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Effects of intracerebroventricular leptin and orexin-A on the baroreflex control of renal sympathetic nerve activity in conscious rats fed a normal or high-fat diet

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Running title: Leptin and orexin-A and baroreflex control of RSNA

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Abstract

This study examined the effect of leptin and orexin-A on autonomic baroreflex control in conscious Wistar rats exposed to high-fat (45% fat) or normal (3.4%) diet for 4 weeks. Renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP) and heart rate (HR) were monitored during the generation of baroreflex gain curves and acute volume expansion (VEP). I.C.V. leptin (1μg/min) increased RSNA in the normal diet group (0.31±0.04 vs. 0.23±0.03mV/s) and MAP in the high-fat diet group (115±5 vs. 105±5mmHg, $P<0.05$). Orexin-A (50ng/min) increased RSNA, HR and MAP in the high-fat diet group (0.26±0.03 vs. 0.22±0.02mV/s, 454±8 vs. 417±12beats/min, 117±1 vs. 108±1mmHg) and the normal diet group (0.18±0.05 vs. 0.17±0.05mV/s, 465±10 vs. 426±6beats/min, 116±2 vs. 104±3mmHg). Baroreflex sensitivity for RSNA was increased during I.C.V. leptin by 50% in the normal diet group, compared to 14% in the high-fat diet group ($P<0.05$). Similarly, orexin-A increased baroreflex sensitivity by 56% and 50% in the high-fat and normal diet groups, respectively (all $P<0.05$). During I.C.V. saline, VEP decreased RSNA by 31±5% ($P<0.05$) after 10min and the magnitude of this response was blunted during I.C.V. infusion of leptin (17±2%, $P<0.05$) but not orexin-A in the normal diet group. RSNA response to VEP was not changed during I.C.V. leptin or orexin-A in the high-fat diet group. These findings indicate possible central roles for leptin and orexin-A in modulating the baroreflexes under normal or increased fat intake in conscious rats and potential therapeutic approaches for obesity associated hypertension.

Key words: Leptin, orexin-A, baroreflex, volume expansion, hypertension, high-fat diet.

Introduction

Obesity is a pathophysiological state which is reaching epidemic proportions in many developed nations. It is frequently associated with hypertension, insulin resistance, hyperinsulinaemia and hyperlipidaemia. [1] Obese individuals are not only at increased risk of cardiovascular accidents such as myocardial infarcts and strokes, but the dyslipidaemia and hypertension also initiates kidney damage, which eventually leads to end-stage renal failure. [2] It was previously suggested that there is an activation of the sympathetic nervous system in hypertensive obese patients that is associated with higher plasma levels of catecholamines. [3]

The regulation of energy homeostasis is a complex process which involves the interaction of a number of neuropeptides such as leptin and orexin-A at different areas of the brain, most importantly the lateral hypothalamic area (LHA) and paraventricular nucleus (PVN). However, the neural mechanisms involving these neuropeptides also has a major impact on the autonomic control of a number of organs, including the kidney. [4] Leptin, for example, plays a key role in central nervous system-mediated control of food intake and energy homeostasis. [5] It causes weight loss primarily by reduction of adipose tissue and sparing of lean body mass. In addition, leptin causes a centrally-mediated and long lasting increase in sympathetic nerve activity to brown adipose tissue, the adrenal gland, lumbar musculature, and also to the kidney. [6,7] Indeed, the leptin receptor is highly expressed in the PVN, LHA, arcuate, dorsomedial, and venterolateral nuclei. [8,9] It is particularly significant that firing of cells within the PVN is increased by leptin in vivo. [10] A significant question arising from these reports and yet to be addressed is whether leptin can modify or modulate the normal baroreceptor reflex mechanism in response to challenges to the cardiovascular system.

Orexins, or hypocretins, in contrast to leptin, play an important role in energy homeostasis by stimulating food intake and promoting appetite. [11] They are abundantly and specifically expressed in neurons which originate in the LHA and project not only into the cerebral cortex and thalamus, but also into the brainstem. Earlier studies showed that mRNA for the two orexin receptors, orexin-1 and orexin-2, are widely distributed in the brain, specifically in the hypothalamus and the PVN. [4] In relation to this, orexins cause a dose-dependent and centrally mediated increase in blood pressure, heart rate and renal sympathetic nerve activity. [12-14]

Interestingly, it was reported that orexin overexpression and/or upregulation of orexin receptor 2 promoted resistance to diet- induced obesity and the central activation of these receptors inhibited metabolic changes in obesity. Therefore, in this study, it was hypothesised that central leptin blunts, while orexin-A enhances, the RSNA and HR baroreflex sensitivity and RSNA response to volume expansion (VEP) during normal fat intake and are exaggerated in a high-fat diet state. This was tested by evaluating the high- and low-pressure baroreflex regulation of RSNA before and following intracerebroventricular (I.C.V.) infusion of either leptin or orexin-A and measuring RSNA to the kidney in conscious freely moving rats fed either a normal diet or a diet with a high fat content.

Results

Body weight

At onset of the acute study, after 4 weeks of feeding regimens, there was a significant increase in body weight in the high-fat fed group compared to the normal diet group (300±14 vs. 260±10g). Although the baseline and follow-up body weight was not recorded in this study, a similar dietary protocol using the same diet and supplier was utilised in a previous study from this group and resulted in a significant increase of baseline body weight in high-fat diet group by almost 46% compared to their normal diet counterparts. [15]

High pressure baroreflex study

Baseline values of cardiovascular variables in experimental groups

Table 1 shows the baseline values of mean arterial pressure (MAP), heart rate (HR) and RSNA recorded during the acute study in normal and high-fat diet fed rats before and immediately after an I.C.V. injection of saline, leptin or orexin-A. There were no changes in MAP, HR or RSNA before or after I.C.V. injection of saline. However, leptin I.C.V. caused a significant increase in MAP in both normal and high-fat diet fed rats. There was also a modest increase in RSNA in both diet groups but did not reach statistical significance (P=0.05). Orexin-A I.C.V., on the other hand, caused a significant increase in MAP, HR and RSNA irrespective of the dietary state of the rats.

1. High pressure baroreflex control of RSNA

Figure 1 illustrates HR, MAP, and integrated RSNA signals from an individual normal diet fed rat subjected to the baroreflex gain curve protocol. The I.V. injection of bolus doses of phenylephrine or sodium nitroprusside caused a transient increase or decrease in

MAP, respectively, together with decreases and increases in both HR and RSNA, respectively.

The effect of I.C.V. saline on RSNA baroreflex gain curves

Figure 2 illustrates the effect of I.C.V. saline on the baroreflex gain curves generated in normal diet fed rats. The I.C.V. saline infusion (Figure 2a,b) had no significant effect on the maximum gain (after vs. before, 5.37±0.72 vs. 4.81±0.53), range of the curve (*A*1), the gain coefficient (*A*2), the range mid-point blood pressure (*A*3) or the minimum asymptote of the curve (*A*4). However, there was a small but significant decrease in the saturation point of the baroreflex gain curve (Table 2).

The effect of I.C.V. leptin on RSNA baroreflex gain curves

Figure 3 illustrates the averaged baroreflex function curves (Figure 3a,c) and the sensitivity of the relationship between RSNA and MAP, as expressed by the maximal absolute gain (Figure 3b,d), in both normal and high-fat diet fed rat groups. In the normal or high-fat diet groups (Figure 3a,c), the range of the curve (*A*1) was not changed significantly after I.C.V. leptin, but the gain coefficient of the baroreflex curve (*A*2) was significantly greater ($P<0.05$) by 50% in the normal diet group and by 14% in the high-fat diet group (Table 2). There was also a significant increase ($P<0.05$) in the range mid-point blood pressures (*A*3) in the normal diet and high-fat diet groups. The minimum asymptote of the RSNA curves (*A*4) were not different before and after I.C.V. leptin infusion in both diet groups. The threshold of the baroreflex curve was significantly increased in normal and high-fat diet groups, by 11% and 15%, respectively, after I.C.V. leptin (Table 2). Finally, the maximal absolute gain was similar before and after leptin I.C.V. infusion in both diet groups (Figure 3b).

The effect of orexin-A on RSNA baroreflex gain curves

The effect of I.C.V. orexin-A infusion on the baroreflex gain curves generated in the normal and high-fat diet fed groups is shown in Figure 3. In the normal as well as in the high-fat diet groups, the I.C.V. orexin-A infusion (Figure 3c,d) had no significant effect on the range of the curve (*A*1) or the minimum asymptote of the curve (*A*4). However, the gain coefficient (*A*2) was significantly increased ($P<0.05$) by 56% and 50%, respectively, following I.C.V. orexin-A administration in both normal and high-fat diet groups (Table 2). Likewise, the range mid-point blood pressure (*A3*) and the threshold of the baroreflex curve were significantly increased ($P<0.05$) after I.C.V. orexin-A but with no meaningful changes in the saturation point. The maximum absolute gain (Figure 3d) was greater in normal (8.52±1.86 vs. 6.15±1.67%/mmHg) and high-fat diet (11.61±2.76 vs. 7.72±1.60%/mmHg) groups by some 39% and 50%, respectively, following I.C.V. orexin-A administration.

2- High pressure baroreflex control of heart rate

Following orexin-A I.C.V., the response range (*A1*) of the HR baroreflex was significantly higher in the high-fat diet rats (Diet $P<0.05$) compared to normal diet rats. However, the other baroreflex curve parameters of HR were similar between the two diet groups. Meanwhile, the I.C.V. infusion of leptin increased the minimum asymptote of the RSNA curves (*A*4) significantly in both diet groups (Table 3).

Low pressure baroreflex study

The effect of I.C.V. saline on the sympatho-inhibitory response to volume expansion (VEP)

The effect of I.C.V. saline in normal diet rats on the RSNA response to VEP is presented in Figure 4. There was a significant ($P<0.05$) reduction in RSNA after 10 min VEP of some 30% which was not changed after I.C.V. saline administration.

The effect of I.C.V. leptin on the sympatho-inhibitory response to VEP

The time course for the decrease in the RSNA due to VEP over time in the presence of I.C.V. leptin or orexin-A in the normal and high-fat diet fed rat groups is shown in Figure 5. There was a significant ($P<0.05$) decrease in RSNA at the end of 10 min of VEP by 31% in the normal diet group (Figure 5a). I.C.V. leptin infusion blunted the RSNA response to VEP by some 48% ($P<0.05$) compared to RSNA response before I.C.V. leptin (after vs. before, 85±2 vs. 69±2%). On another hand, the high-fat diet group (Figure 5a) showed a decrease in RSNA by 19% from baseline. The magnitude of the RSNA response to VEP in the high-fat diet group was smaller compared to normal diet group by some 39% but this effect did not reach statistical significance (Diet $P=0.07$). I.C.V. leptin administration in the high-fat diet group had no effect on the magnitude of the decrease in RSNA in response to VEP (after vs. before, 84±2 vs. 81±3%).

The effect of I.C.V. orexin-A on the sympatho-inhibitory response to VEP

There was a significant decrease in RSNA by almost 31% in response to VEP after 10 min of VEP in the normal diet group (Figure 5b). I.C.V. orexin-A administration had no significant effect on the magnitude of the decrease in RSNA due to VEP. Similarly, the decrease in RSNA in response to VEP (27%, $P<0.05$) in the high-fat diet group was not changed after I.C.V. orexin-A.

Discussion

The main new finding of the present study was that central leptin administration blunted the sympatho-inhibitory response to VEP for rats in the normal diet state but it had no effect for rats in a high-fat diet state. Moreover, I.C.V. orexin-A enhanced the high-pressure baroreflex sensitivity under both normal and high-fat diet fed states. These findings indicate a possible central role for leptin and orexin-A systems in modulating the baroreflex mechanism under normal or increased fat intake in conscious rats. The RSNA response to VEP after I.C.V. leptin was blunted in the normal diet group to a magnitude comparable to that seen in the high-fat diet group. These findings point to a possible involvement of leptin in the impaired baroreflex mechanism to high fat diet as seen before in obese rats [16] and humans. [17]

Leptin has a number of actions within the body to control energy status and nutritional homeostasis. [18] Moreover, previous studies have pointed to the effects of central leptin in increasing sympathetic activity and blood pressure in mice, rats and rabbits after I.C.V. administration. Dunbar et al. [19] showed an increase in blood pressure by almost 15% within 45 minutes of I.C.V. leptin infusion in anaesthetised rats. Furthermore, I.C.V. leptin in mice caused a significant increase in RSNA for up to 150% of its basal level. [20] Similarly, an increase in RSNA, HR and blood pressure was seen following I.C.V. infusion of leptin in conscious rabbits. [21,22] The present study showed an increase in the baseline levels of arterial blood pressure following I.C.V. leptin administration. The sympatho-excitation due to leptin is hypothesized to be mediated via melanocortin receptors as blockade of these receptors inhibited this effect. [2,7] Furthermore, an aldosterone-dependent hypertensive state was also proposed in leptin-induced hypertension in female mice. [23] Leptin also caused increased vascular sensitization to angiotensin II via a central mechanism which involves upregulation of inflammatory cytokines. [24] Therefore, the reported effects of leptin on blood pressure are

mediated by a central mechanism that involves hyperactivation of both sympathetic nervous and renin-angiotensin-aldosterone systems (RAAS).

The I.C.V. leptin administration in the present study blunted the renal sympatho-inhibitory response to VEP. Indeed, leptin through its central effect on the nucleus tractus solitarius (NTS) was found to impair other sympatho-inhibitory responses, such as the cardiovagal baroreflex. [25] This blunting effect of leptin on the baroreflex could partly explain the sympatho-excitation seen in obesity. [26] Leptin was also shown to play an important role in the renal excretion mechanism of sodium through a dose dependent mechanism. [27] In general, circulating leptin promotes sodium excretion under normal physiologic levels and is therefore an essential factor in extracellular fluid volume and blood pressure regulation. [28] However, the impairment of these effects by leptin in obesity, as well as its stimulatory effects on the sympathetic nervous system and RAAS results not only in sodium retention, but also cardiac remodelling. [29] The effect of leptin on the normal baroreflex mechanism, as demonstrated by impaired low-pressure baroreflex in this study, can also contribute to increased blood pressure in obesity by enhancing the responsiveness of the kidney to sympathetic activity, leading to sodium and water retention. [30] However, in this study, we did not investigate renal function changes in response to ICV administration of leptin and orexin-A. These responses to a defective leptin system in high-fat diet might explain the impaired sympatho-inhibitory response to VEP in other obese rat models. [15,16]

The effect of leptin on the baroreflex mechanism could explain the effects of leptin on arterial blood pressure. The present study showed a significant enhancement in the baroreflex sensitivity represented by the gain coefficient (*A2*) of the MAP vs. RSNA relationship in normal and high-fat diet rats following I.C.V. leptin. However, the magnitude of this increase

in baroreflex sensitivity was smaller in the in high-fat diet group (14%) than in the normal diet group (50%). This result supports previous reports that have linked leptin to an altered baroreflex control mechanism in the obese state. [31,32] A blunted baroreflex sensitivity was reported in leptin receptor-deficient, diabetic, obese mice, indicating that leptin enhances the baroreflex sensitivity. [5,33] In contrast, Arnold et al., [25] showed that central leptin administration impaired the baroreflex sensitivity for initiation of bradycardia in response to increases in blood pressure. Hausberg et al., [34] nonetheless, reported no changes in the baroreflex sensitivity during I.C.V. leptin administration in anesthetized male Sprague-Dawley rats, although RSNA was elevated in their study. Therefore, data regarding the effects of leptin on the autonomic control of sympathetic activity and blood pressure are conflicting possibly as a result of experimental design and depth and type of anaesthetic used. Our data point to a baroreflex sensitivity enhancing effect of leptin as demonstrated by increased gain coefficient (*A2*) of the baroreflex curve during I.C.V. leptin administration in both normal and high-fat diet rats. However, the magnitude of this enhancement is less in the high-fat group, either due to already increased endogenous basal leptin levels as shown before, [35-38] or due to increased baseline baroreflex sensitivity. Further studies utilising temporal changes in leptin levels in the high-fat diet model and its effect on the baroreflex, possibly dose-dependent, is therefore warranted.

Similar to leptin, neurons containing orexin-A are linked to the cardiovascular regulatory network that involves the hypothalamus and areas in the brain stem, such as the NTS, that are essential for maintaining blood pressure homeostasis. [39-41] This study showed a significant increase in MAP, HR and RSNA following I.C.V. orexin-A in normal and high-fat diet rats. Indeed, central administration of orexin-A in conscious rats in a previous study was associated with increased blood pressure and RSNA. [4] More importantly, microinjection

of orexin into the rostral ventrolateral medulla (RVLM) in conscious rats resulted in increased blood pressure and heart rate. [42] Similarly, in chloralose:urethane anesthetised rats, orexin-A increased blood pressure and sympathetic activity when injected into the RVLM. This was further evident in studies using orexin blockers which showed a decrease in blood pressure in spontaneous hypertensive rats and pointed to orexin as an important treatment target in hypertension. [43]

With regard to the baroreflex mechanism, central orexin-A administration has been shown in previous studies to enhance the high pressure baroreflex control of splanchnic sympathetic nerve activity in anesthetised rats. [44,45] I.C.V. orexin-A in the present study enhanced the baroreflex sensitivity in normal and high-fat fed diet. These studies collectively show that central orexin-A plays a key role in the baroreflex control mechanism under basal conditions by sensitizing baroreflex related neural pathways. However, this study is one of the first to show the effects of central orexin-A specific to the baroreflex mechanism itself in high-fat diet conscious rats. In a previous study in obese Zucker rats, microinjection of orexin-A into the paraventricular nucleus (PVN) resulted in increased blood pressure and RSNA.[46] The sympatho-excitatory response to central administration of orexin-A in the mentioned study was related to upregulation of orexin-A type 1 receptors in the PVN in the obese state. Based on these findings, the data of the present study suggest that an enhanced baroreflex sensitivity due to orexin-A provides an important functional mechanism for increased blood pressure and RSNA in the obese state, that is possibly due to increased sensitivity to orexin-A in brain region where orexin-A receptors are expressed, namely the PVN and RVLM.

The I.C.V. injection of orexin-A in the present study was not associated with meaningful changes in the sympatho-inhibitory response to VEP either in normal or high-fat diet fed groups. This indicated that the role played by orexin-A in modifying the sympathetic outflow is manifested during acute blood pressure changes more than cardiopulmonary plasma volume changes. This suggestion is in line with the notion that the cardiovascular effects of central orexin are both dose-dependent and site-specific. [47]

This study has some limitations. The effect of leptin and orexin-A on the baroreflex mechanism was examined in a short term high-fat diet model and data might not reflect an obese state. In this regard, the present study did not report data regarding adiposity index, temporal changes in body weight or serum levels of leptin. However, a previous study from this group utilising the same dietary protocol and diet supplier to the present study presented this model as an obese rat model with a significant increase in body weight at the end of the 4 weeks study period. [15] Moreover, a previous study utilised a comparable high-fat diet (46.1% fat by calories) and showed a significantly increased visceral adipose tissues weight and serum leptin levels in high-fat compared to normal diet group. [38]

In conclusion, central leptin and orexin-A are both involved in the normal baroreflex mechanism under basal conditions. However, during obesity, the role of these neuromodulators become subtly altered possibly due to a change in receptors population in the brain regions involved in mediating these responses. The change in the central levels or receptor response to leptin during obesity might have a significant impact on the normal baroreflex control of blood pressure. Similarly, the enhanced high-pressure baroreflex sensitivity due to orexin-A in the high-fat diet state points to an important treatment target in hypertensive obese patients.

Materials and methods

Male Wistar rats (7 weeks of age) were purchased from animal supplier (Harlan, UK). They were maintained in the Biological Services Units at University College Cork for at least one week before going into a 4-week dietary regimen. The rats received either regular laboratory diet (3.4% fat) or a high-fat diet (45% saturated fat, SDS, Essex, UK) for 4 weeks with *ad libitum* access to tap water. This high-fat diet regimen has been utilised before by this group and others and is associated with increased body weight, fat and protein. [15,48,49] All procedures were performed in accordance with national guidelines and the European Community Directive 86/609/EC and approved by the University College Cork Local Animal Experimentation Ethics Committee.

Implantation procedures

The implantation of I.C.V. cannula and all other techniques and approaches in this study were according to previous reports from this lab. [50,51] Rats were anaesthetised with sodium pentobarbital (60 mg/kg, I.P.), supplemented if necessary with maintenance doses based on pedal withdrawal reflex. All procedures were done under aseptic conditions and using sterilised surgical tools and instruments. The right femoral artery was exposed using a small incision in the groin region and cannulated using 0.58 mm internal diameter cannula for recording mean arterial blood pressure (MAP) and heart rate (HR). The jugular vein was also exposed and cannulated using the same cannula as the one used for the femoral artery. The cannulae from the femoral artery and jugular vein were tunnelled subcutaneously to exit between the ears. The rat head was mounted on a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA) and the skull was exposed. A hole was drilled in the skull for unilateral placement of a stainless-steel guide cannula into the right cerebral ventricle (Coordinates: 1.0

mm posterior to bregma, 2.5 mm lateral to the midline, and 2.55 mm ventral to the surface of the dura) as previously described. [52] The correct positioning of the guide cannula into the lateral ventricle was confirmed using Evans blue dye at the end of the study. [53]

The left kidney was exposed through a flank incision and the renal sympathetic nerves adjacent to the renal artery were isolated, placed on bipolar recording electrodes and sealed into place with a silicone adhesive (Kwik-Cast & Kwik-Sil, World Precision Instruments, Hertfordshire, UK). The recording electrodes were taken subcutaneously to exit between the ears. Rats then received buprenorphine hydrochloride (3 μg per 100 g body weight, S.C.) to provide analgesia before being taken to their home cages. The rats were monitored for any adverse effects in their cages until they gained consciousness. A tether connected to a swivel system was attached to the cannulae and recording electrodes. This system allowed continuous recording of MAP, HR and renal sympathetic nerve activity (RSNA) with minimal disruption to the animal. The patency of the venous and arterial cannulae were maintained by flushing them every day with heparin (100 and 300 i.u./ml, respectively) in sterile saline solution. The animals were allowed at least 3 days to recover before acute experiments were commenced.

Blood pressure was measured using a fluid filled pressure transducer (Spectromed, Oxnard, CA, USA) attached to an amplifier. Raw RSNA signal was amplified with a gain of 100000 and high- and low-pass filters were set at 0.2 and 2 kHz. Both pulsatile blood pressure and RSNA signals were displayed on an oscilloscope and digitized with a sampling frequency of 1 kHz. All data were stored on a computer for further off-line analysis using LabVIEW software (National Instruments, Austin, TX, USA).

Baroreflex and Volume Expansion Tests

Baroreflex function curves of RSNA and HR were determined during the infusion of phenylephrine (50 μg/ml) to increase blood pressure and sodium nitroprusside (50 μg/ml) to decrease blood pressure. Infusion was done manually to deliver 0.2 ml over 40 s with an approximate change in blood pressure by 50 mmHg. The RSNA value corresponding to the maximum increase in blood pressure during phenylephrine infusion was used as the minimum RSNA value and was subtracted from all readings taken that day before (baseline) and during the baroreflex function curves. The baseline RSNA value (3.5 min recording before any intervention) was taken as 100% and all values were calculated as percentages of this value. [51,54,55] This ameliorated any differences in day to day absolute values within each group of rats due to variance of multifibre recordings and surgical skills. The baroreflex gain curves of RSNA and HR were generated using a four-parameter logistic equation. [56] The average values of RSNA or HR were calculated for each 2-mmHg change in blood pressure using the 1-s values stored on a computer. This allowed calculation of the range (A_1) over which the baroreceptors operate, the sensitivity or curvature coefficient of the relationship (A_2), the mid-point mean blood pressure of the curve (A_3) and the lowest point to which the RSNA or HR could be driven (A_4).

Saline volume expansions (9 ml/kg) equivalent to 15% of estimated plasma volume (60 ml/kg) [57] were infused over 10 minutes in normal and high-fat diet fed rats, and the respective responses of RSNA were assessed. Initial tests demonstrated that the animals showed no signs of alerting or discomfort in response to this magnitude and rate of infusion. MAP, HR and RSNA were measured immediately before, during, 10 min and 30 min after volume expansion.

Experimental protocol

The experiments took place 1 hour after the rats had been connected to the transducers and nerve amplifiers and when they were in a quiet resting state.

The protocol was carried out on 35 rats which were divided into the following groups:

Group I (n=7): This is a time control group, in which Wistar rats received a normal laboratory diet and an I.C.V. infusion of saline at 60 μl/h (normal CSF formation was estimated to be 1 μl/min). [58] The baroreflex test (n=6) and VEP (n=6) were performed in the resting state before I.C.V. saline administration and also three hours later.

Group II (n=7): In this group, normal Wistar rats received a normal laboratory diet. On the day of experiment, a baroreflex test (n=7) and VE (n=6) were performed in the resting state and at least one hour later, the animals were given an I.C.V. bolus dose of 5 μg leptin (over 5 min), followed by an I.C.V. infusion at 10 μg/h. A second baroreflex test and VEP were then performed three hours later, analogous to Group I. This dose of leptin and duration of infusion was based on previous reports demonstrating that the maximum effect of leptin on sympathetic outflow became maximal after three hours when given either centrally or systemically.[59]

Group III (n=7): Wistar rats in this group received a high-fat diet containing 45% of fat (Special Diet Service, Essex, UK) for four weeks before the experiment. On the day of experiment, baroreflex test (n=7) and VEP (n=5) were performed in the resting state and at least one hour later, the animals were given an I.C.V. bolus dose of 5 μg leptin (over 5 min), followed by an I.C.V. infusion at 10 μg/h. A second baroreflex test and VEP were then performed three hours later, analogous to Groups I and II.

Group IV (n=7): Wistar rats received a regular laboratory diet as part of this group. On the day of experiment, baroreflex test (n=7) and VEP (n=7) were performed in the resting state and two hours later, the animals were given an I.C.V. infusion of orexin-A (50 ng/μl) at rate of 60 μl/h. A second baroreflex test and VEP were then performed approximately 10 min later. This shorter time-frame compared with the leptin administration was used as previously demonstrated that I.C.V. administration of orexin-A in the rat caused a maximal increase in basal RSNA within 10 min.[60]

Group V (n=7): In this group, Wistar rats received a high-fat diet containing 45% of fat (SDS, Essex, UK) for four weeks before the experiment. Baroreflex test (n=7) and VEP (n=7) were performed in the resting state and two hours later, the animals were given an I.C.V. infusion of orexin-A (50 ng/μl) at rate of 60 μl/h. A second baroreflex test and VEP were then performed approximately 10 min later, analogous to Group IV.

Statistical analysis

The mean values of all data were calculated from individual rats, and are presented as means ± S.E.M. On each day, the background or minimum noise level in the renal nerve signal was determined as that obtained during the peak increase in blood pressure in response to the bolus dose of phenylephrine, and this value was subtracted from all readings. A two-tailed paired *t*-test was used to compare the baroreflex curve parameters and the baseline values of MAP, HR and RSNA in the time control group before and after I.C.V. saline. A repeated-measures two-way ANOVA was utilized to detect the differences between each individual variable being measured (each of the parameters (*A1-A4*), the maximum gain and the baseline values of MAP, HR and RSNA) before and after I.C.V. drug administration, between the two different diet groups. The comparison of the mean percentage reduction in RSNA from the

baseline was performed using repeated-measures one-way ANOVA followed by Dennett's *post hoc* test and corrected using the Geisser–Greenhouse application to indicate any differences during volume expansion or recovery period from baseline. To compare the sympatho-inhibitory response to VEP before and after I.C.V. drug administration of different diet groups, mean % reduction of RSNA during 10 minutes of VEP was analysed by repeated-measures two-way ANOVA. Significance was taken when $P<0.05$.

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Authors' Contributions

All authors participated in the interpretation of the studies and analysis of the data and review of the manuscript. CH performed the experiments as well as collected the data. SAM and JC contributed to statistical analysis and reviewed the final draft. MHA participated in data analysis and wrote the manuscript. CH and EJJ contributed to conception and design of the experiments. All individuals who made contributions to this study are included.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Table legends:

Table 1: Baseline values of MAP, HR and RSNA obtained before and three hours after I.C.V. saline, leptin or orexin-A in normal and high-fat diet fed Wistar rats. ***** P<0.05 After vs. Before I.C.V. infusion of leptin or orexin-A of same diet group. I.C.V., intracerebroventricular; MAP, mean arterial pressure; RSNA, renal sympathetic nerve activities; HR, heart rate; Before, before I.C.V. infusion; After, after I.C.V. infusion.

Table 2: Baroreflex parameters obtained before and three hours after I.C.V. saline, leptin or orexin-A in normal and high-fat diet fed Wistar rats. ***** P<0.05 compared with before I.C.V. infusion of leptin or orexin-A of same diet group. I.C.V., intracerebroventricular; MAP, mean arterial blood pressure; RSNA, renal sympathetic nerve activity; $\boldsymbol{A_1}$ the range over which the baroreceptors operate; $\boldsymbol{A_2}$ the sensitivity or curvature coefficient of the relationship; $\boldsymbol{A_3}$ the range mid-point mean blood pressure of the curve; $\boldsymbol{A_4}$ the lowest point to which the RSNA could be driven.

Table 3: Baroreflex parameters of heart rate obtained before and three hours after saline, leptin or orexin-A I.C.V. in normal and high-fat diet fed Wistar rats. ***** P<0.05 compared with before I.C.V. infusion of leptin or orexin-A of same diet group. † P<0.05 between diet groups. I.C.V., intracerebroventricular; MAP, mean arterial blood pressure; HR, heart rate; $\boldsymbol{A_1}$ the range over which the baroreceptors operate; $\boldsymbol{A_2}$ the sensitivity or curvature coefficient of the relationship; $\boldsymbol{A_3}$ the range mid-point mean blood pressure of the curve; $\boldsymbol{A_4}$ the lowest point to which the RSNA could be driven.

Figures legends:

Figure 1: Representative recording of heart rate (HR), mean arterial blood pressure (MAP) and integrated renal sympathetic nerve activity (RSNA). PE, Phenylephrine; SNP, sodium nitroprusside.

Figure 2: Baroreflex curves for RSNA **(*a*)** and maximum gain **(*b*)** before and after I.C.V. administration of saline in time control group of normal diet fed rats.

Figure 3: Baroreflex curves for RSNA **(*a, c*)** and maximum gain **(*b, d*)** before and after I.C.V. administration of leptin **(*a, b*)** or Orexin-A **(*c, d*)** in normal and high-fat fed rats. Data are presented as mean±S.E.M. and analysed using repeated measures two-way ANOVA. ***** $P<0.05$ After vs. Before I.C.V. leptin or orexin-A in each group.

Figure 4: The change in RSNA over time in response to volume expansion (VEP) before and after I.C.V. administration of saline in the time control group of normal diet fed rats.

Figure 5: The change in RSNA over time in response to volume expansion (VEP) before and after I.C.V. administration of leptin **(*a*)** or orexin-A **(*b*)** in normal and high-fat fed rats. Data are presented as mean±S.E.M. and analysed using repeated measures two-way ANOVA. ***** $P<0.05$ compared with baseline within each drug and diet group. † $P<0.05$ After vs. Before I.C.V. leptin or orexin-A administration.

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