Central Leptin And Resistin Combined Elicits Enhanced Central Effects On Renal Sympathetic Nerve Activity

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New Findings

What is the central question of this study?

Leptin and resistin act centrally to increase renal sympathetic nerve activity (RSNA). We also used Fos protein to quantify the number of activated neurons in the brain. We investigated whether resistin and leptin combined could induce a greater response than each alone.

2. What is the main finding and its importance?

Leptin and resistin, combined, induces (i) a greater increase RSNA increase than with either drug alone. This correlated with a greater number of activated neurons in the arcuate nucleus, than with either drug alone.

Abstract

Leptin and resistin act centrally to increase renal sympathetic nerve activity (RSNA). We investigated whether resistin and leptin combined could induce a greater response than each alone. MAP, HR and RSNA were recorded before and for 3 hours after intracerebroventricular saline (control) (n=5), leptin (7 µg; n=5), resistin (7 µg; n=4) and leptin administered 15 minutes after resistin (n=6). Leptin alone and resistin alone significantly increased RSNA ($74 \pm 17\%$, $50 \pm 14\%$ respectively) ($P < 0.0001$ compared to saline). When leptin and resistin were combined there was a significantly greater increase in RSNA ($163 \pm 23\%$) compared to either drug alone ($P < 0.0001$). Maximum responses in MAP and HR were not significantly different between groups. We also used Fos protein to quantify the number of activated neurons in the brain. Compared to controls, there were significant increases in numbers of Fos-positive neurons (i) in the arcuate and hypothalamic paraventricular nuclei when leptin or resistin were administered alone, or combined, and (ii)

in the lamina terminalis when leptin or resistin were combined. Only in the arcuate nucleus was the increase significantly greater compared to either drug alone. The findings show that leptin and resistin, combined, induces (i) a greater RSNA increase, and (ii) a greater number of activated neurons in the arcuate nucleus, than with either drug alone. Since leptin makes an important contribution to the elevated RSNA observed in obese/overweight conditions, the increased levels of leptin and resistin may mean the contribution of leptin to the elevated RSNA in those conditions is enhanced.

INTRODUCTION

Leptin is a well known hormone released from fat tissue and acts centrally to influence metabolic and cardiovascular functions (Elmqvist *et al.*, 1997; Mark *et al.*, 1999; Mark *et al.*, 2002; Rahmouni *et al.*, 2005). It can increase renal sympathetic nerve activity (RSNA) and this action may make an important contribution to the abnormally elevated RSNA observed in obesity or overweight conditions (Haynes *et al.*, 1997b; Prior *et al.*, 2010).

Resistin, also a hormone released from fat tissue, has recently been shown to act centrally to elicit similar effects as leptin on RSNA (Kosari *et al.*, 2012). Both leptin and resistin increase RSNA through central mechanisms that involve phosphatidylinositol 3 kinase (PI 3-kinase) (Rahmouni *et al.*, 2003; Kosari *et al.*, 2012). Thus, these two hormones appear to act through similar mechanisms to increase RSNA and this raises the question of whether combined administration of leptin and resistin could elicit greater increases of RSNA. This could have important implications since plasma leptin and resistin levels correlate with adiposity

(Shimizu *et al.*, 1997; Steppan *et al.*, 2001; Azuma *et al.*, 2003; Rajala *et al.*, 2004), and both are elevated in obesity and overweight conditions. Thus, the first aim of the present study was to measure RSNA in response to leptin administered in the presence of resistin compared to the responses induced by leptin and resistin alone.

Although there is considerable knowledge of the brain nuclei activated by leptin, there has not been a systematic investigation of those activated by resistin. Since similar mechanisms appear to be mediating the central actions of leptin and resistin, we wondered whether similar areas in the brain were activated and whether an enhanced effect observed when both were combined could be correlated with specific brain nuclei. We focussed on autonomic brain nuclei known to influence cardiovascular function. We used the protein, Fos, as a marker of increased neuronal activity to quantify the number of activated neurons in each brain nucleus and compared their distribution after administration of resistin with that after administration of leptin given alone and the combination of leptin and resistin.

METHODS

Ethical approval

All the experimental protocols were performed in accordance with the Prevention of Cruelty to Animals Act 1986 (Australia). The procedures conform to the “Guiding Principles for Research Involving Animals and Human Beings” and the guidelines set out by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 2013 (National Health and Medical Research Council of Australia) and were approved by the RMIT University Animal Ethics Committee.

Procedures

General

Male Sprague-Dawley rats (Av body weight=481±19 gms) were obtained from ARC animal resources centre (W.A, Australia). Rats were housed at 23°C with a 12-h light/dark cycle and allowed free access to standard rat chow and water. On the day of the experiment, anesthesia was induced using isoflurane gas (initially 5% then lowered to 2.5%) in O₂. A catheter was inserted into the femoral vein, so that anesthesia could be maintained with intravenous urethane (1.4–1.6 g/kg initially, followed by supplemental doses of 0.05 ml of a 25% solution, as required). The depth of anesthesia was maintained to ensure the absence of corneal and pedal reflexes. The femoral artery was cannulated for monitoring arterial blood pressure. Mean arterial pressure (MAP) and heart rate (HR) were calculated from the arterial pressure pulse using LabChart (ADInstruments, NSW, Australia). Rats were kept on a heating pad for the duration of the experiments to maintain body temperature at approximately 37.5 °C.

Renal sympathetic nerve activity

After a flank incision, the left kidney was exposed retroperitoneally. With the aid of an operating microscope, the renal nerve was carefully dissected free of surrounding tissue under mineral oil and placed onto the bared tips of two Teflon-coated silver wire electrodes.

RSNA was amplified using a low-noise differential amplifier (models ENG 187B and 133, Baker Institute, Victoria, Australia), filtered (band pass 100–1,000 Hz), rectified, and integrated at 0.5 s intervals. The signal was recorded using a PowerLab data acquisition system (ADInstruments, NSW, Australia).

Microinjections into the lateral brain ventricle (ICV)

Each animal was placed prone, and the head was mounted in a Stoelting stereotaxic frame, such that bregma and lambda were positioned on the same horizontal plane. For exposure of the dorsal surface of the brain, a hole (2 mm diameter) centred 0.7 mm caudal to bregma and 1.8 mm lateral to the midline, was drilled into the skull. Pressure ejection was used to make unilateral microinjections using a fine glass micropipette (50–70 μm tip diameter) inserted into the lateral brain ventricle (stereotaxic coordinates: 0.7 mm caudal to bregma, 1.8 mm lateral to midline, and 3.7–3.9 mm ventral to the surface of the dura). The volume injected was 5 μl and was determined by using calibrated markings on the micropipette. After the microinjection, the micropipette was left in place for 1 min. At the end of the experiment a small amount of pontamine sky blue was microinjected using the same coordinates to confirm microinjection into the lateral ventricle.

Experimental protocols

MAP, HR and RSNA were measured before and for three hours after administration of saline vehicle (n=5), leptin (7 μg ; n=5), resistin (7 μg ; n=4) and the combination of both resistin and leptin (n=6) (leptin was administered 15 minutes after resistin). The doses of each hormone were chosen on the basis of previous studies (Rahmouni *et al.*, 2009; Kosari *et al.*, 2011).

Immunohistochemistry for Fos

At the end of the experiment, the animals were overdosed with pentobarbitone (325 mg iv) and then perfused through the heart with 200 ml of 0.1 M phosphate buffered saline (PBS, pH 7.2), followed by 200 ml of 4% *para*-formaldehyde in 0.1 M PBS). The brains were then carefully removed, post-fixed in the same fixative (for 3 hours) and left overnight in 0.1 M PBS containing 20% sucrose.

Serial coronal sections (40 µm thick) of the brain were cut using a cryostat (Leica, CM1900, Germany). One in five sections was collected, placed onto gelatine-coated slides, dried for 2 hours at room temperature and then processed immunohistochemically to detect the protein Fos using standard immunohistochemical procedures. In brief, endogenous peroxidase activity was destroyed by incubating with 0.5% H₂O₂ for 30 minutes. The sections were then incubated in 10% normal horse serum (NHS) for 60 minutes prior to 0.5% Triton X-100 (30 minutes) to facilitate antibody penetration. The sections were then incubated in primary antibody raised in the rabbit against Fos (1:400, rabbit polyclonal IgG , c-Fos (K-25): sc-253, Santa Cruz Biotechnology, CA, USA) for 24 hours at room temperature. The sections were then incubated for 2 hours with biotinylated anti rabbit secondary antibody raised in goat, (1:400, Sigma Aldrich, Australia) and subsequently with Extravidin – peroxidase complex (1:400, Sigma Aldrich, Australia) for 1 hour. In between all steps the sections were washed three times (for 5 minutes each time) with PBS. Then the sections were incubated in 0.05% 3,3'-diaminobenzidine hydrochloride (DAB; sigma Aldrich, Australia) in PBS for 10 minutes. The reaction was initiated by adding 5 µl of 30% hydrogen peroxide and terminated

by washes with fresh PBS. Finally the sections were allowed to dry and were coverslipped with Depex.

The investigators understand the ethical principles under which the journal operates and that their work complies with this animal ethics checklist.

Statistical analysis

The average resting levels of MAP and HR in the different groups of animals prior to drug administration were compared between groups using one-way ANOVA. The integrated RSNA was calculated over a period of 1-2 minutes at each time point (ie before and every fifteen minutes after injection) and expressed as a percentage of the resting level prior to the injections. Changes in MAP, HR and RSNA over time were compared between groups in each experimental series by using two-way ANOVA. The average of the last 30 minutes of the observation period was used to calculate the maximum changes and these were compared between groups using one-way ANOVA.

Fos-positive cell nuclei were visualised and counted unilaterally, except in the midline structures (raphe pallidus, dorsal raphe, median preoptic nucleus and organum vasculosum of the lamina terminalis), using bright field illumination at 200x magnification. At least two sections for each brain nucleus were examined. The average number of Fos-positive cell nuclei per section was calculated for each brain nucleus in each animal and comparisons between the groups were performed using one-way ANOVA. When an overall significant difference between groups was detected, comparisons were made between individual groups,

using Holm-Sidak's test for multiple comparisons. All results are expressed as means $\pm$ sem. $P < 0.05$ was considered to be statistically significant. PRISM software was used.

RESULTS

Effects of ICV injection of leptin and resistin on RSNA

Intracerebroventricular (ICV) injection of leptin alone and resistin alone increased RSNA significantly compared to saline (control). Leptin alone increased RSNA reaching a maximum of approximately 75% between 2-3 hours after administration ($p < 0.0001$ compared to saline) (figure 1). Resistin increased RSNA significantly compared with saline and followed a similar time course to that of leptin reaching a maximum level of approximately 50% ($p < 0.0001$ compared to saline). When leptin and resistin were administered, RSNA increased dramatically to a maximum of approximately 165%, which was significantly greater compared to the responses of either drug alone ($P < 0.0001$) (figure 1).

Effects of ICV injection of leptin and resistin on MAP and HR.

Leptin alone induced a maximum increase in MAP of 4 ± 3 mmHg by the end of the observation period and this was not significantly different from saline control (5 ± 2 mmHg) (figure 2). Resistin increased MAP by 11 ± 1 mmHg and followed a similar time course to that of leptin and it was not significantly different compared to saline (figure 2). When leptin and resistin were administered, the increase in MAP was not significantly different to the responses of either drug alone or control (figure 2).

Similarly, there was no significant difference in the responses in HR between the groups. By the end of the observation period, neither leptin nor resistin induced a statistically significant change in HR compared to saline (figure 2). When leptin and resistin were administered, the change in HR was not significantly different to the responses of either drug alone or control (figure 2).

The average resting MAP and HR prior to drug administration are shown in table 1. One way ANOVA analysis showed there was no significant differences between the groups.

Effects of leptin and resistin, alone and in combination, on Fos-positive cell nuclei

Lamina Terminalis

In the organum vasculosum of the lamina terminalis (OVLT), and in the median preoptic (MnPO) nucleus, the number of Fos-positive cell nuclei following leptin or resistin alone were not significantly different compared to control (figures 3 and 4). However, when leptin and resistin were administered, there was a significant increase in Fos-positive cell nuclei by two-fold compared to the saline control ($P < 0.05$) (figures 3 and 4).

Arcuate nucleus

In the arcuate nucleus, leptin and resistin alone significantly increased the number of Fos-positive cell nuclei by approximately 50% and 100%, respectively, compared to control (figure 5). When leptin and resistin were administered, the increase in Fos-positive cell nuclei

was significantly greater than in control ($P<0.001$) and compared to each drug alone ($P<0.001$) (figure 5).

Paraventricular and supraoptic nuclei

In the hypothalamic paraventricular nucleus leptin and resistin alone induced a significant two-fold increase in the number of Fos-positive cell nuclei compared to control ($P<0.05$) (figures 5 and 6). When leptin and resistin were administered, the increase in the number of Fos-positive cell nuclei was also significantly greater than control but did not differ from the result observed with leptin or resistin alone. In the supraoptic nucleus, the number of Fos-positive cell nuclei following each treatment was not statistically significant between groups (figure 5).

Dorsal Raphe and Medulla Oblongata

In the dorsal raphe, the rostral ventrolateral medulla (RVLM), and in the nucleus tractus solitarius (NTS), there was no significant difference in the number of Fos-positive cell nuclei between the treatments (figure 7). In the raphe pallidus, following leptin or resistin alone the average number of Fos-positive cell nuclei was not significantly different from controls. However, when leptin and resistin were administered, the number of Fos-positive cell nuclei was significantly reduced compared to leptin or resistin alone ($P<0.05$), but was not significantly different from control (figure 7).

DISCUSSION

In the present study we found that when leptin was administered ICV after resistin, there was a significantly greater increase in RSNA compared to the response elicited by either hormone alone. We also determined in a more systematic way than has been previously investigated, the distribution of activated neurons in the brain following resistin. Compared to saline control we found that resistin significantly increased the number of activated neurons in arcuate and hypothalamic nuclei, which was similar to leptin. We also found that in the OVLT, median preoptic nucleus, arcuate nucleus, and in the hypothalamic paraventricular nucleus the number of Fos-positive cell nuclei was increased significantly more when leptin was administered after resistin compared to control. However, only in the arcuate nucleus was the increase in the number of activated neurons significantly greater than when each hormone was administered alone. In the RPA, by contrast, the number of Fos-positive cell nuclei was significantly reduced when leptin was administered after resistin compared to that observed after each hormone alone.

In the present study, ICV leptin alone increased RSNA, as did resistin, confirming previous studies (Dunbar *et al.*, 1997; Haynes *et al.*, 1997a). In contrast to leptin alone, the increase in RSNA induced by leptin was at least doubled when it was administered in the presence of resistin. This finding could have important implications in conditions in which leptin and resistin are elevated such as in obesity. In this condition RSNA is abnormally elevated (Prior *et al.*, 2010), and since leptin is believed to be an important mediator of the increase in RSNA in that condition (Haynes *et al.*, 1997b; Prior *et al.*, 2010), the presence of resistin could contribute to leptin's key role in elevating RSNA in obesity.

We did not explore the responses of reversing the order of leptin and resistin since we were particularly interested in the effects of leptin on RSNA as we plan to investigate this interaction in obesity. The mechanisms involved in the enhanced response may involve (i) activation of separate populations of cells (as discussed later) and (ii) a population of cells that can be activated by both leptin and resistin. In the latter case, the intracellular transduction pathways resulting in the increased RSNA utilised by each hormone involves phosphatidylinositol 3-kinase (Rahmouni *et al.*, 2003; Kosari *et al.*, 2012). It is likely that both possibilities contribute and the present findings investigating the distribution of Fos would support this.

In contrast to the RSNA response, the maximum changes in MAP and HR following the combination of leptin and resistin, were not significantly greater than the responses observed when leptin or resistin were administered alone. This is somewhat surprising given the dramatic effect on RSNA. It is possible that anaesthesia may have an influence on our ability to detect changes in MAP. Alternatively, the greater sympatho-excitatory effect is selective for the renal output and may be insufficient to elicit acute increases in blood pressure. Reflex responses could also contribute to attenuating an increase in blood pressure.

In the present work we describe the distribution of activated neurons following resistin administration more extensively than has been performed in the past (Tovar *et al.*, 2005; Singhal *et al.*, 2007; Kosari *et al.*, 2011). We have also, for the first time, compared the distribution with that induced by leptin alone and following leptin plus resistin in combination. Resistin induced a significant increase in Fos-positive cell nuclei in the arcuate,

and paraventricular nuclei in the hypothalamus. In other brain regions examined we could not detect a statistically significant difference compared to control. In the PVN and ARC our present data agrees with previous work (Singhal *et al.*, 2007; Kosari *et al.*, 2011).

In the present study, we found that leptin also significantly increased the number of Fos-positive cell nuclei in the arcuate nucleus and PVN, which agrees with previous studies that have investigated the distribution of activated neurons following leptin administration (van Dijk *et al.*, 1996; Woods & Stock, 1996; Elias *et al.*, 2000), or studies in which leptin was microinjected into specific brain nuclei (Montanaro *et al.*, 2005), or studies that determined the distribution of leptin receptors (Mercer *et al.*, 1996).

We could not detect statistically significant increases in Fos-positive cell nuclei in the medulla oblongata following leptin administration. This is despite evidence that leptin can influence cardiovascular and respiratory regulation by acting within nuclei present in the dorsal and ventral medulla oblongata (Bassi *et al.*, 2015a; Bassi *et al.*, 2015b; Bassi *et al.*, 2016). We administered leptin into the lateral ventricle and it is likely that the hormone spread to more caudal brain regions including the medulla oblongata. Thus, it is possible that under the present conditions, we did not have the sensitivity to detect significant increases in Fos-positive neurons in those medullary nuclei, and we do not exclude actions of the hormones in the medulla.

In contrast to leptin, there have not been studies that have microinjected resistin into brain nuclei nor has the receptor for resistin been identified. Thus, the distribution of activated

neurons may provide some insight into potential sites in which resistin acts, though it must be recognised that the protein Fos is a marker of increased neuronal activity rather than a marker of neurons directly activated by resistin.

When leptin and resistin were administered, the numbers of Fos-positive cell nuclei in the lamina terminalis, ARC and in the PVN were significantly greater than in controls. We found that only in the ARC was the increase in the number of Fos-positive cell nuclei significantly greater than when either hormone was administered alone. This result suggests that in the ARC the neurons activated by leptin include a population that is separate from the population activated by resistin. In contrast, a similar increase in the number of Fos-positive cell nuclei in the PVN was observed following each hormone alone and after leptin and resistin were combined, suggesting that the same population was activated by leptin and resistin.

The PVN receives projections from the ARC (Baker & Herkenham, 1995), so it is perhaps surprising that the number of Fos-positive cell nuclei was not greater in the PVN following the administration of leptin and resistin compared to each alone, given the dramatic increase in the ARC following both hormones combined. This anomaly may be explained when one considers that Fos protein is a marker of increased neuronal activity but not a measure of the level of increased activity in a neuron. Thus, in the PVN the level of neuronal activity may have increased dramatically when leptin and resistin were administered, without a marked increase in the number of neurons activated. Thus, it is possible that the same neuronal population was activated by the administration of leptin and resistin but to a much higher level than that observed after the administration of each hormone alone.

In contrast to all regions examined, the administration of leptin and resistin resulted in a significant decrease in the number of Fos-positive cell nuclei in the RPA compared to either hormone administered alone. This suggests a negative interaction is occurring within this nucleus. Since the RPA is important in the regulation of sympathetic nerve activity to brown adipose tissue (Morrison *et al.*, 1999), the present findings are in agreement with studies that show that leptin has sympatho-excitatory actions on brown adipose tissue, whilst resistin has opposing inhibitory effects on sympathetic nerves innervating brown adipose tissue (Rahmouni *et al.*, 2009; Kosari *et al.*, 2011). In the present study, we note that there was no evidence that resistin alone reduced basal Fos production in the RPA.

The physiological responses that may be attributed to the increase in activated neurons in the ARC, lamina terminalis and the PVN are unknown. The ARC is known for its involvement in dietary intake and cardiovascular regulation. This nucleus is well recognized as a key brain nucleus mediating leptin's anorexigenic actions (Cowley *et al.*, 2001). Resistin administered acutely also reportedly reduces food intake (Tovar *et al.*, 2005). Thus, the present observation of enhanced neuronal activity in the ARC induced by the combination of leptin and resistin could be manifested functionally as a change in energy balance.

The ARC is also important in eliciting cardiovascular responses (Sapru, 2013), and taken together with the present functional data on RSNA, the enhanced neuronal activity in the ARC could be related to the greater increase in RSNA observed when leptin and resistin were

administered compared to the responses to each hormone alone. Further studies are needed to explore these possibilities.

In a study using resistin over-expressing mice crossed with mice lacking the low density lipoprotein receptor, leptin levels were elevated, suggesting leptin resistance (Asterholm *et al.*, 2014). Thus, as part of their report, the authors investigated the distribution of pSTAT3, a marker of neurons activated by leptin, in the arcuate nucleus in vitro following leptin alone and in the presence of resistin. They reported that pSTAT3 levels induced by leptin were reduced by resistin (Asterholm *et al.*, 2014). At first sight, our present data following the administration of leptin in the presence of resistin appears to contrast those observations. However, the results may be reconciled in that pSTAT3 is a marker of neurons that were activated directly by leptin, whilst the presence of Fos is indicative of neurons whose activity increased (not necessarily by direct effects of the hormones on the neuron). Nonetheless, our observations on RSNA shows that the combination of leptin and resistin results in a greater renal sympatho-excitatory actions than following either hormone alone.

The OVLT and MnPO form part of the lamina terminalis and have been shown to have polysynaptic connections to sympathetic nerves innervating the kidneys (Sly *et al.*, 2001). Those brain nuclei are also well known to play key roles in osmoregulation and body fluid homeostasis (Sly *et al.*, 2001). Whether the marked increase in RSNA observed when leptin and resistin were combined is related to the greater increase in the number of Fos-positive cell nuclei in the lamina terminalis awaits further investigation. Similarly, we are not aware of studies investigating the effects of leptin or resistin on thirst.

Limitations of the study

One limitation of this study is that we used anaesthetized rats. The presence of Fos is affected by anaesthesia, where basal levels are usually higher, than in conscious animals (Oldfield *et al.*, 1994; McKinley *et al.*, 1995). The results in the present study agree with that. Thus, it may be more difficult to detect changes from control under anesthetized conditions, so we may have underestimated the brain nuclei activated. Nonetheless, the fact that increases in Fos production were observed suggests that the areas identified are important.

Fos is a marker of increased neuronal activity, but does not indicate the level of increased activity. Thus, in areas where the increase in Fos-positive cell nuclei following the administration of leptin and resistin were not greater than after each hormone alone, does not necessarily indicate there was not a greater level of activity in that brain nucleus.

Also, in the present work we studied the effects of acute administration of the two hormones. We observed the responses for three hours and though clearly long enough to see changes in RSNA, we did not see significant effects on MAP of leptin or resistin. It has been previously reported that leptin administered acutely increases MAP (Rahmouni & Morgan, 2007). One difference between the present work and that study is that the increase in MAP in response to ICV leptin required more than three hours to be clearly observed, although RSNA was elevated earlier. Thus, it would also be interesting to observe the effects of leptin and resistin following chronic administration. This could be particularly relevant to obesity where chronically elevated plasma leptin levels make an important contribution to the hypertension

and the elevated RSNA associated with obesity (Abramson *et al.*, 2011; Simonds *et al.*, 2014).

Increases in RSNA can induce anti-natriuretic responses (DiBona, 1985). In the present study we did not monitor changes in natriuresis or diuresis. Given the increase in RSNA we observed, one could expect an anti-natriuretic response. Intravenously administered leptin has natriuretic actions, which are mediated by peripheral mechanisms (Jackson & Herzer, 1999). Thus, central and peripheral actions of leptin produce opposing actions on natriuretic responses. Whether this contributes to the time that it takes to observe pressor responses with leptin is unknown, but it appears to be insufficient to prevent the chronic pressor action of leptin.

In conclusion, the present study investigated the effects of acute central administration of leptin and resistin, alone and in combination, on RSNA, MAP and HR. Leptin alone and resistin alone increased RSNA. When leptin was administered 15 minutes after resistin, there was a significantly greater increase in RSNA compared to the responses to either hormone alone. This may be important in conditions like obesity where RSNA is abnormally elevated and both leptin and resistin levels are increased. The brain nuclei involved in mediating the enhanced increase in RSNA are not known but may involve the lamina terminalis, arcuate nucleus and the hypothalamic PVN.

Legends

Figure 1. Percent changes in renal sympathetic nerve activity (RSNA) from baseline levels over time after intracerebroventricular injection of leptin (n=5) (7µg/5µl), resistin (n=4) (7µg/5µl), control (saline, n=5) (5µl) and the combination of leptin and resistin (n=6). (*P<0.0001 compared to control; #P<0.0001 compared to leptin alone or resistin alone).

Figure 2. Changes in mean arterial pressure (MAP) and heart rate (HR) over time induced by the intracerebroventricular administration of leptin (n=5) (7µg/5µl), resistin (n=4) (7µg/5µl), Control (saline, n=5) (5µl) and the combination of leptin and resistin (n=6).

Figure 3. Average numbers of Fos-positive cell nuclei per section in the organum vasculosum of lamina terminalis (OVLT) and median preoptic nucleus (MnPO) from rats administered intracerebroventricular saline (control) (n=5) (5µl), leptin (n=5) (7µg/5µl), resistin (n=4) (7µg/5µl) and leptin combined with resistin (n=5). *P<0.05 compared to control.

Figure 4. Photomicrographs showing Fos-positive cell nuclei in the organum vasculosum of the lamina terminalis (OVLT) of rats following centrally administered saline (5µl; control), leptin (7µg), resistin (7µg) and leptin combined with resistin. Bar, 200 µm. Dashed lines outline the OVLT.

Figure 5. The average number of Fos-positive cell nuclei per section in the arcuate (ARC), paraventricular (PVN) and supraoptic (SON) nuclei after intracerebroventricular injection of saline (5µl; control) leptin (n=5) (7µg/5µl), resistin (n=4) (7µg/5µl), and leptin combined

with resistin (n=5). *P<0.05, **P<0.01, ***P<0.001 compared to control; #P<0.001 compared to leptin and resistin alone.

Figure 6. Photomicrographs showing Fos-positive cell nuclei in paraventricular nucleus (PVN) of rats following centrally administered saline (5µl; control), leptin (7µg), resistin (7µg) and leptin combined with resistin. Bar, 200 µm. Dashed lines outline the PVN. Abbreviations, Fx, fornix.

Figure 7. Average number of Fos-positive cell nuclei per section in the dorsal raphe (DR), rostral ventrolateral medulla (RVLM), nucleus tractus solitarius (NTS) and raphe pallidus (RPA) following saline (control) (n=5) (5µl), leptin (n=5) (7µg/5µl) resistin (n=4) (7µg/5µl) and leptin combined with resistin (n=5). *P<0.05 compared to leptin or resistin alone.

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Author Contributions

The work was performed in the laboratory of EB.

EB devised study, wrote manuscript, analysed data, interpretation of data

HH and NA contributed to manuscript, performed experiments, analysed data interpretation of data

TJ and MS contributed to the manuscript and interpretation of data

All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Table. 1. Resting mean arterial pressure (MAP) and heart rate (HR) prior to intracerebroventricular injection of saline (n=5), leptin (n=5), resistin (n=4) and the combination of leptin and resistin (n=6) in anesthetised rats.

	Saline	Leptin	Resistin	Leptin + Resistin
MAP (mmHg)	93.5 ± 4.1	79.7 ± 1.6	82.1 ± 3.7	90.5 ± 4.2
HR (beats/min)	360 ± 7	336 ± 11	342 ± 10	323 ± 16

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