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Adrenalectomy impairs insulin-induced hypophagia and related hypothalamic changes

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Abstract

Adrenalectomy (ADX) induces hypophagia and glucocorticoids counter-regulate the peripheral metabolic effects of insulin. This study evaluated the effects of ADX on ICV (lateral ventricle) injection of insulin-induced changes on food intake, mRNA expression of hypothalamic neuropeptides (insulin receptor (*InsR*), proopiomelanocortin, cocaine and amphetamine-regulated transcript (*Cart*), agouti-related protein, neuropeptide Y (*Npy*) in the arcuate nucleus of the hypothalamus (ARC), corticotrophin-releasing factor in the paraventricular nucleus of the hypothalamus) and hypothalamic protein content of insulin signaling-related molecules (insulin receptor substrate (IRS) 1, protein kinase B (AKT), extracellular-signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), protein tyrosine phosphatase-1B (PTP1B) and T cell protein tyrosine phosphatase (TCPTP)) Compared with sham animals, ADX increased the hypothalamic content of pJNK/JNK, PTP1B and TCPTP, as well as decreased mRNA expression of *InsR*, and corticosterone (B) treatment reversed these effects. Insulin central injection enhanced hypothalamic content of pAKT/AKT and *Cart* mRNA expression, decreased *Npy* mRNA expression and food intake only in sham rats, without effects in ADX and ADX + B rats. Insulin did not alter the hypothalamic phosphorylation of IRS1 and ERK1/2 in the three experimental groups. These data demonstrate that ADX reduces the expression of *InsR* and increases insulin counter-regulators in the hypothalamus, as well as ADX abolishes hypophagia, activation of hypothalamic AKT pathway and changes in *Cart* and *Npy* mRNA expression in the ARC induced by insulin. Thus, the higher levels of insulin counter-regulatory proteins and lower expression of *InsR* in the hypothalamus are likely to underlie impaired insulin-induced hypophagia and responses in the hypothalamus after ADX.

Key Words

- adrenal cortex
- insulin receptor
- neuropeptides
- insulin signaling
- hypothalamus

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Introduction

Bilateral adrenalectomy in rodents is a well-established experimental model to investigate the mechanisms underlying changes on energy homeostasis, as hypophagia and body weight loss, which are also classical symptoms observed in primary adrenal insufficiency in humans (Freedman *et al.* 1985, Oelkers 1996, Uchoa *et al.* 2009*a*,*b*). The hypophagic effect induced by ADX is associated with changes in the expression of hypothalamic neuropeptides involved in the regulation of food intake, as evidenced by increases in the expression of the anorexigenic

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neuropeptide corticotrophin-releasing factor (*Crf*) in the paraventricular nucleus of the hypothalamus (PVN) (Uchoa *et al.* 2010) and reduction in the expression of the orexigenic neuropeptides neuropeptide Y (*Npy*) and agouti-related protein (*Agrp*) in the arcuate nucleus of the hypothalamus (ARC) (Uchoa *et al.* 2012).

In addition to glucocorticoids, insulin is another peripheral factor that acts in the central nervous system (CNS) to regulate energy homeostasis (Plum *et al.* 2005). Insulin secretion is proportional to the body weight and adiposity and is stimulated by food intake (Trayhurn *et al.* 1995, Peiser *et al.* 2000, Germano *et al.* 2008, Uchoa *et al.* 2010, Panchal *et al.* 2011). Plasma insulin access the brain by penetrating the blood–brain barrier through a receptor mediator and saturable transporter (Baura *et al.* 1993). In the CNS, insulin receptors (*InsR*s) are expressed in the hypothalamus, especially in the nuclei involved in the regulation of energy homeostasis, as ARC, PVN, dorsomedial (DMH) and ventromedial (VMH) nuclei of the hypothalamus (van Houten *et al.* 1980, Werther *et al.* 1987, Unger *et al.* 1989). Indeed, insulin injection in different regions of the hypothalamus, as well as in the lateral or third ventricle, reduces food intake and body weight gain (Brief & Davis 1984, McGowan *et al.* 1990, Rorato *et al.* 2017). It is known that phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) signaling pathways mediate insulin effects in the CNS. The PI3K pathway is responsible for most metabolic effects of insulin, while the MAPK pathway is involved in the regulation of gene expression and, together with the PI3K pathway, in the control of cell growth and differentiation (De Meyts 2000). In addition, insulin-induced hypophagia is associated with changes in the expression of hypothalamic neuropeptides (Schwartz *et al.* 1992, Benoit *et al.* 2002, Li *et al.* 2002, Chan *et al.* 2005, Honda *et al.* 2007). On the other hand, it is known that molecules that counter-regulate insulin signaling are more activated or expressed in different models of obesity and insulin resistance, including protein tyrosine phosphatases (PTPs), protein tyrosine phosphatase 1B (PTP1B) and T cell protein tyrosine phosphatase (TCPTP) and c-Jun-N-terminal kinase (JNK) (Belgardt *et al.* 2010, Xu *et al.* 2014).

Additionally, the interplay between insulin and glucocorticoids has been extensively explored, since peripheral metabolic effects of insulin are counter-regulated by glucocorticoids and ADX enhances insulin sensitivity in peripheral tissues (Caro & Amatruda 1982, Weinstein *et al.* 1995, Haluzik *et al.* 2002, Long *et al.* 2003). Since few studies have reported the role of glucocorticoids on the central effects of insulin on energy homeostasis, the present study was designed to evaluate the effects of ADX on central injection of insulin-induced changes on food intake, hypothalamic neuropeptides and insulin signaling molecules.

Experimental procedures

Animals

Male Wistar rats, from the Animal Facility of the Campus of Ribeirao Preto, University of Sao Paulo, Brazil, weighing 230–280 g, were individually housed at a controlled temperature (23 ± 2°C) and light–darkness cycle (light from 06:00 to 18:00 h). Animals had *ad libitum* access to pelleted rat chow and water, unless otherwise specified. To improve habituation to the laboratory environment, the rats were handled daily for 7 days preceding the experiments. All experimental procedures were conducted between 14:00 and 18:00 h and were approved by the Ethical Committee for Animal Use of the School of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil (protocol number 092/2011).

Intracerebroventricular (icv) cannulation and bilateral adrenalectomy

Animals were deeply anesthetized with intraperitoneal (IP) injections of a mixture of ketamine (72 mg/kg) and xylazine (11.2 mg/kg), administered as a cocktail consisting of 45% ketamine (100 mg/mL), 35% xylazine (20 mg/mL) and 20% 0.15 M NaCl at a dose of 0.16 mL/100 g of body weight. Then, animals were placed in a stereotaxic instrument (Kopf, model 900) with bregma and lambda in a horizontal plane. A stainless-steel guide cannula (10.0 mm long, 0.6 mm o.d., 0.4 mm i.d.) was implanted into the right lateral ventricle using coordinates from the atlas of Paxinos and Watson (1997): 0.6 mm caudal to bregma, 1.5 mm lateral to the sagittal suture and 3.5 mm below the skullcap. The cannula was fixed to the cranium using dental acrylic resin and two jeweler's screws. A 30-gauge metal obturator filled the cannula except during the injections. Cannula placement was verified by the influx of saline (NaCl 0,9%), through a saline column, during the cannula implantation surgery.

Immediately after icv surgery, animals were subjected to bilateral ADX and sham surgeries. Surgeries were performed via a dorsal midline approach with a single incision in the skin and small bilateral incision through

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the muscle layer at the angle between the last rib and vertebral column. In adrenalectomized rats, the kidney was readily seen, and the adrenal gland found at the top of the kidney was visualized and totally removed. Animals were given 0.9% saline with 0.5% ethanol, without (ADX) or with corticosterone diluted in 0.5% ethanol at a concentration of 25 mg/L (ADX+B) (Uchoa *et al.* 2009*a*). Sham-operated animals underwent similar surgical procedures, but the adrenal glands were not removed, and animals were given tap water with 0.5% ethanol to drink. After surgeries, the rats received a prophylactic injection of penicillin (50,000 U, i.m.) and could recover for 7 days, during which they were handled daily. Plasma corticosterone levels were determined by radioimmunoassay (Castro *et al.* 1995).

Microdissection, total RNA isolation and semi-quantitative real-time PCR

A set of sham, ADX and ADX+B rats was killed by decapitation for the determination of the relative expression of insulin receptor (*InsR*) mRNA in the hypothalamus (PVN, ARC, VMH and DMH). Another set of experimental animals was submitted to vehicle or insulin administration, as previously described, and decapitated 120 min after for the determination of the relative mRNA expression of neuropeptides involved in the regulation of food intake in the PVN and ARC. The brains were collected under RNAse-free conditions and kept at −80°C until total RNA extraction. The target brain structures were obtained in a cryostat from two consecutive thick coronal sections (1500 µm each, from coordinates 0.6–2.1 mm and 2.1–3.6 mm posterior to bregma), according to the rat brain atlas (Paxinos & Watson 1997). The PVN and ARC were isolated from the first and second thick sections, respectively, using a stainless-steel punch needle with 1.5 mm of internal diameter. DMH and VMH were punched from the second section, using a stainless-steel punch needle with 1.0 mm of internal diameter. Tissue samples were transferred to a microtube containing RNA later reagent (Ambion, USA) until RNA isolation. Total RNA was isolated from each micropunched hypothalamic tissue sample using TRIzol reagent (Invitrogen®) according to the manufacturer's protocol. The RNA concentration in each sample was determined using a UV spectrophotometer, and 500 ng of RNA were used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system. The TaqMan® Gene Expression Assays (Applied Biosystems) used in this study were Rn00690703_m1 (Insulin receptor), Rn 01431703_g1 (*Agrp*), Rn 01410145_m1 (*Npy*), Rn 00595020_m1 (*Pomc*), Rn 00567382_m1 (*Cart*) and Rn 01462137_m1 (*Crf*). Each PCR reaction was performed in duplicate. Water (instead of cDNA) was used as a negative control. Housekeeping gene (beta actin) was run for each cDNA sample. The determination of gene transcript levels in each sample was obtained by the ΔΔCT method. For each sample, the threshold cycle (Ct) was determined and normalized to the average of the housekeeping genes (ΔCt = CtUnknown − CtHousekeeping genes). The fold-change of mRNA expression in the unknown sample relative to the calibrator group was calculated as $2^{-\Delta\Delta Ct}$, where ΔΔCt = ΔctUnknown − ΔCtCalibrator (Livak & Schmittgen 2001). Housekeeping genes were used to normalize the amount of RNA used in each reaction. To get the relative quantification, described in arbitrary units, appropriate calibrator groups were used as control to compare (1) the effect of treatment (vehicle or insulin) on mRNA expression in each group (sham, ADX, ADX+B); (2) the effect of glucocorticoid sufficiency or not (sham, ADX, ADX+B) on mRNA expression in vehicle- and insulin-treated animals. To attain the first and second analysis, the respective vehicle group and sham-vehicle or sham-insulin group were used as calibrator. Data are shown as mRNA expression relative to the calibrator group, respectively.

Western blotting analysis of medial basal hypothalamus/immunoblot analysis

Hypothalamic fragments were dissected out (thickness: 2.7 mm) from an area 1.0 mm lateral to the midline at the anterior border of the optic chiasm and the anterior border of the mammillary bodies. The medial basal hypothalamus samples were extracted using Triton-X 100 (1%), Tris–HCl pH 7.4 (100 mM), sodium pyrophosphate (100 mM), sodium fluoride (100 mM), EDTA (10 mM), sodium orthovanadate (10 mM), PMSF (2 mM), aprotinin (0.2 mg/mL) and leupeptin (0.2 mg/mL), at 4°C, 15,000 ***g*** for 40 min. Aliquots of the lysates containing 50 µg of protein were denatured in Laemmli sample buffer (6% SDS, 30% glycerol, 0.02% bromophenol blue, 200 mm Tris–HCl (pH 6.8) and 250 mm mercaptoethanol), at 95°C for 5 min. Samples were blotted onto nitrocellulose membrane. Nonspecific binding was prevented by immersing the membranes in blocking buffer (10% BSA in Tris-buffered saline-Tween 20, TBS-T) for 90 min at room temperature. The membranes were then exposed

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overnight to the primary antibodies: rabbit anti-IRS-1 (1:5000, Cell Signaling # 2390); rabbit anti-phospho IRS-1 tyr1222 (1:1500, Cell Signaling # 3066), rabbit anti β-actin (1:1000, Cell Signaling # 8457), rabbit anti-AKT (1:10000, Cell Signaling # 4691); rabbit anti-phospho AKT S473 (1:1500, Cell signaling # 9271), rabbit anti-JNK (1:5000, Cell Signaling # 9252), rabbit anti-phospho JNK (1:1000, Cell Signaling # 9251), goat anti-PTP1B (0,15 µg/mL, AF3954 – R&D Systems) and rabbit anti-TCPTP (1:3000, AB180714-Abcam). The blots were rinsed in TBS-T and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1:5000, Cell Signaling # 7074) or donkey anti-goat IgG-B (1:10000, Santa Cruz SC2042) for 1 h at room temperature. Antibody–antigen complexes were visualized by detecting enhanced chemiluminescence by ECL detection system (Amersham Biosciences) in digital images using Quantity One 4.5.0 software (Bio-Rad). To normalize pIRS, pAKT and pJNK levels, total IRS1, total AKT and total JNK were used, respectively. B-actin was used to normalize PTP1B and TCPTP. Data are expressed as percentage of the calibrator group, and, similarly to the relative quantification of mRNA, to get the relative quantification, appropriate calibrator groups were used as control to compare: (1) the effect of treatment (vehicle or insulin) on protein content in each group (sham, ADX, ADX+B); (2) the effect of glucocorticoid sufficiency or not (sham, ADX, ADX+B) on protein content in vehicle- and insulin-treated animals. To attain the first and second analysis, the respective vehicle group and sham-vehicle or sham-insulin group were used as calibrator, respectively.

Experimental protocols

Effects of ADX and B treatment on the mRNA expression of insulin receptor in the ARC, PVN, DMH and VMH and corticosterone plasma levels

Animals were subjected to either sham or ADX surgery, and half of the ADX animals received B in their drinking fluid. Animals were individually housed in plastic cages. Seven days after surgery, food was withdrawn at 16:00 h and, at 17:30 h, a first set of sham, ADX and ADX+B animals (n=7–9 per group) were decapitated for trunk blood and brain tissue collection, corticosterone plasma levels were determined by radioimmunoassay and *InsR* mRNA was determined by real-time PCR.

Effects of the central injection of insulin on food intake in sham, ADX and ADX + B animals

Immediately after icv surgery, animals (n=5–7 per group) were subjected to either sham or ADX surgery, and half of the ADX animals received B in their drinking fluid. Animals were individually housed in hanging cages. Seven days after surgery, food was withdrawn at 16:00 h, and 90 min later they received an icv injection of vehicle (0.9% NaCl/5 µL) or insulin (12 µM/5 µL). Fifteen minutes after the injections, food was reintroduced, and food consumption was determined after 1, 2, 4, 14 and 22 h of food access.

Effects of the central injection of insulin on the hypothalamic levels of insulin signaling-related molecules in sham, ADX and ADX + B animals

Immediately after icv surgery, animals (n=5–10 per group) were subjected to either sham or ADX surgery, and half of the ADX animals received B in their drinking fluid. Animals were individually housed in hanging cages. Seven days after surgery, food was withdrawn at 16:00 h, and 90 min later, they received an icv injection of vehicle (0.9% NaCl/5 µL) or insulin (12 µM/5 µL). Fifteen minutes after the injections, sham, ADX and ADX+B animals were decapitated for brain tissue collection, and the levels of hypothalamic insulin signaling molecules were determined by Western blotting analyses.

Effects of the central injection of insulin on the mRNA expression of hypothalamic neuropeptides in sham, ADX and ADX + B animals

Immediately after icv surgery, animals (n=5–10 per group) were subjected to either sham or ADX surgery, and half of the ADX animals received B in their drinking fluid. Animals were individually housed in hanging cages. Seven days after surgery, food was withdrawn at 14:00 h, and 90 min later, they received an icv injection of vehicle (0.9% NaCl/5 µL) or insulin (12 µM/5 µL). Sham, ADX and ADX+B animals were decapitated 120 min after the injections for brain tissue collection, and mRNA expression of hypothalamic neuropeptides was determined by real-time PCR.

Statistical analysis

The data are presented as means±S.E.M. The normal distribution and homogeneity of the data were tested. One-way ANOVA (*InsR* mRNA results, mRNA results and protein levels, in the comparison among sham, ADX and ADB+B groups in each treatment) and two-way ANOVA (food intake), followed by Newmann–Keuls (or Kruskal–Wallis test) *post hoc* test and Student *t*-test or Mann–Whitney test (mRNA results and protein levels, in the comparison between vehicle and insulin treatments

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in each experimental group) were used when appropriate. Pearson or Spearman correlation tests were used for the analysis of correlation between corticosterone plasma levels and *InsR* mRNA expression. Differences were considered significant at $P<0.05$.

Results

ADX decreases insulin receptor mRNA expression and increases the levels of insulin counter-regulatory proteins in the hypothalamus

ADX decreased ($P<0.05$) *InsR* mRNA expression in the ARC (Fig. 1A), PVN (Fig. 1B), DMH (Fig. 1C) and VMH (Fig. 1D), compared to sham group. Corticosterone treatment reversed this response in the PVN and VMH and it partially reversed it ($P<0.05$) in the ARC and DMH. Sham group showed higher ($P<0.05$) corticosterone concentrations than ADX+B group (10.1±0.5 µg/dL vs 7.0±0.7 µg/dL), and both groups showed higher ($P<0.05$) values than ADX group (0.45±0.02 µg/dL). A positive and significant correlation was observed between plasma corticosterone levels and insulin receptor mRNA expression in the ARC (Fig. 2A, $P=0.0036$, $r=0.55$), PVN (Fig. 2B, $P<0.0001$, $r=0.85$), DMH (Fig. 2C, $P<0.0001$, $r=0.73$) and VMH (Fig. 2D, $P=0.02$, $r=0.45$). Compared to sham animals, ADX increased ($P<0.05$) hypothalamic levels of PTP1B (Fig. 3A), TCPTP (Fig. 3B) and pJNK/JNK (Fig. 3C), and corticosterone treatment partially reversed the effect on pJNK and normalized the levels of PTP1B and TCPTP.

Adrenalectomy abolishes insulin-induced hypophagia

ADX/vehicle animals showed decreased ($P<0.05$) food intake, when compared to sham/vehicle and this response was reversed by B treatment after 1, 2, 4, 14 and 22 h. Central injection of insulin decreased ($P<0.05$) food intake in sham rats after 1 h, with no effects in ADX rats. However, insulin did not change food consumption at the other times evaluated. Though a nonsignificant trend was observed in the ADX+B group, corticosterone treatment did not rescue the hypophagic effect of insulin (Fig. 4).

Adrenalectomy reduces insulin signaling in the hypothalamus

The comparison with vehicle injection showed that central insulin enhanced ($P<0.05$) hypothalamic pAKT/AKT protein content only in the sham group, with no effects in ADX and ADX+B animals (Fig. 5B), but insulin treatment did not alter hypothalamic pIRS1/IRS1 levels in the three experimental groups (Fig. 5A). Central insulin injection did not alter protein levels of pERK1/2/ERK1/2 in sham, ADX or ADX+B groups (Fig. 5C), compared to vehicle injection. After vehicle microinjection, there was no difference in the relative amount of hypothalamic pIRS1/IRS and pAKT/AKT among sham, ADX and ADX+B groups (Fig. 6A and C). In insulin-treated animals, there was a reduction ($P<0.05$) in the hypothalamic levels of pIRS1/IRS and pAKT/AKT of ADX and ADX+B groups, compared with sham animals (Fig. 6B and D). In vehicle- or insulin-treated animals, hypothalamic levels of

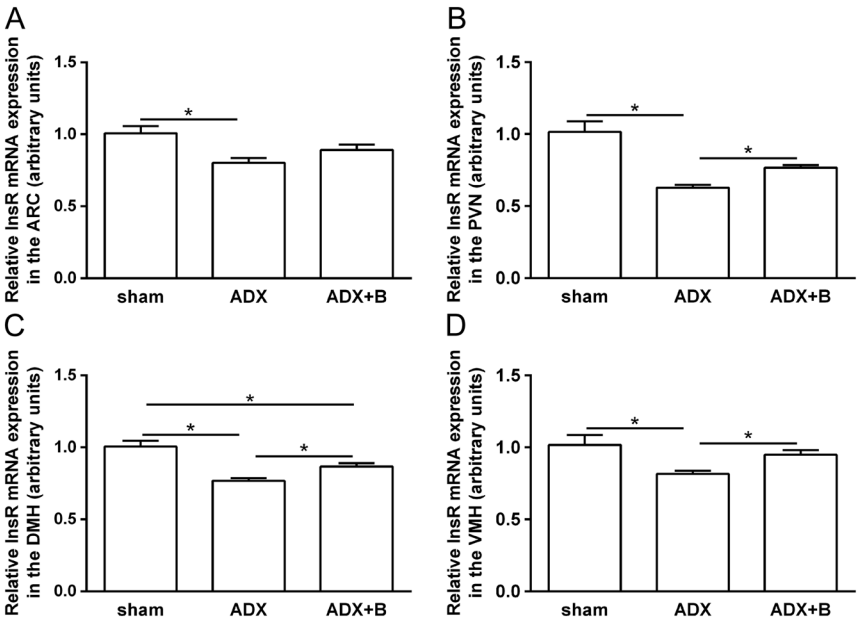

Figure 1
Relative expression of insulin receptor mRNA in the arcuate (ARC) (A), paraventricular (PVN) (B), dorsomedial (DMH) (C) and ventromedial (VMH) (D) nuclei of the hypothalamus of sham, ADX, and ADX + B animals (n = 7-10 rats/group). Data are shown as means ± S.E.M. *$P < 0.05$.

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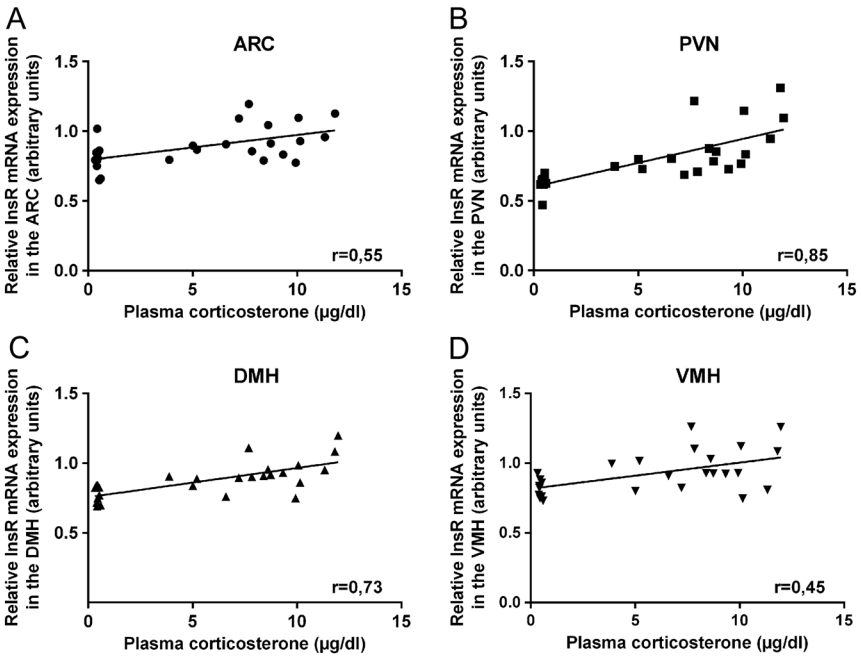

Figure 2
Correlation between corticosterone plasma levels and relative expression of insulin receptor mRNA in the arcuate (ARC) (A), paraventricular (PVN) (B), dorsomedial (DMH) (C) and ventromedial (VMH) (D) nuclei of the hypothalamus of sham, ADX and ADX + B animals (n = 7–10 rats/group). Pearson or Spearman correlation analyses were ARC (P = 0.0036, r = 0.55), PVN (P < 0.0001, r = 0.85), DMH (P < 0.0001, r = 0.73) and VMH (P = 0.02, r = 0.45).

pERK1/2/ERK1/2 were not different among sham, ADX and ADX+B groups (Fig. 6E and F).

Adrenalectomy impairs insulin-induced changes in the expression of hypothalamic neuropeptides

Central insulin injection was able to enhance (P<0.05) *Cart* mRNA expression (Fig. 7B) and to reduce *Npy* mRNA expression (Fig. 7C) in the ARC only in the sham group, with no effects in ADX and ADX+B animals, but insulin treatment did not change hypothalamic mRNA expression of other neuropeptides in the three experimental groups (Fig. 7). In vehicle-treated animals, ADX reduced (P<0.05) *Pomc*, *Cart*, *Npy* and *Agrp* mRNA expression in the ARC, but it increased (P<0.05) *Crf* mRNA expression in the PVN, compared to sham group, while corticosterone replacement was able to reverse (P<0.05) these effects (Fig. 8A, C, E, G and I). In insulin-treated animals, *Cart* mRNA expression in the ARC was reduced (P<0.05) in ADX and ADX+B groups, compared to sham group (Fig. 8D). In insulin-treated animals, expression of *Crf* mRNA in the PVN was also augmented in the ADX group, with no changes in *Pomc*, *Npy* and *Agrp* mRNA expression (Fig. 8B, F and H).

Discussion

The present study addressed the mechanisms by which glucocorticoids are involved in the central treatment of insulin on food intake. First, we observed that the ADX group had increased levels of insulin signaling

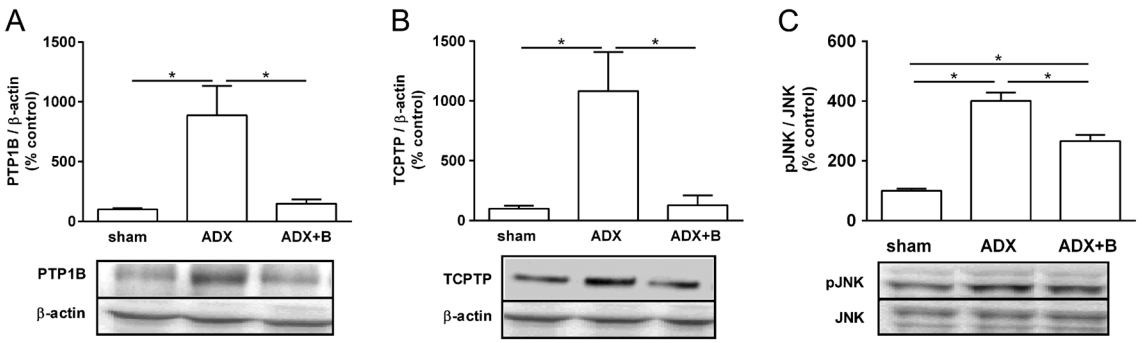

Figure 3
Percentage of protein tyrosine phosphatase-1B (PTP1B) (A), T cell protein tyrosine phosphatase (TCPTP) (B) and Phospho-c-Jun N-terminal Kinase (pJNK) (C) levels in the mediobasal hypothalamus of sham, ADX and ADX + B animals (n = 5–8 rats/group). Data are shown as means ± S.E.M. *P < 0.05.

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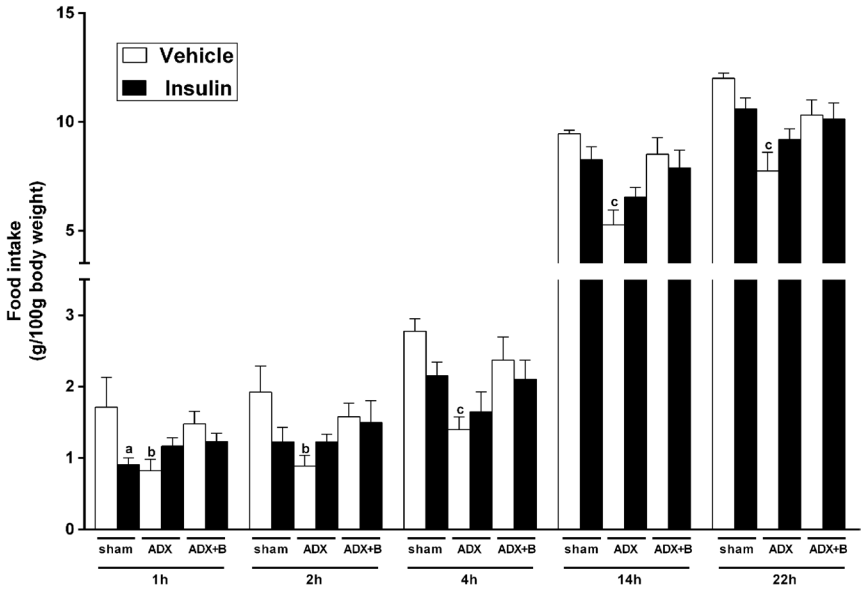

Figure 4
Food intake (g/100 g) after 1, 2, 4, 14 and 22 h of food access (n = 5–7 rats/group) of sham, ADX and ADX + B animals treated with central vehicle (0.9 NaCl/5 µL) or insulin (12 µM/5 µL). Data are shown as means ± S.E.M. $^{a}P < 0.05$ vs insulin, $^{b}P < 0.05$ vs respective ADX group, $^{c}P < 0.05$ vs respective ADX and ADX + B groups.

counter-regulators pJNK, PTP1B and TCPTP, and reduced expression of *InsR* in the hypothalamus. Moreover, hypothalamic expression of *InsR* mRNA was shown to be positively correlated with plasma corticosterone levels, and hypophagia induced by central insulin injection was absent after ADX. Finally, ADX abolished the insulin-induced pAKT and *Cart* mRNA expression and the decrease of *Npy* mRNA expression in the hypothalamus.

In vehicle animals, ADX-induced increase in PVN *Crf* mRNA and reductions in *Pomc*, *Cart*, *Npy* and *Agrp* mRNA in the ARC is well established in the literature, since ADX is known to reduce the expression of orexigenic neuropeptides *Npy* and *Agrp* (Savontaus *et al.* 2002, Uchoa *et al.* 2012) and to increase the expression of anorexigenic neuropeptide *Crf* (Uchoa *et al.* 2010). In addition, the reduction of *Pomc* and *Cart* induced by ADX had already been reported by different works, probably to due lower levels of plasma insulin and leptin in these animals (Savontaus *et al.* 2002, Germano *et al.* 2007, Uchoa *et al.* 2012). As observed, corticosterone treatment was not effective to restore all the effects induced by ADX, and this phenomenon may be ascribed to the experimental design, since rats eat and drink mostly during the night. As the experiments were conducted at the end of the day, it is likely that circulating plasma levels of corticosterone of ADX + B animals were not as high as sham group because they stayed the last hours without drinking fluid, although B was available throughout the day. This hypothesis is confirmed by the plasma levels of corticosterone, where sham group had upper corticosterone concentrations than ADX + B group, and both groups showed higher values than ADX group. In addition, it cannot be ruled out the possibility that other hormones of adrenal glands, as mineralocorticoids, could also participate on these responses. In fact, replacement with mineralocorticoid to ADX rats, in addition to glucocorticoid replacement

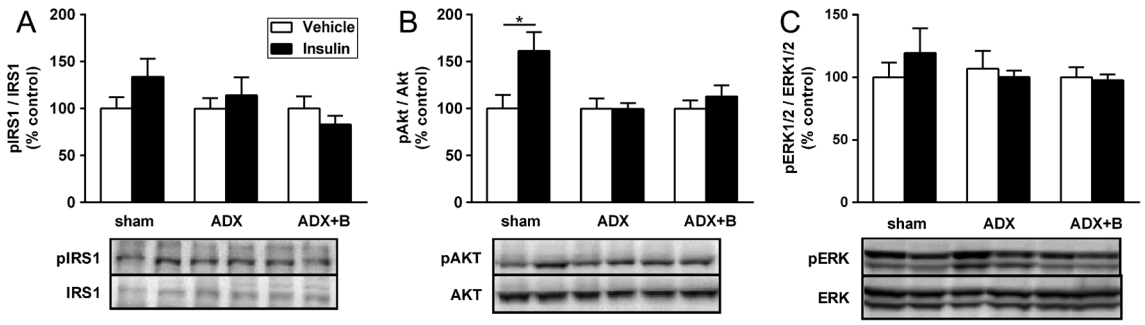

Figure 5
Levels of phospho-insulin receptor substrate (IRS) 1 (A), phospho-protein kinase B (AKT) (B) and phospho-extracellular-signal-regulated kinase (ERK1/2) (C) in the mediobasal hypothalamus of sham, ADX and ADX + B animals 15 min after the icv injection of vehicle (0.9% NaCl/5 µL) or insulin (12 µM/5 µL) (n = 5–8 rats/group). Vehicle in each group was used as calibrator. Data are shown as means ± S.E.M. $^{*}P < 0.05$.

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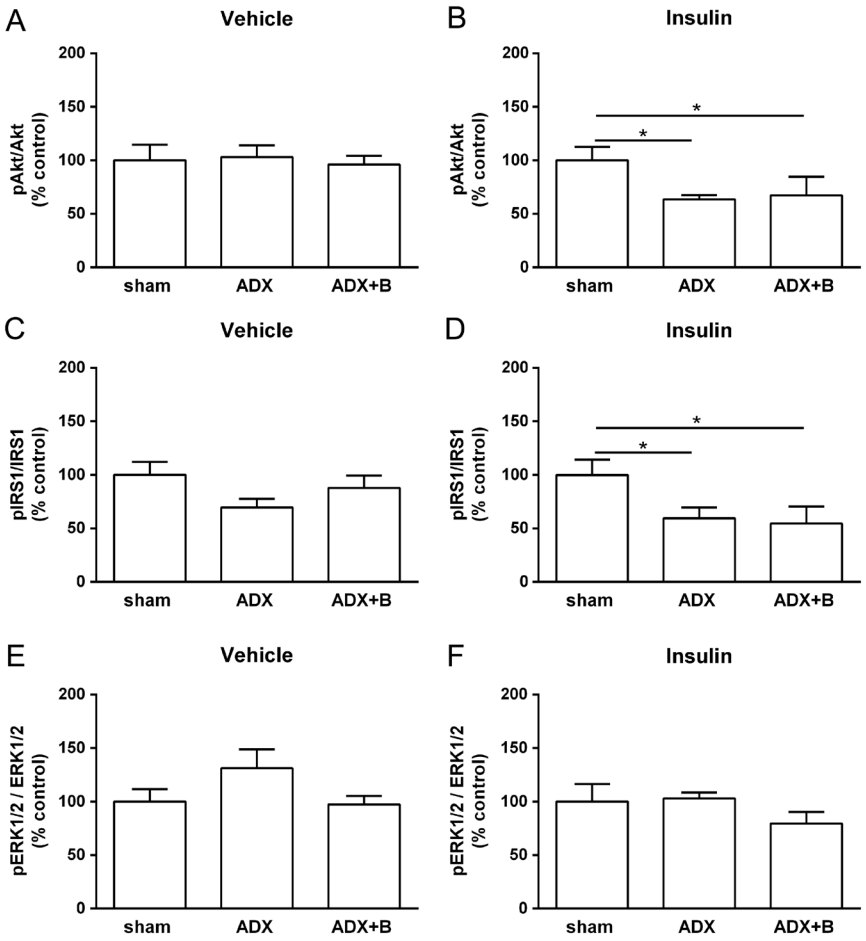

Figure 6
Levels of phospho-insulin receptor substrate (IRS) 1 (A), phospho-protein kinase B (AKT) (B) and phospho-extracellular-signal-regulated kinase (ERK1/2) (C) in the mediobasal hypothalamus of sham, ADX and ADX + B animals 15 min after the icv injection of vehicle (0.9% NaCl/5 µL) (A, C and E) or insulin (12 µM/5 µL) (n = 5–11 rats/group) (B, D and F). (n = 5–8 rats/group). Respective sham group was used as calibrator in each treatment. Data are shown as means ± S.E.M. *P < 0.05.

(aldosterone + cortiscosterone), was effective in boosting food intake and body weight gain beyond that of only corticosterone replacement (Devenport *et al.* 1983, 1985). In addition, aldosterone replacement to ADX animals restored daily caloric intake, specially lipid intake, and body fat deposition, and this preferential role in fat balance is most clearly seen during the later hours of the feeding cycle (Devenport & Devenport 1982, Tempel & Leibowitz 1989). Altogether, these data demonstrated that, besides corticosterone, aldosterone is another adrenal hormone in controlling body weight gain and food intake in rats.

Insulin-induced reduction in food intake and increase in the phosphorylation of AKT in the hypothalamus observed in sham animals is supported by previous work of Niswender *et al.* (2003), who reported that hypophagic effect of central treatment of insulin is mediated by PI3K pathway, which recruits IRS/AKT cascade in the hypothalamus. Although no significant trend in insulin treatment to increase IRS1 phosphorylation, sham animals showed higher levels of pIRS1 levels than ADX and ADX + B animals after insulin treatment, suggesting that IRS1 may contribute to hypophagia induced by insulin in sham group. However, as IRS2 has been suggested to have pivotal role in insulin effects on energy homeostasis, it is also reasonable to suggest that IRS2 may also be involved in insulin-induced activation of IRS/PI3K/AKT signaling pathway (Pardini *et al.* 2006). In addition, insulin-induced hypophagia in sham group was associated with enhancement of *Cart* mRNA expression in the ARC as well as reduction of *Npy* mRNA in this nucleus. Indeed, insulin is known to decrease *Npy* expression in the ARC and its release in the PVN (Schwartz *et al.* 1991, 1992, Wang & Leibowitz 1997), as well as to increase *Cart* expression in the hypothalamus (Honda *et al.* 2007). Moreover, diabetic animals, with insulin deficiency, also show reduced *Cart* expression in the ARC (Li *et al.* 2002), reinforcing the present findings. Additionally, studies from the literature reported that insulin was also able to increase *Pomc* and *Crf* expression in the hypothalamus and reduce *Agrp* expression; thus, the lack of changes in the expression of *Pomc*, *Agrp* and *Crf* mRNA after central treatment with insulin may be due to experimental differences (Benoit *et al.* 2002, Chan *et al.* 2005).

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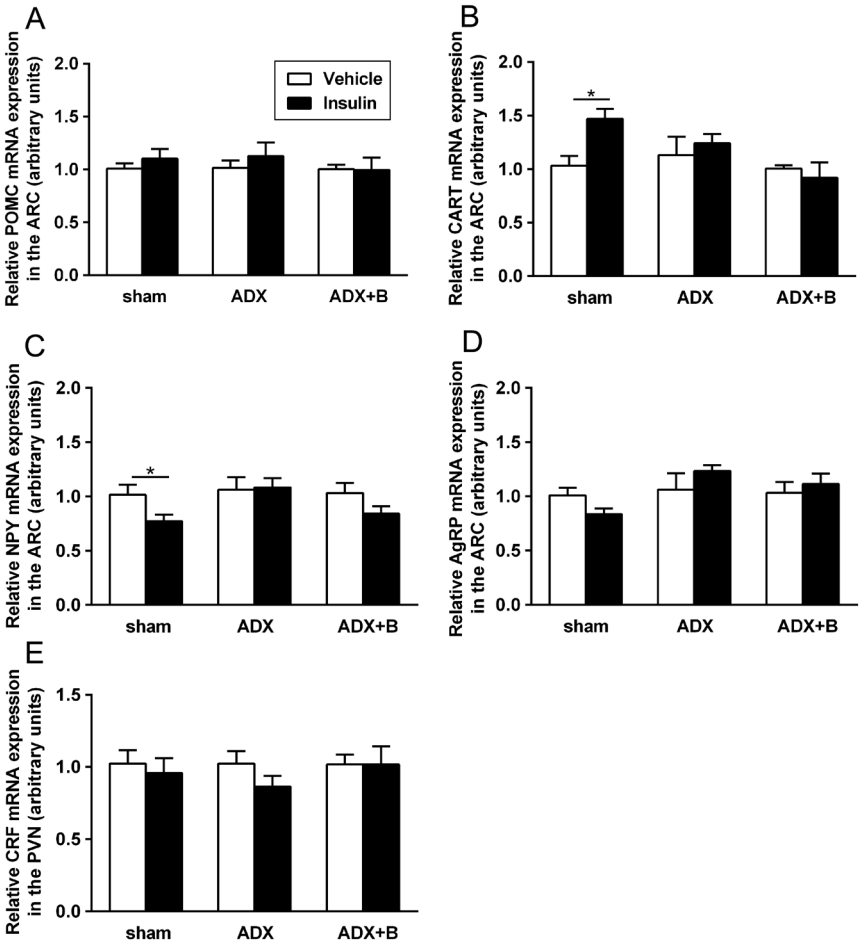

Figure 7
Relative mRNA expression of proopiomelanocortin (*Pomc*) (A), cocaine and amphetamine-regulated transcript (*Cart*) (B), neuropeptide Y (*Npy*) (C), agouti-related protein (*Agrp*) (D) in the arcuate nucleus of the hypothalamus (ARC) and corticotrophin-releasing factor (*Crf*) (E) in the paraventricular nucleus of the hypothalamus (PVN) of sham, ADX and ADX + B animals 120 min after the icv injection of vehicle (0.9% NaCl/5 µL) or insulin (12 µM/5 µL) (n = 5–11 rats/group). Vehicle in each group was used as calibrator. Data are shown as mean ± S.E.M. *P < 0.05.

It is known that insulin effects, both in the periphery and CNS, have been classically ascribed to insulin binding to its receptor in the membrane, a tyrosine kinase receptor which autophosphorylate itself in the internal surface of the cell upon insulin binding, and thereby activates its intrinsic tyrosine kinase activity, resulting in phosphorylation of IRS proteins on tyrosine residues. This phosphorylation can activate PI3K and/or MAPK pathways (Niswender & Schwartz 2003, Plum *et al.* 2005, Vogt & Brüning 2013). Activated PI3K rapidly mediates the phosphorylation of PI 4,5-biphosphate to PI 3,4,5-triphosphate, a key signaling intermediate that recruits and activates downstream molecules, including phosphoinositide-dependent kinase 1, which in turn phosphorylates and thus activates the kinase AKT to elicit downstream signaling events, as activation and/or repression of target genes encoding hypothalamic neuropeptides involved in the regulation of food intake (Niswender & Schwartz 2003, Plum *et al.* 2005, Vogt & Brüning 2013). In this context, Yang *et al.* (2010) demonstrated, by means of eletrophysiological approaches, that insulin-induced reduction in the activity of *Npy* neurons in the ARC is mediated by IRS/PI3K/AKT pathway, but not by MAPK signaling, reinforcing that insulin-mediated activation of the MAPK/ERK pathway in the control of food intake and energy homeostasis is not well established. On the other hand, Mayer and Belsham (2009) have shown, using hypothalamic neuronal cell line, that insulin regulates *Npy* and *Agrp* expression through MAPK pathway but not by IRS/PI3K/AKT signaling. Accordingly, it can be suggested that insulin is likely to reduce food intake by recruiting hypothalamic IRS/PI3K/AKT pathway, and thus modulates hypothalamic expression of neuropeptides involved in the control of food intake, stimulating the anorexigenic neuropeptide *Cart* and reducing the orexigenic neuropeptide *Npy*.

Impaired insulin signaling in the hypothalamus, as observed by the absence of phosphorylation of AKT and changes in the expression of *Cart* and *Npy* mRNA in response to insulin treatment in ADX animals, is likely to underlie the lack of anorexigenic effect of insulin after ADX. Indeed, one possible mechanism for this impairment of insulin effects after ADX may be ascribed to lower

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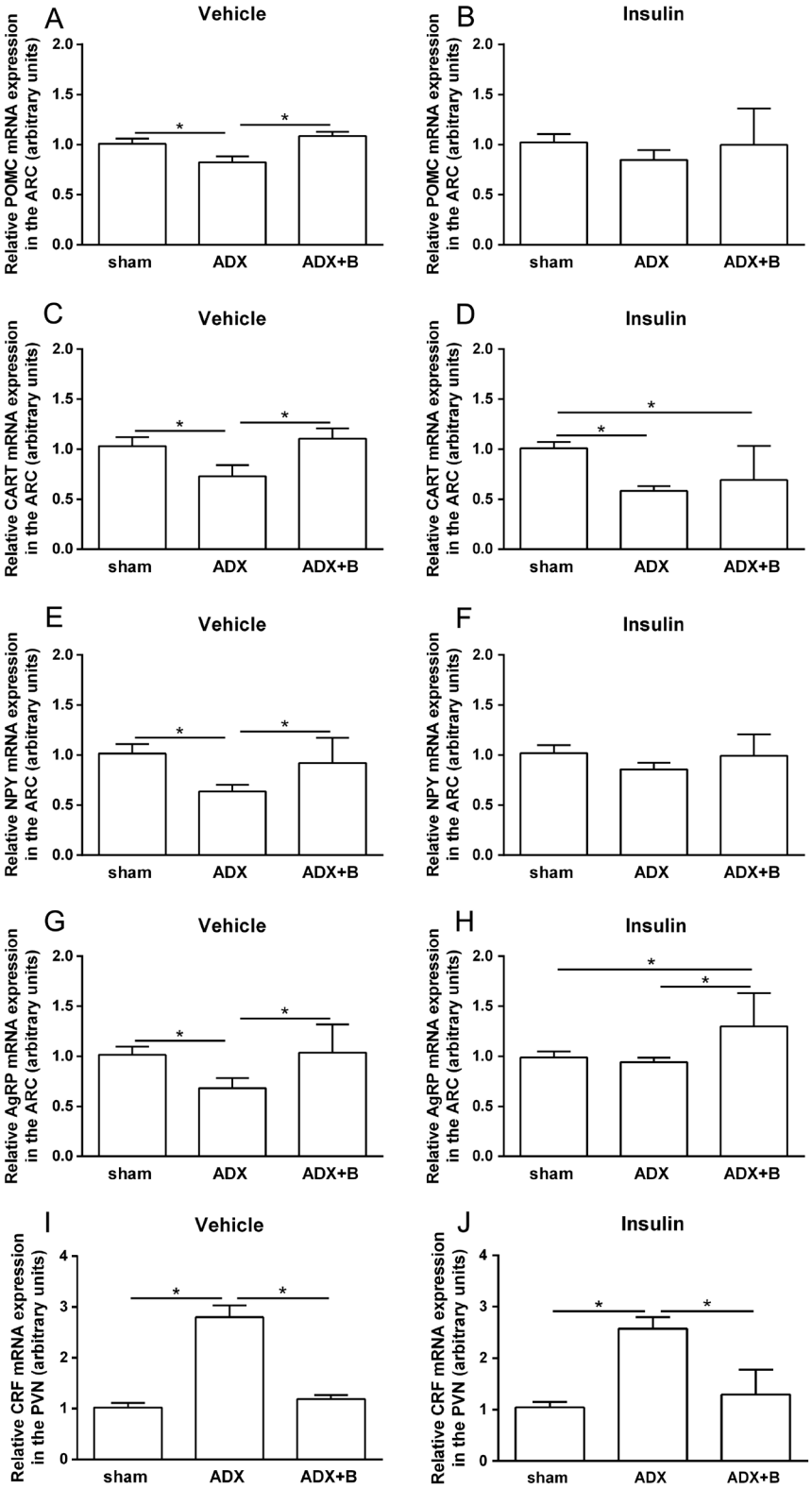

Figure 8
Relative mRNA expression of proopiomelanocortin (*Pomc*) (A), cocaine and amphetamine-regulated transcript (*Cart*) (B), neuropeptide Y (*Npy*) (C), agouti-related protein (*Agrp*) (D) in the arcuate nucleus of the hypothalamus (ARC) and corticotrophin-releasing factor (*Crf*) (E) in the paraventricular nucleus of the hypothalamus (PVN) of sham, ADX and ADX + B animals 120 min after the icv injection of vehicle (0.9% NaCl/5 µL) (A, C, E, G and I) or insulin (12 µM/5 µL) (n = 5–11 rats/group) (B, D, F, H and J). Respective sham group was used as calibrator in each treatment. Data are shown as mean ± S.E.M. *P < 0.05.

expression of *InsR* in the hypothalamus in ADX animals, which might compromise the IRS/PI3K/AKT pathway. In this context, the reduction of *InsR* mRNA expression in different nuclei of hypothalamus is supported by *in vitro* experiments demonstrating that glucocorticoids increased *InsR* expression in different cell lines, as well by *in vivo* data

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that showed that the glucocorticoid prednisone increased the number of *InsR* on circulating monocytes in normal subjects (Beck-Nielsen *et al.* 1980, Iwama *et al.* 1987, McDonald *et al.* 1987, Mamula *et al.* 1990, Desbuquois *et al.* 1993). Accordingly, the positive correlation between plasma corticosterone levels and *InsR* mRNA expression in the hypothalamus indicates that glucocorticoids seem to stimulate the expression of *InsR*, not only *in vitro*, but also in *in vivo*, as suggested in the current study. In addition, glucocorticoids were shown to mediate phosphorylation of retinal AKT induced by stress (Forkwa *et al.* 2014). Thus, it is likely that circulating glucocorticoids are required for hypothalamic expression of *InsR* and consequently for insulin-induced activation of AKT signaling pathway and changes in neuropeptides expression in the hypothalamus, as well as for the anorexigenic actions of insulin.

On the other hand, Chavez *et al.* (1997) demonstrated that ADX enhanced the sensitivity to central insulin effects on food intake, since continuous infusion of insulin in the third ventricle was able to reduce food intake only in ADX animals, without effects in the adrenal-intact group. The difference between these data and the present study might be due to methodological and protocol differences, such as injection site and duration of the treatment. In addition to insulin, the interaction of ADX with other peripheral hormones, as leptin and CCK, was described in previous studies. Concerning leptin, the interaction with ADX remains controversial, since several studies have pointed different results. Pioneer studies in this field have pointed that the absence of glucocorticoids increases the brain's sensitivity to leptin to decrease food intake and body weight (Zakrzewska *et al.* 1997, Madiehe *et al.* 2001), while another study of Arvaniti *et al.* (1998) showed that the ability of leptin to inhibit body gains in energy and fat is not dependent on the presence of corticosterone. On the other hand, another study reports that leptin has a more pronounced effect on weight loss in ADX rats with CORT replacement than it does on ADX rats without CORT replacement (Gemmill *et al.* 2003). Concerning CCK, similarly to the interaction with insulin in the present manuscript, ADX was shown to abolish CCK-induced hypophagia, probably because CCK could not further reduce food intake more than ADX has already reduced, to preserve the energy homeostasis of the organism (Uchoa *et al.* 2009*a*). In addition, CCK-induced activation of NTS neurons, but, interestingly, it did not further reduce food intake in the ADX group, demonstrating that the unchanged food intake in ADX animals following CCK administration was dissociated from the increased Fos expression in NTS neurons (Raboin *et al.* 2006, Uchoa *et al.* 2009*a*). Overall, these responses occur in an attempt to preserve energy homeostasis in the presence of glucocorticoid deficiency.

It is known that PTP1B and TCPTP are PTPs that attenuate insulin signaling by dephosphorylating tyrosine phosphorylated in insulin receptor or IRS (Zhang *et al.* 2015), while JNK phosphorylates serine residues of IRS-1, blocking the interaction between the IRS-1 domain and the insulin receptor (Aguirre *et al.* 2000, Belgardt *et al.* 2010). Thus, higher levels of the counter-regulators of insulin signaling, pJNK, PTP1B and TCPTP observed after ADX may also induce the impaired actions of IRS/PI3K/AKT pathway in ADX animals. Accordingly, glucocorticoids have been demonstrated to reduce the phosphorylation of JNK in different tissues, since JNK has pro-inflammatory effects, while glucocorticoids are classically known to exert anti-inflammatory actions (Adcock & Caramori 2001, Motta *et al.* 2015). However, this is the first work to demonstrate that ADX increase the levels of PTP1B and TCPTP. Though it is well established in the literature that glucocorticoids counter-regulate insulin actions in energy homeostasis in peripheral tissues (Olefsky 1975, Haluzik *et al.* 2002, Long *et al.* 2003), the present study demonstrates for the first time that glucocorticoid withdrawal induced by ADX affects insulin signaling molecules in the hypothalamus. Thus, we postulate that the mechanisms by which adrenalectomy abolishes hypothalamic responses to insulin treatment involve the reduction in the expression of *InsR* and increases in the expression of counter-regulators of insulin signaling pJNK, PTP1B and TCPTP in the hypothalamus. These alterations lead to an impaired AKT signaling pathway as well as changes in neuropeptides expression (*Npy* and *Cart*) in the hypothalamus, then abolishing hypophagia induced by insulin (Fig. 9).

Finally, it is important to mention that insulin increased *Cart* mRNA expression and reduced *Npy* mRNA expression in the ARC, and that ADX abolished these effects; therefore, this nucleus could be considered as a key area for the effects of insulin on food intake regulation. However, changes in the medial basal hypothalamus were also detected after ADX, as enhancement of pJNK, PTP1B and TCPTP as well as reductions in the expression of insulin receptor in other nuclei than ARC, suggesting that these other regions are also important to explain the results. Food intake is regulated by a complex circuitry comprising different hypothalamic nuclei as other extra-hypothalamic area (Morton *et al.* 2006); therefore, signaling pathways from other sites than ARC, such as PVN and NTS could be also affected by ADX. To ascertain

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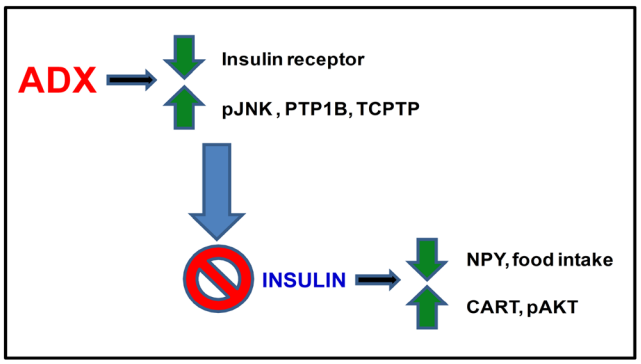

Figure 9
Schematic diagram demonstrating that adrenalectomy (ADX) reduces the expression of insulin receptor and increases the levels of the counter-regulators of insulin signaling phosphorylated c-Jun N-terminal kinase (pJNK), protein tyrosine phosphatase-1B (PTP1B) and T cell protein tyrosine phosphatase (TCPTP), which are likely to yield impairment of insulin-induced phosphorylation of protein kinase B (pAKT) in the hypothalamus, insulin-induced changes mRNA expression of cocaine and amphetamine-regulated transcript (*Cart*) and neuropeptide Y (*Npy*) in the arcuate nucleus of the hypothalamus and hypophagia induced by insulin. A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0217.

this complex neuron network, specific or conditioned gene manipulation would help to better dissect the most prominent neuronal pathway involved in the impaired sensitivity to insulin after ADX in future studies.

In summary, the most remarkable outcome of the present study was that central administration of insulin requires the presence of intact adrenal to reduce food intake, in addition to the mechanisms underlying this interaction between glucocorticoid and insulin action in the hypothalamus. In this context, it is noteworthy that reduced expression of *InsR* and enhanced levels of its counter-regulator molecules, JNK, PTP1B and TCPTP in the hypothalamus may underlie the mechanisms by which ADX impairs insulin-induced recruitment of IRS1/AKT signaling pathway. These effects may be important to counteract the effects of glucocorticoid deficiency on food intake to prevent a more profound hypophagia and to preserve the homeostasis of the animals.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Statement of ethics
All experimental procedures were approved by the Ethical Committee for Animal Use of the School of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil (protocol number 092/2011).

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