitatory responses. [189] Moreover, RVLM neurons can be excited by PVN angiotensin receptors activation, however the directionality of this pathway remains to be clarified. [172] It has also been shown that PVN neurons terminals, corticotrophin releasing factor containers, are found in the RVLM and bilateral microinjection of the latter increases BP. [125] All of this validates the importance of the PVN-RVLM axis.

The discharge pattern of sympathetic efferents innervating the heart, kidney or blood vessels of the skeletal muscles and splanchnic area, is highly correlated with the one from C1 neurons. These cells were thought to be responsible for regulating sympathetic vasomotor pathways, through adrenaline release in the spinal cord, however scientific progress has made clear that both C1 and non-C1 neurons also release glutamate. [53; 148; 170; 171]

Experiments with cats and rats have shown that RVLM electrical or chemical (glutamatergic) stimulation causes an increase in BP and HR, whereas RVLM inhibition in conscious rats elicits a chronic fall in BP, HR and SNS activity. As previously mentioned, the RVLM projects to sympathetic

preganglionic neurons and it has been demonstrated in rats that these display some viscerotopic organization according to the type of sympathetic preganglionic neurons it projects to and, this is crucial to the maintenance of basal BP. [47; 120]

Selective destruction of more than 80% of C1 neurons, in the anaesthetized rat, promoted a 10 mmHg reduction in BP, attenuated the sympathetic tonus involved in baroreflex and had little effect on parasympathetic tonus, demonstrating C1 cells contribution to generation of sympathetic vasomotor tone. [82; 114]

Despite numerous studies on RVLM premotor neurons and their known importance for the control of the sympathetic vasomotor tone, the foundation for the constant tonic activity of these neurons hasn't been fully understood yet. One explanatory hypothesis for this matter is that RVLM pre-sympathetic neurons have pacemaker activity. Guyenet stated that premotor neuron intrinsic auto-depolarization determines the ongoing activity of the basal RVLM. However, this pacemaker pattern has only been demonstrated in *in vitro* studies. *In vivo*, fast excitatory synaptic inputs appear to drive the RVLM spiking activity. [27] On the other hand, Barman and Gebber proposed the "network hypothesis", suggesting that RVLM premotor neurons, *in vivo*, depend on excitatory inputs from other brainstem nuclei, operating as part of an oscillating network. [15; 27]

1.3.3. The parabrachial complex

Located in the dorsal lateral pons, the parabrachial complex can be divided into more than 10 different sub-nuclei. These enclose the superior cerebellar peduncle. [68] The superior cerebellar peduncle defines the three major subdivisions of the parabrachial nucleus: the lateral parabrachial nucleus (LPBN), the medial parabrachial nucleus (MPBN) and the ventrolateral Kolliker –Fuse nucleus. [50]

Functionally, this complex mediates thermoregulation, pain and taste processing and is involved in homeostasis plus cardiorespiratory control. [34; 69; 118; 128; 188] Hence, the parabrachial complex is a major brain area that relays the body primary sensory information to autonomic and limbic forebrain areas, such as the hypothalamus and amygdala. [68; 118] Via the parabrachial sub-nuclei, viscero- and somatosensory sensations are converted into basic emotions, which are in turn converted to specific motor behavior. The basic emotions are the source of a "homeostatic behavioral drive". [39; 40; 118]

1.3.3.1. The lateral parabrachial nucleus

The LPBN contains seven sub-nuclei: the internal lateral, superior lateral, extreme lateral, external lateral, central lateral, ventral lateral and the dorsal lateral sub-nuclei. [68] Being recognized as a major relay center to receive information from the NTS related to blood pressure control, thirst or sodium appetite, the LPBN is an essential integrative site which mediates visceral cardiovascular information arising from the brainstem and transfers it to a wide variety of forebrain regions. [50; 68; 90; 166]

Studies have shown that LPBN electrical and chemical stimulation evokes a significant increase in mean arterial pressure (MAP), tachycardia and sympathetic nerve activity. [35] It also attenuates baroreflex inhibition of MAP. [88] Increased levels of circulating angiotensin II or changes in baroreceptor input, activated LPBN neurons. [91]. Additionally, LPBN temporary chemical inactivation induced a pressor response. [88]

Sustained hypertension, due to angiotensin II (AngII) intravenous infusion, was prevented by LPBN ablation, which suggests an interference of neurogenic pressor mechanisms associated with high levels of AngII. [66] However, lidocaine reversible bilateral lesions of the LPBN did not elicit a change in the pressor response to centrally injected AngII. [50; 121] Interruption of the area postrema

projection to the LPBN may avert pressor activity triggered by blood-borne AngII, since LPBN or area postrema ablation prevented hypertension through systemic AngII infusion. [50; 66]

LPBN electrolytic lesions enhanced baroreflex-mediated cardiovascular responses, whereas LPBN stimulation inhibited those same responses. [88; 151] The LPBN is considered to be a key component of the baroreflex pathway mediating coronary constriction and the latter was shown to involve activation of α_1-adrenoceptors. [50; 124]

L-glutamate microinjections into the LPBN increased MAP and activation of these same neurons activated RVLM cholinergic neurons and raised BP via muscarinic receptors. [105; 184] Experimental evidence shows that acetylcholine release in the RVLM contributes to hypertension. [105] Moreover, there's the hypothesis that hypertensive rats with greater cholinergic activity in the RVLM receive cholinergic inputs from LPBN pressor sites, maintaining hypertension. Therefore, the LPBN is likely to play a role in central mechanisms that control hypertension. [105]

1.3.3.2. The Kolliker-Fuse nucleus

The Kolliker-Fuse nucleus (KF) is involved in the control of the respiratory system, pain modulation and cardiovascular system regulation. [60] Anatomical research has demonstrated the relationship of KF with brain areas involved in cardiovascular regulation, such as the RVLM, NTS, cuneiform nucleus, raphe nucleus, periaqueductal gray matter and the intermediolateral column of the spinal cord. [48; 101; 157] Inputs from the RVLM and the commissural NTS arrive at the KF. It is likewise the main source of descending projections from the parabrachial complex to the NTS and RVLM and the intermediolateral column in the thoracic spinal cord. [68]

KF electrical stimulation caused a pressor effect with mild tachycardia. [101] Chan and colleagues showed that KF neurons exhibited a restricted c-Fos expression pattern during phenylephrine-induced activation of baroreceptors. [36] On the other hand, a large number of KF neurons were

activated after stimulation of cardiac sympathetic afferents, which suggests a role for KF in the regulation of central sympathoexcitatory responses during activation of cardiac sympathetic afferents. [59; 80; 107]

Besides its cardiovascular role, the KF is an essential part of the respiratory network which contributes to respiratory pattern formation and breathing adaptation to afferent information. An important role for KF in upper airway resistance and breathing regulation, by influencing respiratory rate and breathing amplitude, has been suggested by studies in anesthetized animals. Bilateral microinjection of muscimol into rats KF led to resting ventilation fall and hypercapnia induced ventilation rise. This implies that KF neurons are critical to the control of ventilation in resting or hypercapnic conditions. [46]

1.3.4. The periaqueductal gray matter

The major functions of the midbrain periaqueductal gray matter (PAG) include pain, analgesia, anxiety, vocalization, lordosis and cardiovascular control. Due to the variety of its interconnections, PAG has a pivotal role in the integration of emotional aspects of cardiovascular regulation. It has reciprocal connections with the lateral hypothalamic nucleus, paraventricular nucleus, medial pre-optic nucleus, amygdala, pre-frontal cortex plus insular cortex and projects to medullary regions responsible for blood pressure and heart rate control. [17]

Midbrain areas stimulation, including PAG, resulted in blood pressure rise. [17] Additionally, stimulation of the dorsolateral PAG increased BP plus muscle blood flow, decreased skin blood flow and produced respiratory changes. [6] Experiments in anesthetized cats showed that PAG stimulation raised BP and expiratory carbon dioxide. [58] Cell bodies activation in distinct PAG regions, by homocysteic acid administration, revealed that PAG is organized in four longitudinal columns: dorsomedial, dorsolateral, lateral and ventrolateral. [17; 29-31]

BP is raised following PAG dorsomedial and dorsolateral columns stimulation. A defensive behavior is also elicited in both cats and rats. Contrarily, stimulation of the PAG lateral and ventrolateral columns produced hypotension and freezing behavior in cats. 10 Hz stimulation of the ventral PAG had a more intense depressor effect when compared to caudal-ventral PAG stimulation. At 100 Hz the number of sites that produce a pressor effect increased. Stimulation of caudal PAG with this frequency generated a pressor effect and tachycardia. Hence, these results suggest that the PAG is dependent on the activity level of the afferent inputs or whether many afferents are simultaneously activated. [17]

2. THESIS RATIONALE & AIM

The heart and vessels are innervated by sympathetic nerves which are tonically active under a variety of conditions. This sympathetic tone is critical for cardiovascular homeostasis including the maintenance of blood pressure within a normal range of values for each individual and behavioural condition. Sympathetic activity, however, is not uniform along the visceral organs and functions which leads to a variety of complex visceral responses in physiological and pathological conditions.

A causal neurogenic component, i.e. sympathetic nervous system hyperactivity, has been implicated in the onset, development and maintenance of hypertension of neurogenic origin. Experimental evidence indicates that the PVN, a major sympathoexcitatory area, tonic effect on sympathetic vasomotor tone control is enhanced in spontaneously hypertensive rats. Recent studies in conscious animals from our laboratory showed a role for PVN in the modulation of peripheral sympathetic activity and blood pressure control through arterial baroreflex and also, for the first time, RVLM involvement in the modulation of sympathetic activity in essential hypertension. [70; 71] These observations were made possible due to a decrease of central sympathetic drive originated by a cellular activity decrease in both PVN and RVLM that was itself induced by the overexpression of potassium channels *hKir2.1*. The latter was genetically promoted through a brain microinjection of a lentiviral vector (LVV-*hkir2.1*) in spontaneously hypertensive rats, which are an animal model for neurogenic hypertension.

Nevertheless, several questions arise from these two important observations: are just these two areas, PVN and RVLM, the sole involved in the sympathoexcitation observed in neurogenic hypertension? What would be, or it is, the involvement of other sympathetic areas of the central autonomic network in this pathological condition? Is the decrease of arterial blood pressure values and sympathetic tone linearly dependent on the decrease of neuronal excitability due to the *hkir2.1* overexpression? Would *hkir2.1* effect on cardiovascular control be similar in normotensive conditions? Is there a cut-off value for peripheral sympathetic tone that determines the effect of central interventions to modulate sympathetic activity?

In fact, the search for brain areas responsible for sympathetic activation in physiological and pathological conditions is still a subject of research. However, it began at Carl Ludwig laboratory with one of his students, Oswjannikow (1871) who found that progressive serial transections between the pons and the obex progressively decrease blood pressure values. [109] Around the same time (1873), Dittmar, working at Ludwig's laboratory, demonstrated that the maintenance of blood pressure values was dependent on the ventral medulla. Later, in 1946, Alexander showed that the fall in blood pressure was the consequence of a decrease in post-ganglionic sympathetic activity making clear that the integrity of the connexions between the brainstem and the spinal cord were vital to keep blood pressure under homeostatic values; and in, 1976, Feldberg and colleagues established that the rostral ventrolateral medulla contains the cell bodies of neurons involved in the sympathetic outflow. [109] Anatomically, RVLM neurons project directly to the intermediolateral cell collumn of the spinal cord where they synapse with the preganglionic sympathetic neurons modulating peripheral sympathetic tone. In anaesthetized normotensive animals, RVLM activity has been extensively pharmacologically modulated with a transient impact on peripheral blood pressure values and arterial baroreflex responses. [109]

The autonomic nervous system also plays a strong and coordinated role in providing the appropriate activity pattern to complement somatic alterations associated to behavioural patterns. [109] The

central neuronal networks underlying the conditioning of these responses include both cortical and subcortical pathways and involve several sympathetic centres culminating with the appropriate level of sympathoexcitation. Also, it is generally thought that the lower brainstem contains neuronal circuits capable of interfering in these processes but there are no studies showing the changes on stress behavioural responses of RVLM decreased excitability in conscious animals. [109] Thus, in the present study, we sought to investigate:

- the changes on blood pressure, sympathetic activity and cardiorespiratory reflexes evoked by the overexpression of potassium *hkir2.1* channels of RVLM in normotensive conditions;
- the role of the decreased RVLM excitability on the autonomic–somatic relationships that condition behavioral responses on conscious normotensive animals;
- and the effect on blood pressure, peripheral sympathetic tone and cardiovascular reflex responses of the reduction of excitability of midbrain and pontine areas in an animal model of essential hypertension.

3. METHODOLOGY

3.1. Ethical considerations

All experimental procedures described in this study were in accordance with the European and Portuguese Law on animal welfare and had the approval of the Ethics Committee of the Faculty of Medicine, University of Lisbon, Portugal.

3.2. Animals

Studies were carried out on Wistar-Kyoto rats (*n*=12), and on Spontaneously Hypertensive rats (SHR, *n*=18) of both sexes and aged >10 weeks. Rats were housed individually at the Faculty of Medicine animal house, in a temperature and humidity-controlled room (20°C and 55%, correspondingly) and synchronized to a 12h-12h light/dark cycle. Animals were fed standard rat chow *ad libitum* and allowed free access to tap water.

3.3. Metabolic evaluation

Prior to microinjection protocol and 59 days after it, animals were individually housed for 24h in metabolic cages and given free access to 200g of standard rat chow plus 250ml of water. Evaluated

parameters included food and fluid intake, urine and feces production and body weight. Water waste was also accounted for.

3.4. Behavioral testing

3.4.1. Handling and habituation

For three consecutive days, animals were placed in the behavior room to enable acclimatization. Light, noise, odor and temperature were controlled to reduce the influence on animal behavior to a minimum. After one hour, rats were handled for 10 minutes by the experimenter.

3.4.2. General locomotor activity

The Open-Field test was used (see Appendix). Animals were placed in the center of a square chamber 80cmx80cmx50cm, 50cm above floor level, for 5 minutes and allowed to freely explore. The apparatus was divided into three virtual zones (central, intermediate and peripheral) for behavior analysis purposes. Scored parameters included: total distance travelled (cm), number of entries, time spent in each virtual area and number of rearings.

3.4.3. Anxiety-like behavior

50cm above floor level, the Elevated-Plus Maze was assembled (see Appendix). Two open arms (50cmx10cm) and two enclosed arms (50cmx10cmx40cm) extended from a central platform (14cmx10cm). Rat was placed on the central platform, facing the open arm, and allowed to freely explore the apparatus during 5 minutes. Animal's performance evaluation was based on the number of entries in open/closed arms, time spent in each of them, time spent on the central platform, travelled distance (cm) and number of rearings.

Behavioral assays were performed on two time points: before bilateral microinjection into the RVLM and prior to cardiorespiratory reflexes assessment. To prevent influence of olfactory cues on animal's

behavior, all used apparatus were thoroughly rinsed with 70% ethanol and dried with paper towels between sessions. Tests were recorded by a video camera, using i-SEC Guarding Recording computer program (version 5.0.1.284).

3.5. Surgical procedures

3.5.1. Implantation of radio-telemetry probes

Subsequently to anesthetic administration (ketamine, 100mg ml^{-1}, and dexmedetomidine, 0.5mg ml^{-1}, IP) a medial laparotomy was performed and the abdominal aorta proximally clamped. By means of a binocular microscope, the radio-telemetry sensor catheter (ca. 0.7 mm, thin-walled thermoplastic membrane) was then inserted into the root of the abdominal aorta, between the renal and iliac arteries. Catheter and artery were bound together with a cellulose patch and tissue adhesive. The radio-telemetric pressure transducer (DSI, USA), a fluid-filled catheter connected to a PA-C40 transmitter, was sutured to the abdominal wall. Internal and skin layers were closed with polyglycolic acid and silk surgical suture, respectively. An anti-inflammatory (carprofen, 4 mg kg^{-1}, SC) and a sedative reverser (atipamazole, 5mg ml^{-1}, IM) were injected at the end of the surgical procedure. Rats had 15 days to recover from the intervention.

3.5.2. Viral vector construction and validation

Lentiviral vector construction was based on previous studies. [56; 71; 180] Briefly, LVV-eGFP, used for the sham-treated group, was a misture of LVTREtight-GFP 5.7×109 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×109 IU in a ratio 1:4. This binary system expresses eGFP. The LVV-*hKir2.1* is a mix of LV-TREtight-Kir-cIRES-GFP 5.4×109 IU and LV-Syn-Eff-G4BS-Syn-Tetoff 6.2×109 IU (ratio 1:4), which expresses eGFP as well as human inwardly rectifying potassium channels of *hKir2.1* type in neuronal cells. Validation of the efficacy of transduction and transgene expression were assessed by Duale *et al.* and included mRNA expression, immunocytochemical and electrophysiological data. [56]

3.5.3. Central Microinjection sites

After catheter implant, animals were randomly divided into two groups according to the microinjection content:

- Group A (n=12), normotensive Wistar rats to address functional responses under RVLM modulation, further subdivided into two subgroups: a experimental group (n=6) microinjected with the LVV-*hKir2.1* and a sham group (n=6) matching sex and age distribution injected with LVV-eGFP;
- Group B (n=18), SHR's that were centrally microinjected with a lentiviral vector (LVV) overexpressing *hKir2.1* potassium channels. These animals were further divided into 3 sub-groups according to the microinjected area: Lateral Parabrachial nucleus (LPBN) (n=6), Kolliker-Fuse nucleus (KF) (n=6) and Periaqueductal Grey Matter (PAG) (n=6). All these central brain regions were compared to sham animals used on previous studies of our laboratory. [70; 71] PVN and RVLM are both sympathoexcitatory areas in which a LVV-*hKir2.1* microinjection provoked a decrease in BP and sympathetic tone in SHR. In these same studies, sham animals were microinjected with LVV-eGFP and BP and sympathetic output did not change. Having been demonstrated that LVV-eGFP did not elicit alterations in cardiovascular variables, we used these animals as a control group for LPBN, KF and PAG experiment following the 3R's rule.

After fined tuned the stereotaxic coordinates for bilateral injections in the selected brain areas RVLM, LPBN, KF and PAG and two weeks following radio-telemetry probes implantation, rats were re-anaesthetized (ketamine, 100mg ml^{-1}, IP; dexmedetomidine, 0,5mg ml^{-1}, IP) and placed on a stereotactic frame (Kopf Instruments, USA) under anesthesia effect. A craniotomy was performed and animals were microinjected bilaterally into the already mentioned brain regions (RVLM - Bregma: -12.5mm, Lateral: 2.1mm, Deep: 8mm; LPBN - Bregma: -9.8mm, Lateral: 2.4mm, Deep: 6.8mm; KF - Bregma: -8.7mm, Lateral: 2.6mm, Deep: 7.8mm; PAG - Bregma: -5.2mm, Lateral: 0.5mm,

Deep: 5.4mm, [136]) with 0.05µl of LVV-eGFP or LVV-*hKir2.1* according to their previously assigned experimental group. The microinjected volume (0.05µl) needed to limit transduction to the confines of the chosen area was also confirmed in previous studies. [70; 71] Rats were allowed to recover and monitored by telemetry every 10 days, for a 60 day period.

3.5.4. Cardiorespiratory evaluation

On the 60th day, animals were re-anesthetized (sodium pentobarbitone, 60mg kg^{-1}, IP) and the trachea, carotid artery, femoral artery and vein were cannulated. Rectal temperature was monitored by a servo-controlled heating blanket (Harvard Apparatus). ECG was recorded, via needle electrodes inserted into the three of the four limbs, and HR was derived from it. Baroreceptor reflex was activated by phenylephrine administration (0.2ml, 25µg ml^{-1} IV; Sigma Aldrich). Retrogradely injected lobeline (0.2ml, 25µg ml^{-1} IV, Sigma Aldrich), through the external carotid artery into the bifurcation of the common carotid artery, triggered the peripheral chemoreceptor reflex. Activation of baroreceptor and peripheral chemoreceptor reflexes was performed twice, with a 5 minute interval separating each stimulus. ECG, HR and BP (systolic, diastolic and mean) were recorded continuously throughout the experiment.

3.6. Morphological studies

After cardiorespiratory reflex evaluation, animals were terminally anesthetized with an overdose of anesthetic (sodium pentobarbitone, 60mg kg^{-1}, IV) and the brain areas of interest in each group were rapidly removed, placed on Tissue-Tek® and frozen with isopentane and liquid nitrogen at -80°C. Coronal sections (18 µm) were cut on a microtome and mounted on slides. By means of an epifluorescence microscope, eGFP-labeled regions were identified and plotted on standardized sections of the Paxinos and Watson atlas. [136]

3.7. Data acquisition and analysis

With a 10 day interval, during a 60 day period, telemetric data was acquired at 1kHz and analyzed with suitable software (LabChart6, Powerlab, ADInstruments). Mean values of HR, BP (systolic, diastolic and mean) and RespR were extracted.

3.7.1. Baro and chemoreceptor reflex evaluation

Quantification of baroreceptor reflex gain (BRG) was accomplished as follows: $BRG=(HR_{basal}-HR_{BPmax})/(BP_{max}-BP_{basal})$ (bpm·mm Hg^{-1}). Chemoreceptor reflex (ChR) was measured as: $\Delta ChR=RespR_{lobeline}-RespR_{basal}$. Respiratory rate (RespR) was derived from the tracheal pressure, before and after stimulation with lobeline.

3.7.2. Overall autonomic cardiovascular output

Mean values of HR and BP (systolic, diastolic and mean) were extracted from telemetric data recordings. To assess sympathetic (Low Frequency [LF] band, 0.15-0.6 Hz of sBP) and parasympathetic (High Frequency [HF] band, 0.6-2.0 Hz of HR) activity over time, 3 min periods of systolic BP and RR interval data were analyzed in the frequency domain (Fast Fourier Transform) using in-house software Fisiosinal. [2; 117; 174]

3.7.3. Respiratory sinus arrhythmia evaluation

Respiratory sinus arrhythmia was quantified as the reason between the longer RR interval of the ECG during expiration and the shorter RR interval during inspiration as stated by Castro and colleagues. [32]

3.7.4. Circadian BP and HR profile

Mean BP and HR values were obtained from telemetric data recordings and compared between light (7AM-7PM) and darks phases of the circadian rhythm.

3.8. Statistical analysis

Comparisons between groups for the same period and also comparisons within the same group, before and after the microinjections were performed. For the statistical analysis, Student's t test for paired and unpaired data for comparisons within and between groups was used. All data were expressed as mean ± SEM and passed the normality test. Significance was taken as $p < 0.05$.

4. RESULTS

4.1. On changes on blood pressure, sympathetic activity, cardiorespiratory reflexes and behavioral responses evoked by the overexpression of potassium *hkir2.1* channels of RVLM in normotensive conditions

4.1.1. LVV-*hKir2.1* influence on 24h mean values of blood pressure and heart rate

Prior to LVV-*hKir2.1* bilateral microinjection in the RVLM of conscious normotensive rats (n=6), basal blood pressure (BP) values were 107±1 mmHg for systolic BP (sBP), 97±1 mmHg for diastolic BP (dBP) and 100±1 mmHg for mean BP (mBP). These were not significantly different from the sham group (n=6) ones (107±1, 90±1 and 96±1 mmHg, respectively; $p>0.05$; figure 4.1). LVV-*hKir2.1* rats showed similar HR values when compared to LVV-eGFP animals (390±3 *versus* 375±3 bpm; $p>0.05$).

Thirty days into the experimental protocol, no significant changes were observed between groups (LVV-*hKir2.1 vs* LVV-eGFP: sBP 109±1 *vs* 108±1 mmHg, dBP 100±1 *vs* 91±1 mmHg, mBP 103±1 *vs* 98±1 mmHg, HR 367±4 *vs* 354±3 bpm; $p>0.05$), but with the intent to assess its persistence, rats were monitored for a further 30 days.

60 days after lentiviral microinjection, values for the LVV-*hKir2.1* group systolic, diastolic and mean blood pressure were 107±1, 99±1 and 101±1 mmHg respectively, showing that LVV-*hKir2.1*

microinjection did not elicit significant changes on blood pressure (p>0.05). Heart rate also remained unchanged through the 60 day period (364±4 bpm; p>0.05). The same happened for LVV-eGFP rats (sBP 107±1 mmHg, dBP 91±1 mmHg, mBP 97±1 mmHg, HR 352±3 bpm; p>0.05).

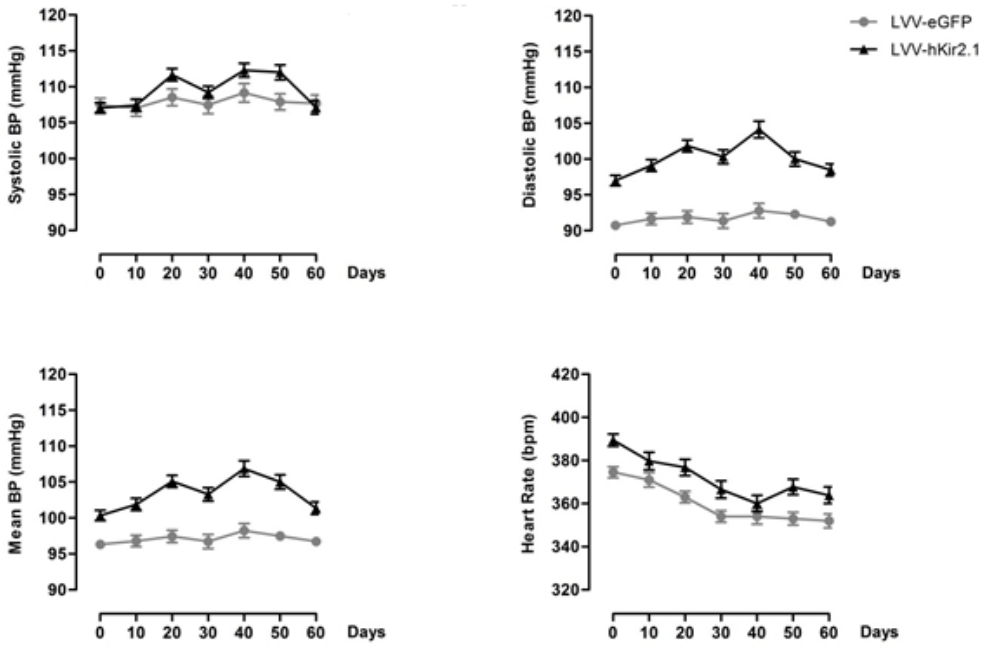

Figure 4.1 – Effect of lentiviral microinjection of LVV-*hKir2.1* (n=6) or LVV-eGFP (n=6) on systolic, diastolic and mean blood pressure and heart rate, for a 60 day period. No significant changes were observed but the higher values of the *hKir2.1* group suggest the interference of other sympathetic areas upon RVLM decrease of excitability in order to reestablish the normal autonomic output (see figure 4.2).

4.1.2. Lentiviral microinjection effect on sympathetic output

Rats microinjected with LVV-*hKir2.1* showed a decreased cardiovascular autonomic outflow during the 60 day period when compared to LVV-eGFP animals. Fast Fourier Transform applied to systolic BP and R-R intervals, revealed a decrease of LF(BP)/HF(RR) (from day 0: 0.087±0.022 to day 60: 0.028±0.027 $mmHg^2ms^{-2}$; p>0.05), due to a fall in LF band power which expresses sympathetic output (from 1.725±0.341 to 0.798±0.358 $mmHg^2/Hz$; p>0.05, figure 4.2). LF was reduced, although not

significantly when compared to LVV-eGFP animals, by 10 days after *LVV-hKir2.1* microinjection but had no effect on blood pressure.

Interestingly, on the 20th day a significant decrease in LF and LF(BP)/HF(RR) was observed on the experimental group (1.016±0.214 mmHg^2/Hz and 0.071±0.061 mmHg^{2}ms^{-2}, respectively; p<0.05). This LF significant statistical difference persisted until day 50. Furthermore, LVV-eGFP basal LF and LF(BP)/HF(RR) values were 1.694±0.295 mmHg^2/Hz and 0.081±0.037 mmHg^{2}ms^{-2}, respectively. 60 days after LVV-eGFP microinjection, LF was 3.009±1.172 mmHg^2/Hz and LF(BP)/HF(RR) was 0.141±0.048 mmHg^{2}ms^{-2}. Statistical significance was found while comparing both groups on the last day of the protocol (p<0.05).

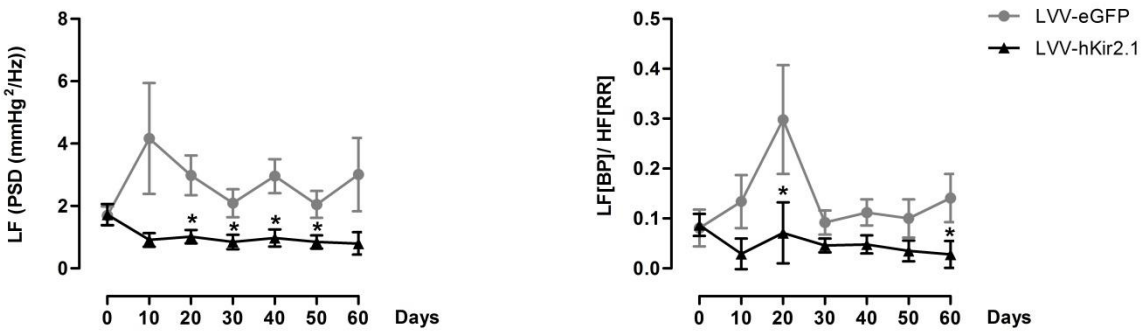

Figure 4.2 – 0 and 10 days intervals after LVV-*hKir2.1* or LVV-eGFP microinjection mean (±SEM) LF and LF(BP)/HF(RR). * p<0.05, statistically significant difference between groups. A significant decrease in autonomic outflow was observed in LVV-*hKir2.1* animals first on the 20th and then on the 60th day.

4.1.3. Blood pressure and heart rate circadian variation

In basal conditions, BP circadian variation profile followed a similar trend, with lower BP values during the light phase relative to the dark one (table 1, p>0.05). HR was lower during light phase (table 1, p>0.05).

On the 60th day, LVV-*hKir2.1* animals showed an increase in systolic, diastolic and mean BP, during both light and dark phases (table 1, $p<0.05$ only for dBP). A decrease in HR was observed in the light phase but not during the dark one ($p>0.05$). LVV-eGFP rats sBP, dBP and mBP values for the light and dark phases remained unchanged until the end of the protocol. However, HR decreased in both light and dark phases (table 1 and figure 4.3 $p>0.05$). Same group comparisons, i.e. comparison between 0 and 60 days of LVV-*hKir2.1* or LVV-eGFP, were not statistically significant ($p>0.05$).

Table 1 – Blood pressure (mmHg) and heart rate (bpm) circadian variation for both groups, before and 60 days after microinjection into the RVLM. Values are expressed as mean±SEM. Abbreviations: sBP, systolic blood pressure; dBP, diastolic blood pressure; mBP, mean blood pressure; HR, heart rate; LVV-*hKir2.1*, Wistar Kyoto rats microinjected with LVV-*hKir2.1*; LVV-eGFP, Wistar Kyoto rats microinjected with LVV-eGFP. *$p<0.05$, statistically significant difference between 0 and 60 days values.

	Light phase				Dark phase			
Group	**sBP**	**dBP**	**mBP**	**HR**	**sBP**	**dBP**	**mBP**	**HR**
Basal conditions								
LVV-*hKir2.1*	107±1	97±1	100±1	385±3	109±2	98±1	102±1	393±3
LVV-eGFP	107±1	90±1	96±1	363±3	108±2	92±1	97±1	387±4
60 days after microninjection								
LVV-*hKir2.1*	122±4	113±4	116±4	316±5	126±4	117±4*	120±4	354±4
LVV-eGFP	106±1	90±1	96±1	334±5	109±2	92±1	98±1	373±5

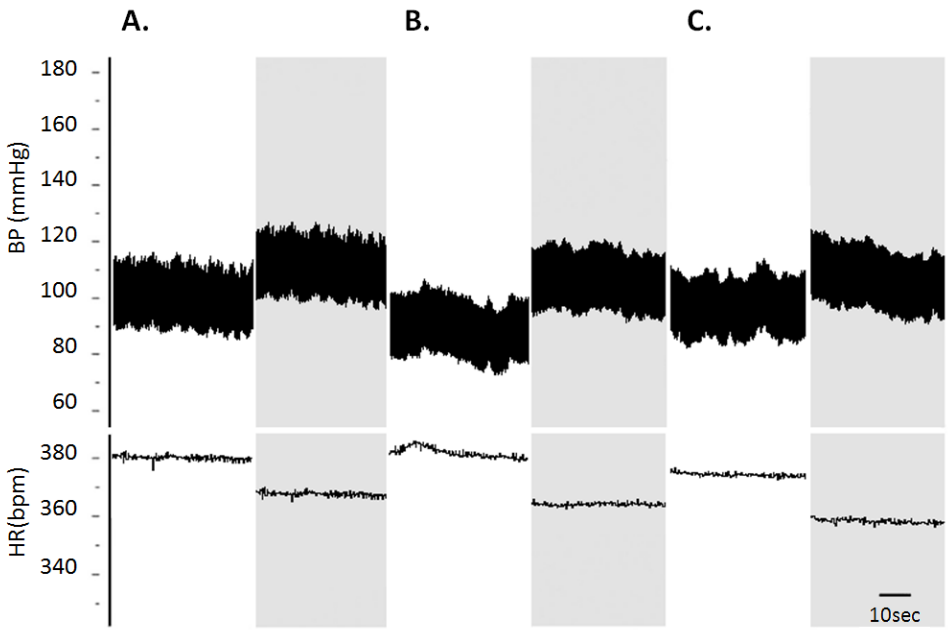

Figure 4.3 – Circadian blood pressure and heart rate raw data: (A) Wistar-Kyoto rat before and **(B)** 60 days after LVV-*hKir2.1* microinjection; **(C)** another Wistar-Kyoto rat 60 days after LVV-eGFP microinjection in the RVLM during light (white) and dark (gray) phases. This raw data serves as an example of rats' circadian rhythm, which is characterized by higher blood pressure values during the night (dark phase) and lower ones during the day (light phase).

4.1.4. Indirect assessment of vagal tonus

Respiratory sinus arrhythmia (RSA) is defined as rhythmic variations in heart rate occurring at the frequency of respiration, leading to an acceleration of the cardiac rate during inspiration and a deceleration during expiration. Is detectable in most individuals but tends to be more pronounced on children and depends on the presence of a normal resting cardiac vagal tone varying linearly with it. In our experiments, RSA was not affected by the lentiviral microinjection. LVV-*hKir2.1* rats presented the same RSA profile on day 0 and 60 (1.11±0.002). Similarly, RSA values for the sham group varied from 1.11±0.002 to 1.12±0.006 (p>0.05) indicating that the vagal tonus was not affected by the decrease in RVLM excitability.

4.1.5. Cardiorespiratory reflex evaluation

Phenylephrine is a selective α_1-adrenergic receptor agonist, thus used to evoke the baroreceptor reflex due to the induced increase in blood pressure when is intravenously injected. In the present work, phenylephrine injection triggered, in both animal groups and as expected, a progressive rise in mean BP accompanied by a progressive fall in HR. No significant changes were observed when comparing the baroreflex gain (BRG) of the experimental group versus the sham treated one (0.48±0.26 *vs* 0.38±0.09 bpm/mmHg; $p>0.05$; figure 4.4).

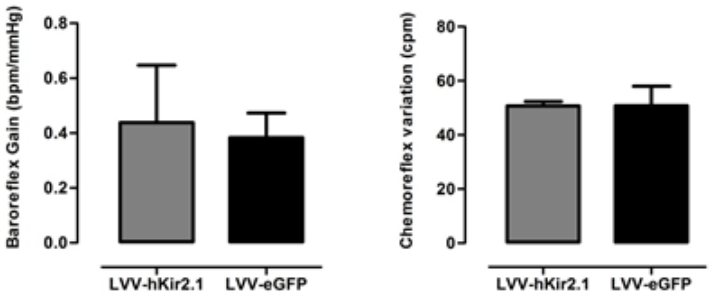

Figure 4.4 – LVV-*hKir2.1* or LVV-eGFP microinjection effect on baroreflex gain and chemoreflex variation, 60 days after microinjection into the RVLM. No significant changes were observed.

Respiratory rate baseline values, 60 days post lentiviral microinjection, in the anesthetized rats were 28±4.3 and 31±3.1 breaths min^{-1} for LVV-*hKir2.1* and LVV-eGFP, respectively. Peripheral chemoreceptor reflex activation through lobeline administration, a nicotine-like drug acting at carotid body type I cells, caused a similar hyperventilatory reflex response on both animal groups (50.7±1.7 *vs* 50.8±7.2 breaths min^{-1}; figure 4.4, $p>0.05$). Additionally, mean BP responses to chemoreflex activation didn't differ significantly between LVV-*hKir2.1* and LVV-eGFP animals (from 90±6 to 100±11 and 92±3 to 108±9 mmHg, correspondingly; $p>0.05$). HR responses remained unchanged (LVV-*hKir2.1*: 199±9 *vs* 198±7 bpm, LVV-eGFP: 188±10 *vs* 179±15 bpm; $p>0.05$).

4.1.6. Metabolic evaluation

Prior and 60 days after bilateral lentiviral microinjection in the RVLM, no significant changes were found in body weight, ingestion, water intake, urine and feces production in both LVV-*hKir2.1* and LVV-eGFP rats (table 2; $p>0.05$). Animals were not subjected to an adjustment period, which could have repercussions on rat's metabolic behavior, thus forming a study limitation.

Table 2 – Metabolic evaluation before and after lentiviral microinjection into the RVLM. Values are expressed as mean±SEM.

Group	Δ Weight (g)	Food (g)	Water (ml)	Urine (ml)	Feces (g)
BASAL CONDITIONS					
LVV-*hKir2.1*	-1.57±2.71	23.14±3.03	25.86±3.60	12.29±1.73	27.86±0.74
LVV-eGFP	-3.50±5.86	16.33±3.18	33.33±6.54	18.00±3.72	26.67±0.88
60 DAYS AFTER MICROINJECTION					
LVV-*hKir2.1*	-1.83±2.26	22.67±0.33	26.17±3.28	16.83±3.25	26.17±0.60
LVV-eGFP	-2.00±6.28	25.75±4.23	36.00±6.83	15.75±4.97	29.00±2.27

4.1.7. Neuroanatomical studies

Microinjection site was located within the RVLM [136] through the detection of e-GFP fluorescence. By means of immunohistochemical studies and confocal microscopy it was confirmed that RVLM neurons expressed eGFP (figure 4.5) and that LVV-*hKir2.1* and LVV-eGFP both contained green fluorescent protein indicating the vector internalization and allowing the estimation of virus dispersion, which was confined to a surface of 0.10 to 0.20 mm around the injection site.

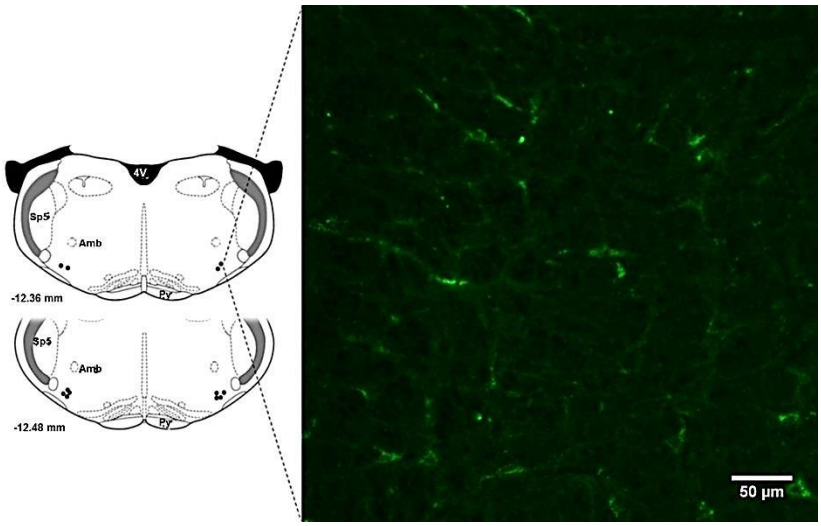

Figure 4.5 - RVLM microinjection sites localization (black circles) plus lentiviral vector-mediated transduction of green fluorescent protein (GFP) in the RVLM. Vector internalization in the neurons is well represented by the light green spots. Right image was acquired on confocal microscope (scale bar: 50µm). Amb, nucleus ambiguous; Py, pyramidal tract; Sp5, spinal trigeminal nucleus; 4V, 4th ventricle.

4.1.8. LVV-*hKir2.1* impact on locomotor activity and exploratory behavior

Behavioral testing despite addressing special learning is an indirect way of stress evaluation. Based on the open-field test (see Appendix), behavior was not significantly different before and after LVV-*hKir2.1* microinjection. Most rearing activity was performed in the periphery zone. No significant differences were found regarding the number of rearings of both groups, prior and post microinjection (day 0 LVV-*hKir2.1*: 22±2, day 60 LVV-*hKir2.1*: 18±3, day 0 LVV-eGFP: 22±5 and day 60 LVV-eGFP: 12±3; p>0.05 for within and between experimental groups). In addition, global locomotor activity, retrieved from total distance travelled, was high indicating that rats had no motor deficit. Animals spent more time in the periphery zone (day 0 LVV-*hKir2.1*: 282±4s, day 60 LVV-*hKir2.1*: 288±4s, day 0 LVV-eGFP: 279±6s and day 60 LVV-eGFP: 287±5s, p>0.05) when compared to the center and intermediate zones of the apparatus (day 0 LVV-*hKir2.1*: 10±3s, day 60 LVV-*hKir2.1*: 10±4s, day 0 LVV-eGFP: 12±5s and day 60 LVV-eGFP: 10±4s; day 0 LVV-*hKir2.1*: 1±0.5s, day 60 LVV-

hKir2.1: 0.3±0.2s, day 0 LVV-eGFP: 2±0.6s and day 60 LVV-eGFP: 1±0.6s respectively, *p*>0.05, figure 4.6).

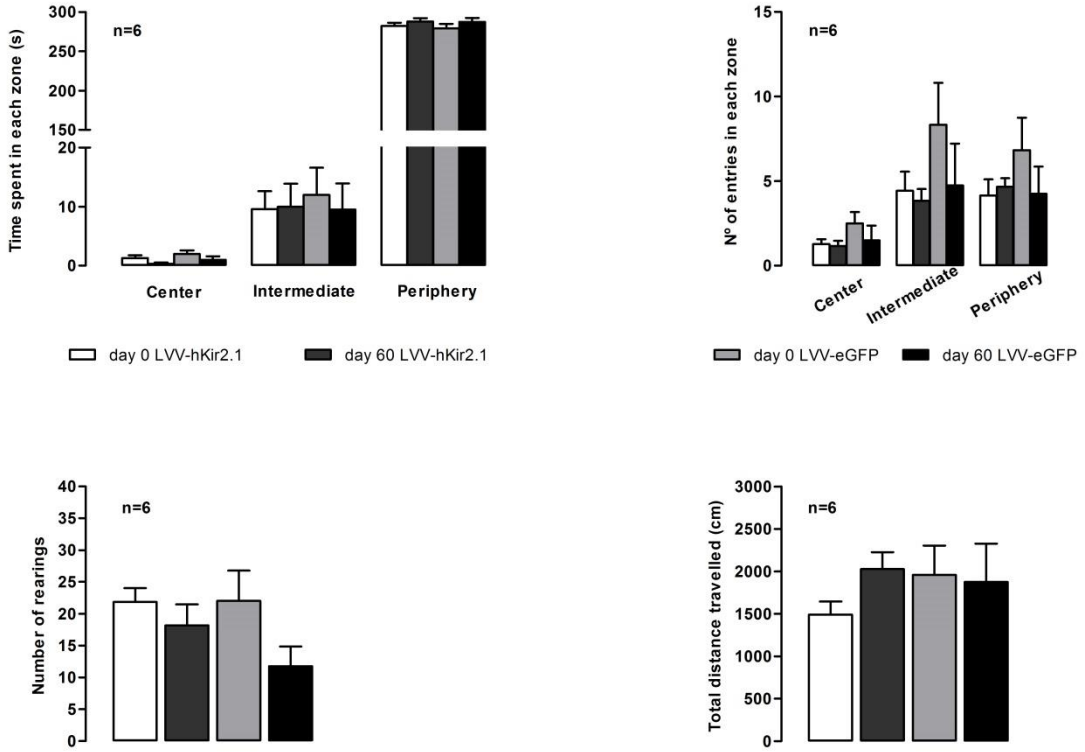

Figure 4.6 – Animals performance in the Open-field test. Time (s) spent and the number of entries in each of the three virtual zones of the apparatus, as well as the total distance (cm) travelled are depicted. The total number of rearings is also shown. Results are expressed as mean±SEM. No significant changes were observed.

As an anxiolytic response measurement, in the elevated-plus maze test (see Appendix) the percentage of time spent in open/closed arms, the number of entries in each of the apparatus four arms and the number of rearings was quantified. No significant changes were found on animal's behavior (figure 4.7). Rats spent less time in the open arms (day 0 LVV-*hKir2.1*: 11±5s, day 60 LVV-*hKir2.1*: 4±4s, day 0 LVV-eGFP: 12±4s and day 60 LVV-eGFP: 4±3s, *p*>0.05) when compared to the closed ones (day 0 LVV-*hKir2.1*: 62±5s, day 60 LVV-*hKir2.1*: 80±6s, day 0 LVV-eGFP: 66±6s and day 60 LVV-eGFP: 80±7s, *p*>0.05). For the remaining time, animals were in the center of the apparatus.

Consequently, the number of entries in the open arms was lower than the closed ones (day 0 LVV-*hKir2.1*: 2±1, day 60 LVV-*hKir2.1*: 1±1, day 0 LVV-eGFP: 3±1 and day 60 LVV-eGFP: 1±1 *vs* day 0 LVV-*hKir2.1*: 7±1, day 60 LVV-*hKir2.1*: 8±1, day 0 LVV-eGFP: 8±2 and 60 LVV-eGFP: 8±3 respectively, $p>0.05$). Exploratory activity did not show any significant changes within same group and between groups' comparisons.

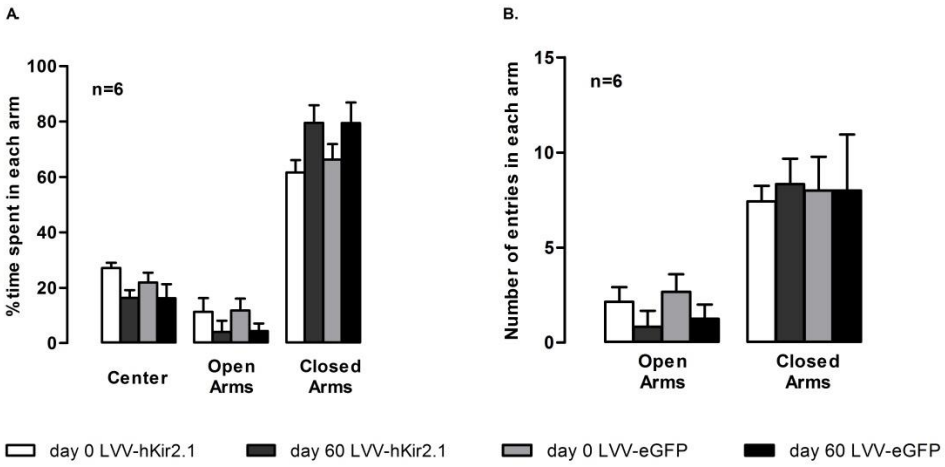

Figure 4.7 – Rats' performance in the Elevated plus-maze. Percentage of time **(A)** spent in open/closed arms and the number of entries **(B)** in each arms group are shown. Results are expressed as mean±SEM.

4.2. On the changes on blood pressure, sympathetic activity and cardiorespiratory reflexes evoked by the overexpression of potassium *hKir2*.1 channels in LPBN, PAG and KF on hypertensive conditions

4.2.1. LATERAL PARABRACHIAL NUCLEUS

4.2.1.1. Microinjection influence on long-term blood pressure control

Prior to LVV-*hKir2.1* bilateral microinjection in the LPBN (n=6), basal blood pressure (BP) values were 161±1, 133±1 and 142±1 mmHg for sBP, dBP and mBP, respectively. Basal BP values for the SHR LVV-

eGFP group (n=6) were 160±2 mmHg for sBP, 135±1 mmHg for dBP and 143±2 mmHg for mBP. All BP values decreased significantly 60 days after microinjection in the LPBN, when compared to the SHR LVV-eGFP (111±1, 104±1 and 107±1 mmHg *vs* 174±10, 149±11 and 157±10 mmHg, respectively n=6, $p<0.05$).

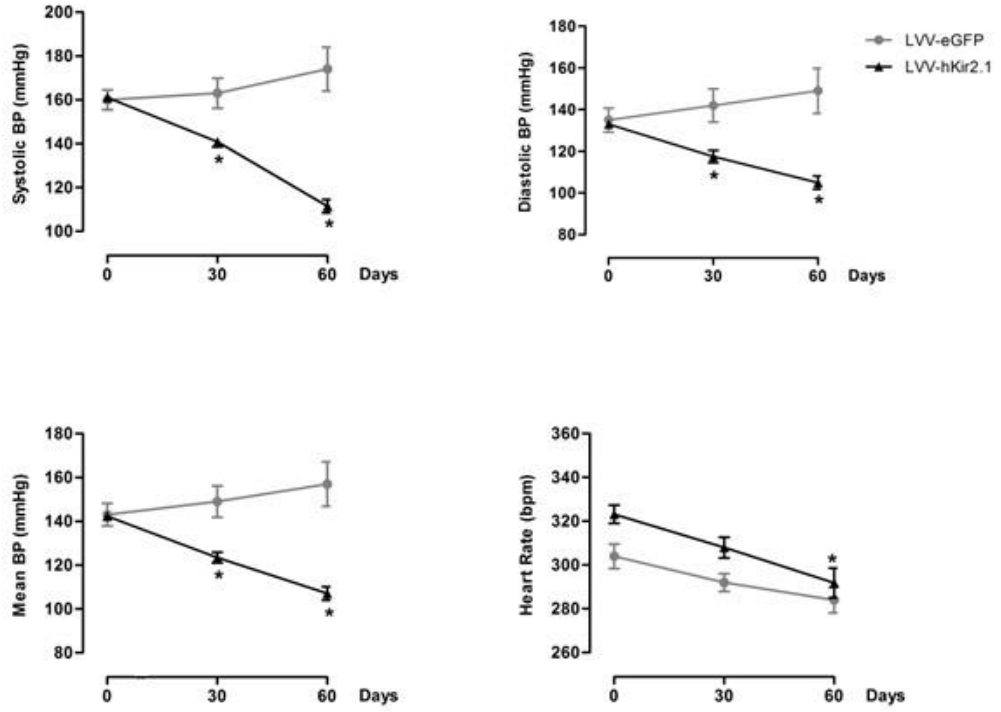

Figure 4.8 – The lentiviral microinjection of LVV-*hKir2.1* in the LPBN evoked a significant decrease on systolic, diastolic and mean blood pressure and heart rate of SHR rats (n=6; * $p<0.05$). LVV-eGFP animals' parameters followed the expected tendency of increased BP values due to the physiological characteristics of the animal model development.

This shows that LVV-*hKir2.1* microinjection elicited a significant change on BP of SHR LPBN (figure 4.8). Heart rate values decreased as well 60 days after microinjection (323±1 *vs* 291±3 bpm, $p<0.0001$).

4.2.1.2. Microinjection impact on sympathetic tone

Fast Fourier Transform application to sBP and interpulse intervals revealed that cardiovascular autonomic outflow of SHR LVV-*hKir2.1* animals significantly decreased 60 days post microinjection both within the same group amongst day 0 and day 60 (SHR LVV-*hKir2.1*: day 0 – 0.11±0.002 $mmHg^2ms^{-2}$; day 60 – 0.05±0.004 $mmHg^2ms^{-2}$, $p<0.05$, n=6) and between groups (SHR LVV-eGFP: day 0 - 0.08±0.02 $mmHg^2ms^{-2}$; day 60 – 0.08±0.003 $mmHg^2ms^{-2}$, $p<0.05$, n=6). Autonomic outflow decrease was due to a fall in sympathetic output expressed by the LF band power (SHR LVV-*hKir2.1:* day 0 - 1.61±0.07 $mmHg^2ms^{-2}$ *vs* day 60 – 0.73±0.04 $mmHg^2ms^{-2}$; $p<0.05$). SHR LVV-eGFP animals did not differ significantly within the group, but did so with the LVV-*hKir2.1* one (SHR LVV-eGFP*:* day 0 – 0.86±0.12 $mmHg^2ms^{-2}$ *vs* day 60 – 0.86±0.19 $mmHg^2ms^{-2}$; $p<0.05$ compared to LVV-*hKir2.1* rats).

4.2.1.3. Blood pressure and heart rate circadian variation

In basal conditions, BP circadian variation profile followed a similar trend, with lower BP values during the light phase relative to the dark one. As expected, HR was lower during the light phase.

On the 60th day, the LPBN LVV-*hKir2.1* animal group showed a non-significant increase in sBP, dBP and mBP accompanied by a decrease in HR, during both light and dark phases (light phase_day 0_sBP- 160±5 mmHg, dBP - 131±6 mmHg, mBP - 141±6 mmHg, HR - 299±13 bpm *vs* day 60_ sBP - 166±6 mmHg, dBP - 131±13 mmHg, mBP - 143±7 mmHg, HR – 277±9 bpm; dark phase_day 0_sBP - 158±1 mmHg, dBP - 127±2 mmHg, mBP - 138±1 mmHg, HR – 325±9 bpm *vs* day 60_ sBP - 176±6 mmHg, dBP - 141±13 mmHg, mBP - 153±7 mmHg, HR – 329±15 bpm, $p>0.05$). Systolic, diastolic and mean BP plus HR light phase values for SHR LVV-eGFP group were 158±4 mmHg, 133±5 mmHg, 148±5 mmHg, 292±6 bpm *vs* 171±11 mmHg, 145±10 mmHg, 154±10 mmHg, 264±5 bpm, for day 0 and day 60 respectively. Dark phase values followed a similar trend ($p>0.05$).

4.2.1.4. Parasympathetic tonus indirect assessment

Respiratory sinus arrhythmia (RSA) has been used as an index of vagal control of the heart. In our research project, RSA was not affected by LVV-*hKir2.1* microinjection into the LPBN. LVV-*hKir2.1* rats presented similar RSA profile on day 0 and 60 (1.07±0.76 *vs* 1.03±0.73, $p>0.05$). Likewise, RSA values for the SHR LVV-eGFP group varied from 1.06±0.01 to 1.03±0.01 ($p>0.05$), which suggests that vagal tonus was not affected by LPBN decreased excitability.

4.2.1.5. Cardiovascular reflexes evaluation

As previously mentioned blood pressure and heart rate both decreased 60 days after LVV-*hkir2.1* microinjection, although the latter did not do so significantly. Thus, we sought to assess if cardiovascular reflexes were also affected by lentiviral microinjection into the LPBN. In both LPBN LVV-*hKir2.1* and SHR LVV-eGFP animal groups, phenylephrine injection elicited a progressive rise in mean BP complemented by a progressive fall in HR. Regarding the baroreflex gain, no significant changes were observed between groups (0.45±0.1 *vs* 0.46±0.05 bpm/mmHg; LPBN LVV-*hKir2.1* and LVV-eGFP correspondingly, $p>0.05$, figure 4.9).

Peripheral chemoreceptor reflex was activated via intravenous injection of lobeline. A similar hyperventilatory reflex response characterized by an increase in the depth and rate of respiration was observed in both animal groups. However, despite the different variation between them, fully statistical significance was not attained (LPBN LVV-*hKir2.1*: Δ19.7±3.7 *vs* SHR LVV-eGFP: Δ31.5±4.5 cpm; $p>0.05$, figure 4.9).

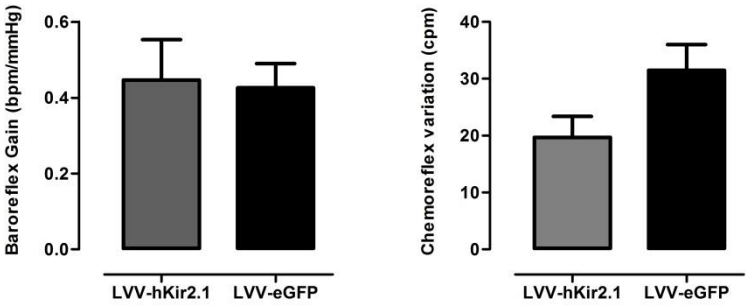

Figure 4.9 - LVV-*hKir2.1* microinjection into LPBN effect on baroreflex gain and chemoreflex variation, 60 days after microinjection. No significant changes were observed.

4.2.2. PERIAQUEDUCTAL GRAY MATTER

4.2.2.1. Microinjection influence on 24h mean values of blood pressure and heart rate

Basal blood pressure values before LVV-*hKir2.1* bilateral microinjection into the PAG (n=6) were 152±1 for sBP, 130±1 for dBP and 137±1 mmHg for mBP. There were not significant differences between SHR LVV-eGFP and PAG LVV-*hKir2.1* animals (figure 4.10). 60 days after lentiviral microinjection, PAG LVV-*hKir2.1* sBP, dBP and mBP were 168±1, 144±5 and 152±4 mmHg correspondingly, demonstrating that lentiviral microinjection did not alter BP values (n=6; $p>0.05$). Heart rate fell significantly ($p<0.05$) thus, accompanying the BP rise.

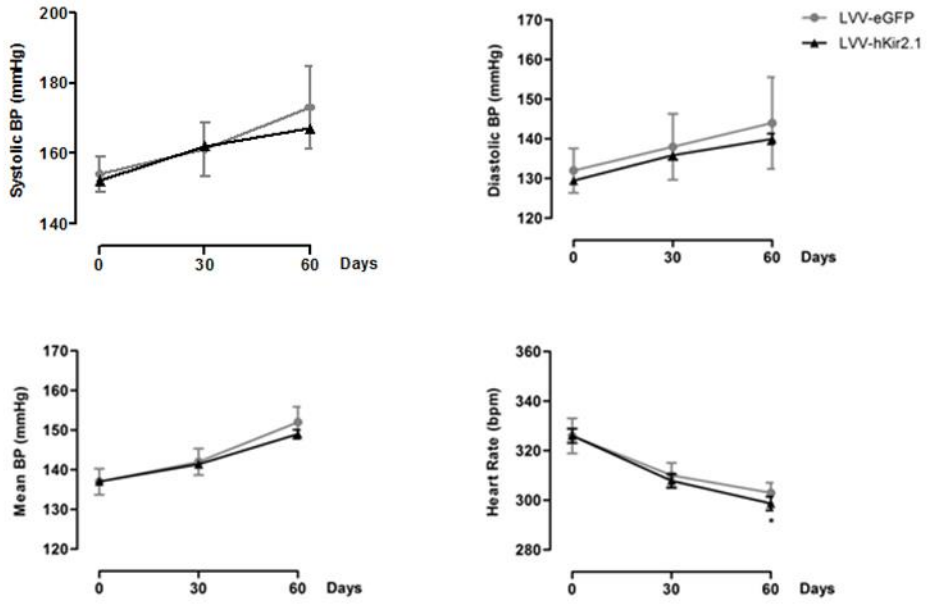

Figure 4.10 - Effect of lentiviral microinjection of LVV-*hKir2.1* in the PAG on systolic, diastolic and mean blood pressure and heart rate, for a 60 day period. * $p<0.05$, comparison between day 0 and day 60 of LVV-*hKir2.1* animals.

4.2.2.2. Lentiviral microinjection effect on autonomic output

Sympathetic tone was assessed indirectly through the application of Fast Fourier Transform to sBP and RR intervals. PAG LVV-*hKir2.1* animals exhibited no change in LF(BP)/HF(RR) ratio, i.e. cardiovascular autonomic outflow was not altered 60 days post LVV-*hKir2.1* microinjection (0.04±0.07 *vs* 0.05±0.02 $mmHg^2ms^{-2}$, $p>0.05$) when compared to LVV-eGFP rats (0.08±0.02 *vs* 0.09±0.03 $mmHg^2ms^{-2}$, $p>0.05$).

4.2.2.3. Blood pressure and heart rate circadian variation

On the 60th day, the PAG LVV-*hKir2.1* animal group showed a significant increase in systolic, diastolic and mean BP, during both light and dark phases (table 3, n=6, $p<0.05$). A decrease in HR was observed on both phases. SHR LVV-eGFP group sBP, dBP and mBP values for the light and dark

phases at 60 days were similar to the one of the PAG LVV-*hKir2.1* group, hence statistical significance was not attained (n=6, $p>0.05$).

Table 3 – Blood pressure (mmHg) and heart rate (bpm) circadian variation for PAG and sham animals, before and 60 days after microinjection. Values are expressed as mean±SEM. Abbreviations: sBP, systolic blood pressure; dBP, diastolic blood pressure; mBP, mean blood pressure; HR, heart rate; LVV-*hKir2.1*, Spontaneously hypertensive rats microinjected with LVV-*hKir2.1* into the periaqueductal gray matter; LVV-eGFP, Spontaneously hypertensive rats microinjected with LVV-eGFP into the paraventricular nucleus of the hypothalamus or rostral ventrolateral medulla.*, ** and *** correspond to significant ($p<0.05$), very significant ($p<0.009$) and extremely significant ($p<0.0005$) difference between day 0 and day 60 PAG rats.

	Light phase				Dark phase			
Group	**sBP**	**dBP**	**mBP**	**HR**	**sBP**	**dBP**	**mBP**	**HR**
Basal conditions								
LVV-*hKir2.1*	149±2	126±3	134±3	309±6	156±3	133±5	141±4	343±8
LVV-eGFP	157±5	130±5	139±5	289±7	160±5	133±6	142±6	316±8
60 days after microinjection								
LVV-*hKir2.1*	164±2***	140±4*	148±3**	284±4***	172±3**	148±7	156±5*	322±5**
LVV-eGFP	170±12	141±12	151±11	268±4	175±11	147±11	156±11	311±3

4.2.2.4. Indirect quantification of vagal tonus

Respiratory sinus arrhythmia, an index of vagal tonus, was not changed by LVV-*hKir2.1* microinjection in the PAG (1.07±0.01 *vs* 1.24±0.1, $p>0.05$). In addition, SHR LVV-eGFP group vagal tonus remained the same (1.05±0.01 to 1.05±0.01 $p>0.05$). Overall, vagal tonus was not affected by the decrease in excitability of PAG.

4.2.2.5. Cardiorespiratory evaluation

Baroreflex gain relates the variations of heart rate with the consequent variations in mean blood pressure. Its calculation is a way of indirectly evaluating the baroreceptor reflex behavior. In the present experiments, a significant fall in the baroreflex gain of the PAG LVV-*hKir2.1* group was observed when compared to the SHR LVV-eGFP one (0.24±0.03 *vs* 0.51±0.05 bpm/mmHg; $p<0.05$, n=6). Contrarily, chemoreflex variation did not differ significantly between both groups (Δ22±5.6 *vs* Δ32.6±5.1 cpm; $p>0.05$, n=6).

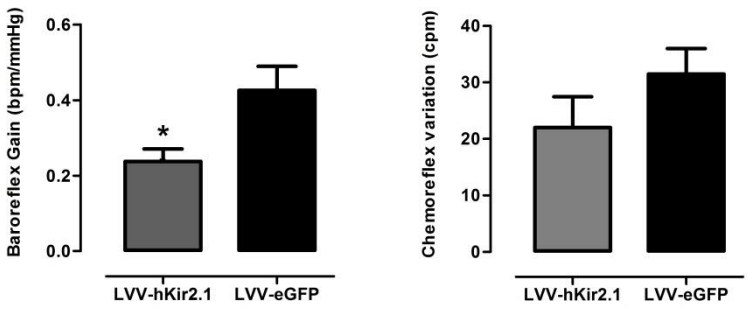

Figure 4.11 - LVV-*hKir2.1* microinjection into PAG effect on baroreflex gain and chemoreflex variation, 60 days after microinjection. Lentiviral administration further impaired baroreflex sensitivity. * $p<0.05$, statistically significant difference between PAG LVV-*hKir2.1* and SHR LVV-eGFP groups.

4.2.3. KOLLIKER-FUSE NUCLEUS

The Kolliker-Fuse nucleus, located in the pons, receives respiratory projections from the NTS and itself sends afferents to the intermediolateral cell column in the spinal cord. Being deeply involved in pontine respiratory control, KF was used as a control area in the present study.

4.2.3.1. Microinjection influence on 24h mean values of blood pressure and heart rate

KF LVV-*hKir2.1* group basal blood pressure values were 150±4 mmHg for sBP, 129±4 mmHg for dBP and 136±3 mmHg for mBP. No significant changes were found between KF LVV-*hKir2.1* and SHR LVV-eGFP group (158±5, 133±6 and 141±6 mmHg, for LVV-eGFP group; $p>0.05$, n=6, figure 4.12) at the beginning of the protocol.

60 days after lentiviral microinjection, KF LVV-*hKir2.1* sBP, dBP and mBP were 164±6, 140±5 and 148±5 mmHg, showing that LVV-*hKir2.1* microinjection did not elicit any change in blood pressure values of SHR KF ($p>0.05$). Heart rate decreased 60 days post microinjection (322±16 *vs* 301±7 bpm, $p>0.05$).

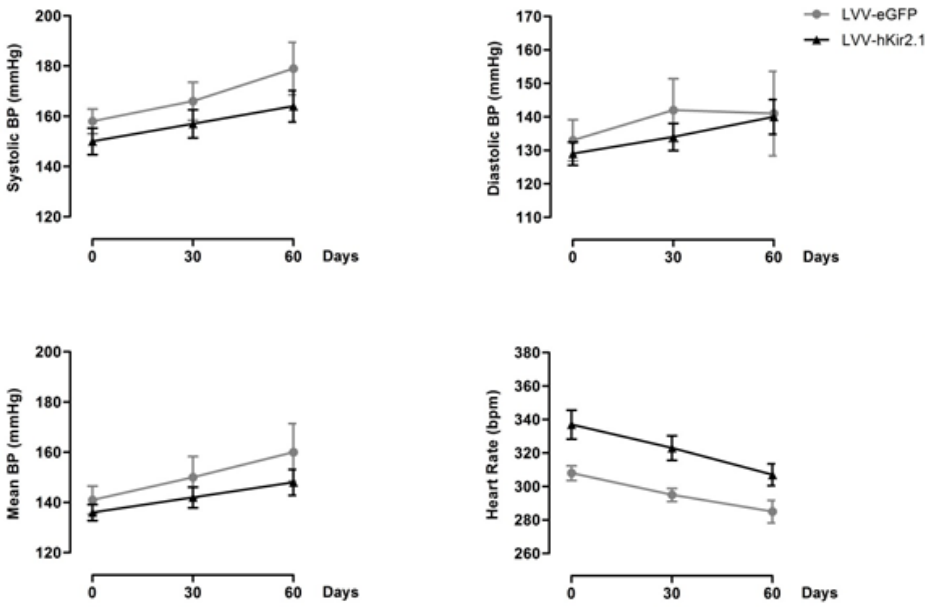

Figure 4.12 - Effect of lentiviral microinjection of LVV-*hKir2.1* in the KF on systolic, diastolic and mean blood pressure and heart rate, for a 60 day period. LVV-*hKir2.1* administration did not reflect itself on blood pressure values. No significant changes were found.

4.2.3.2. Effect of LVV-*hKir2*.1 microinjection on sympathetic output

Rats' microinjected with LVV-*hKir2.1* into the KF showed a notable decreased in sympathetic output expressed by LF band power and measured indirectly through Fast Fourier Transform (KF LVV-*hKir2.1* day 0: 0.84±0.34 $mmHg^2ms^{-2}$ *vs* day 60: 0.46±0.18 $mmHg^2ms^{-2}$ and SHR LVV-eGFP day 0: 0.86±0.14 $mmHg^2ms^{-2}$ *vs* day 60: 0.71±0.16 $mmHg^2ms^{-2}$, n=6, $p<0.05$ for comparisons between groups). Owed to the previous, cardiovascular autonomic outflow also decreased 60 days post microinjection when values were compared within the same group (KF LVV-*hKir2.1* day 0: 0.16±0.1 $mmHg^2ms^{-2}$ and day 60: 0.02±0.01 $mmHg^2ms^{-2}$, n=6, $p<0.05$). Moreover, comparison between day 60 of KF LVV-*hKir2.1* and SHR LVV-eGFP group revealed a significant decrease between groups (SHR LVV-eGFP day 0: 0.06±0.02 $mmHg^2ms^{-2}$ and day 60: 0.06±0.01 $mmHg^2ms^{-2}$, n=6, $p<0.05$).

4.2.3.3. Blood pressure and heart rate circadian variation

As anticipated, lower BP values were observed during the light phase relative to the dark one. HR was higher on the dark phase. 60 days after microinjection into the KF, LVV-*hKir2.1* had no effect on systolic, diastolic and mean BP, therefore all BP values increased on both light and dark phases (light phase LVV-*hKir2.1* day 0: sBP_148±2, dBP_127±3, mBP_134±3 mmHg *vs* LVV-*hKir2.1* day 60: sBP_161±6, dBP_138±5, mBP_146±5 mmHg; dark phase LVV-*hKir2.1* day 0: sBP_152±5, dBP_131±4, mBP_138±4 mmHg *vs* LVV-*hKir2.1* day 60: sBP_166±7, dBP_142±6, mBP_150±6 mmHg; n=6, $p<0.05$). SHR LVV-eGFP values for systolic, diastolic and mean BP showed a similar behavior as those of KF LVV-*hKir2.1* (table 4, n=6, $p>0.05$).

Table 4 – Blood pressure (mmHg) and heart rate (bpm) circadian variation for KF and sham animals, before and 60 days after microinjection. Values are expressed as mean±SEM. Abbreviations: sBP, systolic blood pressure; dBP, diastolic blood pressure; mBP, mean blood pressure; HR, heart rate; LVV-*hKir2.1*, Spontaneously

hypertensive rats microinjected with LVV-*hKir2.1* into the Kolliker-Fuse nucleus; LVV-eGFP, Spontaneously hypertensive rats microinjected with LVV-eGFP into the paraventricular nucleus of the hypothalamus or rostral ventrolateral medulla. * Statistical significance for the comparison between KF day 0 and day 60.

	Light phase				Dark phase			
Group	**sBP**	**dBP**	**mBP**	**HR**	**sBP**	**dBP**	**mBP**	**HR**
Basal conditions								
LVV-*hKir2.1*	148±2	127±3	134±3	322±9	152±5	131±4	138±4	353±8
LVV-eGFP	157±5	131±6	140±5	295±5	160±5	134±6	143±6	321±6
60 days after microninjection								
LVV-*hKir2.1*	161±6*	138±5*	146±5*	294±14*	166±7*	142±6*	150±6*	327±7*
LVV-eGFP	177±10	148±12	158±11	266±5	181±11	153±13	163±12	305±8

4.2.3.4. Indirect assessment of vagal tonus

Indirect assessement of parasympathetic tonus, via respiratory sinus arrhythmia quantification, revealed that lentiviral microinjection into the KF did not elicited any modification on animals vagal tonus (1.12±0.01 *vs* 1.18±0.4, $p>0.05$). SHR LVV-eGFP rats parasympathetic outflow was also unchanged (1.06±0.01 to 1.04±0.01 $p>0.05$).

4.2.3.5. Cardiorespiratory reflex assessment

Baroreflex activation resulted in a progressive rise in mean BP accompanied by a progressive fall in HR. KF and SHR LVV-eGFP baroreflex gain (BRG) did not differ significantly between groups (0.35±0.08 *vs* 0.49±0.06 bpm/mmHg; $p>0.05$), although there seemed to be a tendency towards BRG reduction in the KF LVV-*hKir2.1* group.

On the other hand, KF animals exhibited a significant decrease in chemoreflex variation when compared to SHR LVV-eGFP ones (Δ17.8±1.2 *vs* Δ36.1±4.9 cpm; n=6, $p<0.01$, figure 4.13). This was an expected behavior due to the functional nature of KF in the pontine control of respiration.

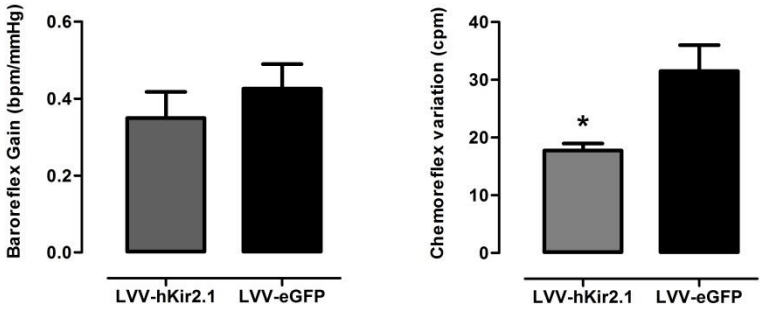

Figure 4.13 - LVV-*hKir2.1* microinjection into KF effect on baroreflex gain and chemoreflex variation, 60 days after microinjection. Lentiviral administration generated a decrease in chemoreflex variation of KF animals, compared to LVV-eGFP ones. *$p<0.01$, value with significant stastitical difference between experimental groups.

5. DISCUSSION

5.1. On the role of the decreased RVLM excitability on the autonomic–somatic relationships that condition behavioral responses on conscious normotensive animals

Data presented in this work show, for the first time, that the modulation of blood pressure values through interventions in the sympathoexcitation, which is a common characteristic in several pathologies, seems to depend on the amplitude of the global sympathetic tone together with the integrated integrity of the central autonomic network. In fact, the decrease of neuronal excitability provoked by the overexpression of *hKir2.1* in RVLM cells of normotensive animals despite evoking a decrease in sympathetic activity, evaluated indirectly by the power of LF band, was unable to evoke significant changes on blood pressure and heart rate. Moreover, the performed cardiorespiratory reflex evaluation, behavior and circadian variations of blood pressure were also not affected by the decrease of sympathetic activity. These results further emphasize the importance of the work of Geraldes and co-workers [70] who recently showed the long-term remodeling of cardiovascular variables and function upon genetic modification of RVLM cells excitability in an animal model of hypertension. In this case, the decrease of sympathetic activity was accompanied by a persistent decrease in blood pressure and heart rate values.

The rostral ventrolateral medulla is a brain center responsible for the generation of sympathetic tonic activity and, thus, for the management of cardiovascular functions and information processing from peripheral nerves plus other brain nuclei. [27] Specific RVLM neurons are essential in mediating reflex inhibition or sympathetic activation to the heart and blood vessels. [47; 144; 167] As an example, experiments have revealed that RVLM electrical or chemical stimulation causes an increase in BP and HR, whereas RVLM inhibition in conscious rats elicits a chronic fall in BP, HR and SNS activity. [47; 120] Selective destruction of more than 80% of C1 neurons, promoted a ≈10 mmHg reduction in BP, attenuated the sympathetic tonus involved in baroreflex and had little effect on parasympathetic tonus. [82; 114] Acute bilateral inactivation or ablation of RVLM neurons in anaesthetized animals triggered a fall in BP and sympathetic activity. Hence, the RVLM significantly contributes to the generation of sympathetic vasomotor tone. [116]

In the present work, however, RVLM activity was not provoked by an acute stimulus of electrical or pharmacological nature but instead, we applied a lentiviral vector with codification for K^+ channels. In this way, and in opposition to other studies, our stimulation type was more specific and physiological as we were able to keep under physiological conditions the cellular environment in the RVLM and did not directly disturb cells from neighboring areas, which activation could also have impact in our results. Likewise, in addition to the manipulation of each cell electrophysiological properties and due to the genetic nature of the intervention, we were able to have a persistent genetic modification of excitability which led to a functional remodeling.

The combination of data from our work with the results of Geraldes *et al*. [70] paper suggests that, despite the decrease in cell excitability in a key area controlling the cardiovascular system being a common feature, it only has a functional effect in the presence of a strong sympathoexcitation; otherwise, other mechanisms putatively with origin in other sympathetic brain areas will try to overcome RVLM decrease of activity. This hypothesis goes in line with the common statement that disautonomy is only observed when the autonomic nervous system is unable to mask its own

dysfunction and with the fact that, in our study, blood pressure values rose, despite non-significantly, after the treatment with the lentiviral vector.

The PVN plus the periaqueductal gray matter (PAG) constitute the descendent anti-nociceptive system, which modulates nociceptive responses. [44; 140; 141] Research shows that RVLM-PAG mutually regulates defensive behaviors including tonic immobility in guinea pigs. [44] Furthermore, it has been suggested that specific neuronal populations within the cerebellum are responsible for mediating exploratory behavior and sensitive to brainstem inhibition. [182] Inhibition of NTS neurons restored exploration behavior in a rodent model for the autism-like behavior. [182] Behavior paradigms, such as the Open-Field Test (OFT) and the Elevated Plus-Maze (EPM) are frequently used to study the anxiety-like behavior in rats, by exploiting rodents' natural aversion to exposed fields.[28] Consequently, we also sought to evaluate if the modulation of RVLM neurons excitability could alter rodents anxiety-like behavior.

After lentiviral microinjection, all LVV rats spent most of the time in the peripheral area of the OFT apparatus (96%) and less time in the central area indicating, according to the literature, an absence of anxiety. [163] Additionally, regarding the EPM, all LVV animals spent more time in the closed arms relative to the open ones of the apparatus. This reflects their natural tendency to avoid exposed and high spaces. [100] All rats spent approximately 1/5 of the test's duration in the central zone, which was taken as the amount of time necessary for the rat to choose between open or closed arm ("decision making" time). [51; 147] The number of entries in the closed arms provides an index of general locomotion. [143] Ferguson *et al*. [65] stated that rats performance tends to increase with age, nonetheless that was not the case in our study mostly because we worked with different age groups from those of Ferguson *et al*..

5.2. On the effect on blood pressure, peripheral sympathetic tone and cardiovascular reflex responses of the reduction of excitability of midbrain and pontine areas in an animal model of essential hypertension

In the present study, we employed the same molecular tool as previously mentioned for the RVLM to address the role of the decreased excitability of LPBN, PAG and KF under hypertensive conditions. KF, located in the parabrachial area is a pontine nucleus that regulates respiration. Despite, the close neuronal and functional relation between the respiratory and cardiovascular functions, KF was used, in this study, as a control area for LPBN and PAG functional responses. Thus, was not surprising the significant variation of chemoreceptor function upon the decrease of sympathetic activity evoked by KF modulation of excitability and the absence of cardiovascular responses.

On the other hand, LPBN and PAG silencing revealed different autonomic mechanisms showing that these areas differently regulate sympathetic activity, and hence, blood pressure. In fact, the established role for these two areas shows that PAG is more involved on the relation of nociception and autonomic behaviour rather than LPBN, which is a major pontine relay for visceral autonomic information with strong neuronal links to the nucleus tractus solitarius, the hypothalamus and the RVLM. An Interesting observation was that the baroreflex gain decreased under PAG silencing mainly due to the decrease in HR which was significantly achieved. Nociceptive processing is influenced by visceral reflexes and pain evoked potentials decrease in hypertensive conditions and upon baroreceptor stimulation. [77] However, pain evaluation was not a purpose of this study but PAG role on sympathoexcitation. In fact, some studies in normotensive animals, showed that PAG stimulation produces a cardiovascular response characterized by sympathetic overactivity. [153] The cardiac baroreflex gain is markedly attenuated after electrolytic lesions in the dorsolateral PAG. [153] This suggests that PAG has a key role in baroreflex mediation and its silencing disrupts blood pressure cardiovascular reflex control.

PAG columns are capable of modulating cardiac sympathetic functions through a series of indirect pathways involving sympathetic premotor neurons found in specific sites of the hypothalamus, midbrain, pons and medulla oblongata. Additionally, PAG major outflow terminates in bulbospinal regions of the RVLM. Therefore, PAG silencing results in sympathetic activity decrease which via its projections less activates adjacent sympathetic areas. [153] Schenberg and colleagues showed that bilateral electrolytic lesion of the PAG evoked a baroreflex gain attenuation. [153] The latter is evidence for PAG's great influence on resting cardiovascular control in SHR's. In our study, PAG silencing also elicited a diminished cardiac baroreflex most probably due to a change in the relation in the autonomic-pain circuits which one of the major centres of integration is PAG as mentioned above.

LPBN decreasing of excitability evoked a sympathetic activity decline, decrease in BP without HR changes, changes in circadian BP and on carotid chemoreflex but not on baroreceptor reflex. Cardiac baroreflex gain is dependent on HR and BP and since there were no changes on HR post lentiviral microinjection, it conditioned the expected baroreflex improvement. Furthermore, previous studies showed that LPBN electrolytic lesions enhanced baroreflex-mediated cardiovascular responses, however that was not the case in the present work. [88; 151] Autonomic information can be relayed by the LPBN to other structures, crucial in autonomic function regulation. LPBN projects to RVLM and according to Kubo *et al*. [106] LPBN pressor sites neurons are involved in the mediation of cholinergic inputs responsible for RVLM pressor responses. Agreeing with these data, the decrease of blood pressure and sympathetic activity could result from the impact of LPBN on RVLM excitability and thus, indirectly conditioning the sympathetic bulbospinal flow to IML by decreasing, for instance, RVLM pacemaker neurons discharging rate. Nevertheless, other studies have shown that LPBN also projects directly, via noradrenergic neurons, to IML in the spinal cord and, thus, directly influencing the tone of sympathetic pre-motor neurons. [45; 72] Consequently, LPBN reduced activity could act on the silencing of sympathetic pre-motor neurons either by tackling RVLM and, hence, indirectly IML neurons or by a direct action on them.

Being LPBN, PAG and KF all simpathoexcitatory nuclei it was not surprising that indirect assessment of autonomic nervous system parasympathetic branch did not reveal any change on vagal tonus, which further highlights the restricted effect of LVV-*hKir2.1* on specific brain areas of interest.

6. CONCLUSIVE REMARKS

The view expressed by Cannon that the sympathetic nervous system is a functionally homogeneous system is no longer accepted. It is now clear that central circuits which control sympathetic nerve discharge are able of originating complex and highly differentiated response patterns. An example is the reflex control of blood pressure but, also, the paradigmatic alert reaction. However, the integrative role of the brain and, particularly, of specific brain areas in the control of visceral functions still remains a mystery.

Regarding the cardiovascular function, the idea of a set-point controller, or a set of them highly organized in the brain regulating arterial blood pressure is not new but remains hypothetical as the neural substrate has yet to be determined. Thus, since neurogenic hypertension is characterized by sympathetic overactivation, it seems appropriate to target central sympathetic areas belonging to the central autonomic network to define which central groups could be involved in the chronic control of blood pressure both in normal and hypertensive conditions.

In the present work, we were able to show the cardiovascular functional consequences of the persistent decreased excitability of LPBN in hypertensive conditions and RVLM in normotension. The

observed responses suggest that LPBN and RVLM have a clearly input on the sympathoexcitation observed in hypertension.

Together with the set point localization, the level of sympathoexcitation needed to disturb the control mechanisms in order to evoke effective changes on blood pressure is not yet defined. Our results from RVLM launched the hypothesis of a threshold for the impact of sympathoexcitation on peripheral variables. The future identification of that threshold will be critical to the passage of a protective sympathoexcitation for the characteristic deleterious sympathetic-excitation of most diseases which is responsible for a number of adverse reactions that facilitate new co-morbidities.

In conclusion, with the present exploratory study, we unravelled the putative contribution of midbrain and pontine central autonomic network areas for the etiology of neurogenic hypertension and provided clues to possible future therapeutic interventions to control sympathoexcitation.

7. APPENDIX

Cardiovascular function is modulated by the balance between sympathetic and parasympathetic neural activity. From the study of hemodynamics, one can be acquainted with the nature of the distress to which the cardiovascular system is exposed as well as the regulatory response to this disturbance. [4; 11] Heart rate variability, referred to as the quantity of physiological spontaneous fluctuations of the cardiac cycle that correspond to physiological sinus arrhythmias in the electrocardiogram, is a well-accepted noninvasive technique used as an index of autonomic function. [11; 87; 115]

Analysis of spontaneously occurring changes in autonomic tone, by means of spectral analysis, allows the overall variance of a biosignal to be split into its various underlying frequency components. Alterations in the neural influences responsible for cardiovascular regulation elicit the variances in the magnitudes of the different frequencies. [93; 169] However, signal stability is a very important requirement for spectral analysis, since the significance of a given frequency component can no longer be well defined once there's physiological changes, which in turn affect the signal modulation. To overcome this limitation, other methodologies of signal processing have been applied to biological signals, in particular to the cardiovascular ones. [57; 174; 175] However, a major question is not yet fully answered: the power of oscillations in frequency bands believed to completely mirror

sympathetic or vagal influence cannot yet be viewed as an absolute measure of sympathetic or parasympathetic outflow. [93]

Neurobehavioral systems at the highest levels of the neuraxis, which include the cerebral cortex, also regulate the autonomic outflow in particular when a behavioral response needs to be elicited to overcome a condition which involves a response beyond the normal homeosthatic levels. In these conditions, rostral brain systems, apart from modulating lower reflex mechanisms, also send descending projections that terminate directly on autonomic source nuclei in the brainstem and spinal cord. The medial prefrontal cortex has been recognized as an important link between anxiety and autonomic function. Selective immunotoxic lesions of basal forebrain cholinergic projections to the medial prefrontal cortex eliminate the exaggerated autonomic reactions in anxiety-related contexts. Furthermore, stress or emotional arousal increases sympathetic activity, however the latter does not change in a uniform way in all sympathetically innervated systems. The level of body awareness depends on various psychologic factors, in which anxiety plays an important role. [20; 94]

Once we modulated central autonomic network nuclei activity, by means of anxiety-like behavioral assessment we sought to find out if decreased excitability in the RVLM had an impact on rats' anxiolytic behavior responses. Thus, behavioral tests applied in this study are considered to be a second indirect autonomic evaluation tool in the conscious and free moving animal.

7.1. Anxiety-like behavioral evaluation

An extensive range of behavioral testing paradigms has been developed, in an endeavor to model human pathological anxiety in rodents. [22; 162; 178] When planning experiments to assess anxiety-like behavior, it is important to keep in mind the experimental history of test subjects, previous test exposure, differences in exploratory motivation, and if the test is to be conducted as part of a test battery or ran as a single behavioral assessment. [131; 137; 146] To control for extraneous variables,

behavioral testing should be avoided on days scheduled for home cages changing, once it can significantly increase general activity and stress levels. [177; 186]

Consistency of experience prior to the test session must be ensured, thus animals should be brought to the testing room, in their home cages, for a minimum of 1 hour prior to the start of behavioral evaluation. Moreover, behavior room lighting, temperature and noise levels should be consistent for all subjects. At the end of each test session, fecal boli and urine are removed and behavioral apparatus surface is wiped with 70% ethanol. Test chamber is allowed to dry completely before starting the following test session. [13]

The Open-Field Exploration Test and the Elevated Plus-Maze Test are regularly used to study the anxiety-like behavior in rats, by exploiting rodents' natural aversion to exposed fields.[28]

7.1.1. Open-Field Exploration Test

The open-field exploration test (OFT), first announced as a measure of emotional behavior in rats, allows to systematically assess novel environment exploration, general locomotor activity and is considered an initial screen for anxiety-like behavior in rodents. Subsequent exposure to the environment (habituation) lessens the anxiogenic response. [12; 43; 139]

The most common open-field design is a large square chamber, from 28x28 cm to 56x56 cm, though several shapes and sizes of OFT arenas for rodents have been used. Behavioral apparatus floor is divided into a grid of equally sized areas for visual scoring of activity by the experimenter. Animal is placed in the chambers' center and allowed to freely explore during the test session (typically 5 min). Completed the test, rat is returned to its home cage. [13]

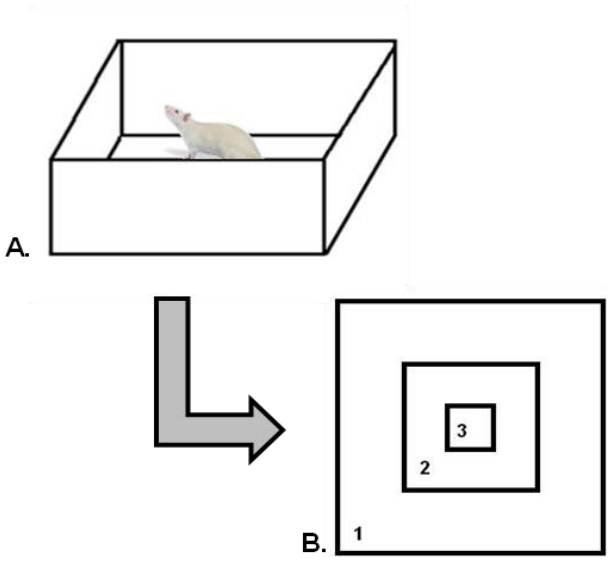

Figure 7.1 – Schematics of the Open-Field test. (A) Tridimensional and **(B)** top view of the apparatus. Three virtual areas are shown in **B**: 1, peripheral; 2, intermediate and 3, central zones. Adapted from Amaro-Leal, 2014 [10].

Movement is OFT outcome of interest and it's influenced by motor output, exploratory drive, sickness, relative time in circadian rhythm, freezing or other fear-related behavior and many other variables. [12; 43] Measures of overall physical motor abilities and interest in the novelty environment can be attained by scoring the distance travelled, the amount of time the rat spends in each of the virtual areas, the number of lines crossed (considered as units of activity), rearing behavior (when rat is on its hind legs, playing or not with his front paws on the wall), defecation and grooming activity. [89; 183] Some studies have considered the number of fecal boli as an outcome measure of anxiety, however defecation is dependent on the last time the animal ate and other confounders, making it ultimately an unreliable parameter. [183]

Rodents usually spend significantly less time exploring the unprotected central area. Conversely, animals tend to greatly explore the periphery of the OFT arena, generally in contact with the apparatus walls. Rats that exhibit more exploratory behavior in the central area are considered to demonstrate low anxiety levels. [89; 183]

7.1.2. Elevated-Plus Maze Test

The elevated-plus maze test (EPM) studies the anxiolytic response to nearly all types of anti-anxiety agents and anxiogenic drugs. [95; 179] Making use of the rats natural tendency to explore novel environments, in the EPM the animals' is given the choice of spending time in open unprotected maze arms or enclosed protected ones, all raised from the floor. Subjects performing this test must have normal ambulatory ability and average levels of exploratory drive. Circadian rhythm, stress, age, gender, strain, changes in housing conditions, prior handling and exposure to previous behavioral test paradigms influence behavioral response in the EPM. [181]

EPM apparatus consists of two sets of opposing arms (30x5 cm), two arms are enclosed with 15 cm high walls and the remaining two are open, extending from a central platform (5x5 cm). [84; 108] Each subject is placed in the mazes' central area and allowed to freely explore the apparatus for 5 min. The number of entries and the amount of time spent in each of the arms (open/closed) are scored. [13; 155]

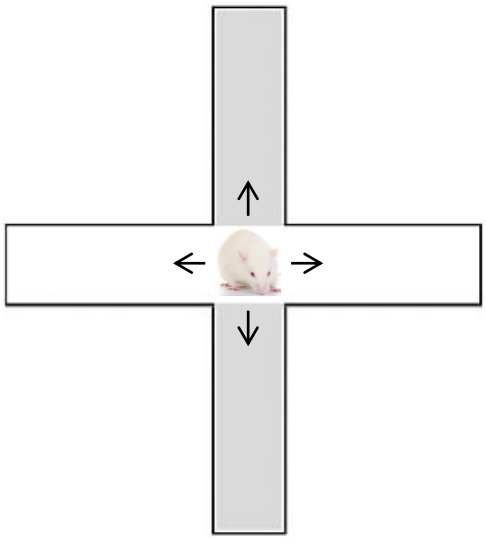

Figure 7.2 – Rodent in Elevated-Plus maze. Shaded and light areas are representative of closed and open arms, respectively. Arrows indicate all possible directions in the apparatus. Adapted from Carl E. Stafstrom, 2006. [168]

Rats favor dark and enclosed spaces and tend to avoid open areas, thus animals exposure to a novel maze alley evokes an avoidance conflict towards the open arms. [138] Consequently, lower time spent in the open arms to total time ratio expresses greater animal's anxiety. [41] Additionally, rodents display an innate fear of heights and being the EPM apparatus raised from the floor level, it adds to the animals' anxiety level. [41]

Finally, studies suggest that anxiety disorders are associated with a shift in autonomic balance towards sympathetic dominance, an over reactivity of the sympathetic system or a generalized hyperattention to environmental stimuli. Given the heterogeneity of cognitive, affective and physiological processes that likely interact in anxiety states, literature remains complex with regard to possible differences among categories of anxiety disorders and autonomic function across the ranges of these states. Further complexity arises as anxiety conditions often show substantial comorbidity with other psychological disorders. There are still reports of the enhanced autonomic reactivity to experimental stimuli in subjects with anxiety disorders, which are in line with the view that anxiety may be associated with exaggerated autonomic reactivity, however this is by no means a universal finding. [20]

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