MARIA FERNANDA CONDES AREIAS

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Orientador (a) : Profa. Dra. Patrícia de Oliveira Prada

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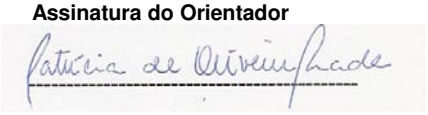

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RESUMO

A insulina tem efeitos anorexigênicos, reduzindo o peso corporal. Entretanto, a maior parte dos estudos teve como foco a ação e sinalização de insulina no hipotálamo. Assim, o **primeiro objetivo do trabalho foi investigar a expressão e grau de fosforilação das proteínas da via de sinalização de insulina (IR/Akt), assim como a modulação da ingestão alimentar após estímulo com insulina na região da amígdala em animais controles.** No **segundo objetivo, investigamos se o bloqueio farmacológico da via da insulina com LY24002 na amígdala alterou a ingestão alimentar em resposta à insulina.** O consumo de dieta hiperlipídica tem sido associado à resistência à insulina no hipotálamo. Assim, o **terceiro objetivo foi investigar se a obesidade induz resistência à insulina nessa região e em adição investigar se a via inflamatória IKK/NFkB e o ER *stress* estavam alterados em amígdala de animais obesos.** Obervou-se que após a injeção de insulina na amígdala, não houve diferença no peso corpóreo após 24 horas em animais controles. Em relação a ingestão alimentar, quatro horas após a injeção de insulina na amígdala, não houve diferença, entretanto, após 8, 12 e 24 horas houve uma diminuição na ingestão alimentar em animais controles. Após o bloqueio da PI3q com o inibidor farmacológico LY (240002), trinta minutos antes da injeção de insulina, observou-se que a insulina não foi capaz de reduzir a ingestão alimentar. Verificou-se, após a injeção de insulina na região da amígdala, um aumento da fosforilação do receptor de insulina (IR) e da AKT em animais controles. Para confirmamação da dissecção da amígdala, testou-se a expressão proteica FKBP5, que é expressa especificamente na região da amigdala. Os animais foram submetidos à dieta hiperlipídica e padrão por 3 meses e realizou-se um ITT (Teste Tolerância Insulina) para verificar a resistência à insulina dos animais. Observou-se que os animais obesos ficaram resistentes à insulina. Em relação ao peso corpóreo, houve um aumento de peso entre os obesos. Já em relação a ingestão alimentar, não houve diferença na ingestão após 4, 8, 12 e 24 horas entre os animais obesos. Após a injeção de insulina na região da amígdala, não observou-se diferença na fosforilação da AKT de animais obesos quando comparada ao controle. Observou-se um aumento da fosforilação da JNK, da IKK alfa e beta, além da PERK e IRE1 no grupo submetido a dieta hiperlipídica quando comparada ao controle. Sendo assim, concluí-se que: o controle da ingestão alimentar é realizado, pelo menos em parte, pela ação da insulina na amígdala; e que o efeito da insulina na amígdala é via IR/PI3q/Akt. Os animais obesos apresentam resistência à ação da insulina na amígdala e esta é associada à redução da ativação da via IR/Akt. Acredita-se também que a dieta hiperlipídica aumenta a ativação, na amígdala, de serinas quinases que participam da via inflamatória e do stress do retículo endoplasmático, o que pode justificar a resistência à insulina.

ABSTRACT

Insulin has anorexigenic effects, reducing body weight. However most part of the studies have focused on hypothalamic insulin signaling. Thus the aim of the study was to investigated if insulin activates IR/Akt pathway in the amygdala and if this activaction controls feeding.Besides this, it was investigated if the inibition of PI3K by LY294002 alters food intake. In obese animals models insulin induce IR/PI3K/Akt pathway is impaired in the hypothalamus. Low grade inflammation characterized at celular level by na activation of IKK/IKB/NF-KB pathway and JNK activation, and also induce of endoplasmatic reticulum (ER) stress are molecular mechanisms involved in insulin resistence in the hypothalamus of obese roedents. So, it was invetigated if high fat diet impairs insulin signaling and action in the amygdala and if low grade inflammation and ER stress were present in this brain region. Food intake is decreased and IR and Akt phosphorylation were increased in response to insulin in the amygdala of control rats. In order to confirm whether the dissections of amygdala were corrected, the membranes with anti-co-chaperone FK506 binding protein 51 (FKBP5) antibody were re-blotted. FKBP51 is expressed in the amygdala region but not in striatum. It was observed the presence of FKBP5 in their membranes, indicating that the dissections of amygdala were appropriated. The administration of LY prior to insulin in the amygdala abolished this effect and also impaired Akt not IR phosphorylation in response to insulin. Body weight was increased in rats fed with HFD compared to control rats. Insulin tolerance test showed that obese rats were insulin resistant in comparison to control rats. Insulin injected in the amygdala did not decrease food intake after 4, 8, 12, 24 h.The IR and Akt phosphorylation were blunted in rats on HFD.The IKK and JNK phosphorylation were also increased in the amygdala of obese rats. In order to evaluate ER stress, it was investigated whether high fat feeding alters the protein expression of RNA-activated protein kinase-like ER resident kinase (PERK) and inositol-requiring kinase (IRE1). PERK and IRE1 protein expression were increased in the amygdala of rats fed a HFD compared to control rats, suggesting increased ER stress in the amygdala of obese rats Summing up, it was suggested that amygdala is an important region for food intake regulation in response to insulin and this regulation is disrupted in obese rats. It was also shown that food intake is regulated in a PI3K/Akt manner in the amygdala similarly to what occurs in the hypothalamus. Besides, data were provided suggesting that obese rats may have low grade inflammation and ER stress in parallel to insulin resistance in the amygdala.

LISTA DE SIGLAS E ABREVIAÇÕES

IR – insulin receptor

AKT – protein kinase B

NFκB - nuclear factor kappa B

IκB - inhibitor of nuclear factor kappa B

IKK- inhibitor of nuclear factor kappa B kinase

ER stress – endoplasmic reticulum stress

PI3K – phosphatidylinositol 3-kinase

FKBP51- peptidyl-prolyl cis-trans isomerase

ITT- Teste Tolerância Insulina

JNK- *c-jun N-terminal kinase*

PERK - eukaryotic translation initiation factor 2 alpha kinase 3 / Pancreatic eIF2-alpha kinase

IRE1- inositol requiring enzyme 1

OMS- Organização Mundial de Saúde

IBGE- Instituto Brasileiro Geografia Estatística

IRS- insulin receptor substrate

Foxo1- Forkhead box O 1

AgRP- Agouti-related peptide

POMC- Pro-opiomelanocortin

JAK2- janus quinase-2

STAT3 - signal transducer and activator of transcription 3

VMH- ventromedial hypothalamus

VTA – ventral tegmental area

NaC- nucleus accubens

Th- tirosina hidroxilase

FMRI – functional magnetic resonance imaging

CeA – central amygdala

SUMÁRIO

1. INTRODUÇÃO

A obesidade é caracterizada por um excesso de gordura acumulada no tecido adiposo e demais órgãos. A Organização Mundial de Saúde (OMS) estimou que em 2008, 1,5 bilhões de adultos com idade superior a 20 anos estavam acima do peso e mais de 200 milhões de homens e 300 milhões de mulheres, aproximadamente 10% da população adulta, eram obesos. Em 2010, 43 milhões de crianças com idade inferior a 5 anos estavam acima do peso (AHIMA, 2011).

No Brasil, dados do IBGE revelam que o país passa por uma transição nutricional, em que a desnutrição infantil se torna cada vez mais rara e a obesidade, a partir da adolescência, cresceu de forma alarmante de 5,7% em 1974-75 para 16,7% em 2002-03 em todas as regiões e extratos econômicos (GUIMARÃES, 2007).

O aumento da obesidade está associado ao aumento de comorbidades relacionadas ao ganho de peso como diabetes, hipertensão, doença coronariana, apnéia do sono e câncer. Além da inatividade física, a obesidade é causada principalmente pelo excesso de ingestão alimentar. Modificações no estilo de vida, na dieta e exercício têm sido recomendadas, porém embora efetivas a curto prazo, as recidivas no ganho de peso são muito frequentes. Além disso, o desenvolvimento de medicamentos para tratar a obesidade vem apresentando baixa eficácia e sérios efeitos colaterais (AHIMA, 2011).

Portanto, o estudo dos mecanismos moleculares relacionados ao desenvolvimento da obesidade é de extrema relevância na atualidade.

O balanço energético é mantido por diversos mecanismos, incluindo sinais metabólicos e hormonais, sinalização celular e molecular e sinais neurais. A identificação de hormônios periféricos como insulina, leptina e outros, assim como nutrientes que atuam em neurônios hipotalâmicos para controlar a homeostase energética tem auxiliado o entendimento dos circuitos neuronais que controlam o peso corporal (BELGARDT et al, 2008; KONNER et al, 2009; PLUM et al, 2005; ROTHER et al., 2008; SANCHEZ-LASHERAS et al, 2010; MORTON et al, 2011; KLOCKENER et al, 2011; KONNER et al, 2011).

A ação da insulina no hipotálamo na regulação do balanço energético tem sido bem descrita na literatura (WHITE, 1997; XU et al., 2005; PLUM et al., 2005). A insulina sinaliza através do seu receptor (IR) que sofre uma modificação conformacional, ativando a subunidade β (WHITE, 1997). Uma vez ativo, este sítio catalisa a fosforilação em tirosina de

proteínas que contém o domínio SH2, como por exemplo, os substratos do receptor de insulina (IRS), principalmente IRS-1 e IRS-2. A fosforilação de IRSs promove a ligação e ativação da enzima fosfatidilinositol-3-quinase (PI3q) que ativa Akt. A Akt ativada fosforila e retira o fator de transcrição Foxo1 do núcleo. A ativação dessas proteínas pela insulina controla o ritmo de disparos neurais (XU et al., 2005), o que é condicionado à ativação de canais de potássio ATP - dependentes. Através do controle do ritmo de disparos neuronais, a insulina modula a liberação de neurotransmissores em sinapses efetoras (PLUM et al., 2005). Além disso, a fosforilação da Foxo1 induzida por insulina tem sido associada com a regulação da expressão dos neuropeptídeos AgRP e POMC, ligados ao controle da homeostase energética. A segunda via pela qual a insulina modula a ingestão alimentar e termogênese depende da ativação da enzima janus quinase-2 (JAK2). Apesar de possuir atividade tirosina quinase intrínseca, o IR é capaz de interagir com, e ativar, a JAK2 principalmente em hipotálamo. Uma vez fosforilada e ativa, a JAK2 recruta e fosforila transdutores-de-sinal-e-ativadores-de-transcrição (STATs), predominantemente STAT3, a qual conecta o sinal da insulina ao controle da transcrição de genes de neurotransmissores envolvidos com o controle da fome e da termogênese.

Estudos realizados em roedores nos últimos 11 anos demonstraram a importância das proteínas da via de sinalização de insulina no sistema nervoso central no controle do balanço energético.

Camundongos *knockouts* para o receptor de insulina especificamente em neurônios do sistema nervoso central desenvolvem obesidade e infertilidade (BRUNNING et al, 2000). Adicionalmente, demonstrou-se que a inibição temporária de IR com oligonucleotídeo antisense em hipotálamo induz hiperfagia e aumento da adiposidade em ratos (OBICI et al, 2002). De maneira similar, a inibição de proteínas da via de sinalização de insulina distais ao IR também está relacionada a alterações na ingestão alimentar. Camundongos *knockouts* para IRS-2 são hiperfágicos e obesos, assim como a inibição da expressão da PI3K ou Foxo1 em hipotálamo alteram a ingestão alimentar de formas variadas (Foxo1, PI3K, VMH e em AgRP) (ROPELLE et al, 2009; BRUNNING et al, 2000).

Embora a maior parte dos estudos tivesse como foco a sinalização de insulina no hipotálamo, recentes evidências têm demonstrado que a insulina tem efeitos diretos em outras regiões do sistema nervoso, tais como área ventral tegmental (VTA), nucleus accumbens (Nac), striatum e amígdala. Regiões, estas conhecidas como parte do sistema

dopaminérgico de recompensa e de regulação da ansiedade (FAROOQI et al, 2007 e TYE et al, 2011).

KONNER et al (2011) demonstraram que o IR é co-expresso com a enzima tirosina hidroxilase (Th) que é um marcador de neurônios dopaminérgicos. Assim, esses autores sugeriram uma potencial ligação entre o controle de ingestão alimentar e o sistema de recompensa através da insulina. Ainda neste artigo, a inativação do IR em células que expressam Th no VTA, através de manipulação gênica, resultou em animais obesos e hiperfágicos (KONNER et al, 2011).

Assim como o VTA, estudos anteriores identificaram a região da amígdala como uma possível área de regulação da ingestão alimentar.

Em humanos, estudos que empregaram ressonância magnética funcional (FMRI) demonstraram que o padrão de resposta na amígdala a estímulos com imagem de alimentos foi similar ao observado no hipotálamo (FLETCHER et al, 2010). Em obesos, observou-se elevada atividade nessa região em resposta a estímulos com imagem de alimentos em indivíduos portadores da síndrome Prader–Willi com obesidade grave (STOECKEL et al, 2008; HOLSEN et al, 2006). A injeção intravenosa de grelina, hormônio orexigênico, aumenta a atividade da amígdala em resposta à imagem de alimentos (MALIK et al, 2008).

Em roedores, estudos demonstraram que lesões na amígdala alteram a ingestão alimentar e o peso corpóreo.

KING et al (1994) verificaram que lesões bilaterais na região postero-dorsal da amígdala resultavam em hiperfagia e excesso de ganho de peso em ratas. Em outro estudo, KING et al (1996) observaram que em paralelo à hiperfagia e ganho de peso, as lesões da amígdala induziam hiperinsulinemia mesmo em ratas alimentadas com dieta padrão.

Mais recentemente, BOGHOSSIAN et al (2009) demonstraram que a administração de injeções de insulina na região do CeA (núcleo central da amígdala) não induziam anorexia em ratos Sprague-Dawley recebendo dieta hiperlipídica. Assim, sugeriram que a amígdala é uma região importante para o controle da ingestão alimentar pela insulina e que animais obesos têm alteração dessa regulação (BOGHOSSIAN et al, 2009). De fato, a amígdala expressa IR em abundância (HAJNAL et al, 1998; BOGHOSSIAN et al 2009), no

entanto, a sinalização desse hormônio ainda não foi investigada nessa região do sistema nervoso central.

Portanto, seria interessante investigar se os mesmos eventos de sinalização insulínica também ocorrem na amígdala e se esta sinalização está envolvida na regulação da ingestão alimentar. Nesse sentido, **o primeiro objetivo do presente estudo é investigar a expressão e grau de fosforilação das proteínas da via de sinalização de insulina, assim como a modulação da ingestão alimentar após estimulo com insulina na região da amígdala em animais controles.** De forma complementar, **o segundo objetivo do estudo é investigar se o bloqueio farmacológico da via da PI3K com (LY24002) na amígdala pode alterar a resposta à insulina quanto à ingestão alimentar e sinalização de insulina intracelular de animais controles.**

O consumo de dieta hiperlipídica tem sido associado à resistência à insulina no hipotálamo (PRADA, et al 2005; PICARDI et al, 2009). Os estudos sugerem que esta resistência é decorrente da redução da ativação da via IRSs/PI3q/Akt nessa região do sistema nervoso (PRADA, et al 2005; PICARDI et al, 2009).

Nas últimas décadas, estudos têm demonstrado que a obesidade produz um estado de inflamação crônica subclínica, caracterizado por níveis circulantes elevados de citocinas pro-inflamatórias e infiltração de macrófagos no tecido adiposo (HOTAMISLIGIL, 2006). Muitas destas citocinas liberadas bloqueiam a ação da insulina em vários tecidos, incluindo hipotálamo. Um dos mecanismos moleculares responsáveis pelo desencadeamento de resistência à insulina é a ativação da via inflamatória IKK/IκB/NFκB (HOTAMISLIGIL, 2006). No hipotálamo, ativação da via IKK/IκB/NFκB e resistência à insulina ocorre devido a IKK*beta* ativada induzir a fosforilação em serina dos IRSs. Este evento bloqueia o sinal da insulina e constitui um dos elementos indutores de resistência hipotalâmica à insulina desencadeada por obesidade (ZHANG et al, 2009). Em paralelo à ativação da via inflamatória, a ativação do *stress* de retículo endoplasmático (RE), por excesso no consumo de calorias, tem sido descrita como outro mecanismo desencadeador de resistência à insulina. O RE induz a ativação da via inflamatória IKK/IkB/NFkB e também da serina quinase JNK (c-Jun N-terminal kinase) que inibem o sinal da insulina (EIZIRIK et al, 2008; HOTAMISLIGIL, 2006, 2010; ZHANG et al, 2009).

Assim, o **terceiro objetivo do presente estudo é investigar a expressão e grau de fosforilação das proteínas da via de sinalização de insulina, assim como a modulação da ingestão alimentar após estimulo com insulina na região da amígdala em animais com obesidade induzida por dieta hiperlipídica.** Se houver redução da ativação das proteínas da via de sinalização de insulina em amígdala de animais obesos, iremos investigar o mecanismo pelo qual ocorre essa redução. Dessa forma, o **quarto objetivo do trabalho é investigar se a via inflamatória IKK/IkB/NFkB e o *stress* de RE estão alterados em amígdala de animais obesos.**

2. OBJETIVOS

1) Investigar a expressão e grau de fosforilação das proteínas da via de sinalização de insulina (IR/ /Akt), assim como a modulação da ingestão alimentar após estímulo com insulina na região da amígdala em animais controles.

2) Investigar se o bloqueio farmacológico da via da PI3K com (LY24002) na amígdala pode alterar a resposta à insulina quanto à ingestão alimentar e sinalização de insulina intracelular de animais controles.

3) Investigar a expressão e grau de fosforilação das proteínas da via de sinalização de insulina, assim como a modulação da ingestão alimentar após estímulo com insulina na região da amígdala em animais com obesidade induzida por dieta.

4) Se houver redução da ativação das proteínas da via de sinalização de insulina em amígdala de animais obesos: investigar a ativação e regulação das proteínas da via inflamatória e do stress do retículo endoplasmático em amígdala de animais obesos.

3. ARTIGO CIENTÍFICO

DIET-INDUCED OBESITY INDUCES ENDOPLASMIC RETICULUM STRESS AND INSULIN RESISTANCE IN AMYGDALA OF RATS

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KEY WORDS: insulin, amygdala, Phosphatidylinositol 3-kinase, obesity, inflammation, endoplasmic reticulum stress

ABSTRACT

Insulin acts in hypothalamus decreasing food intake (FI) by IR/PI3K/Akt pathway. This pathway is impaired in obese animals and endoplasmic reticulum (ER) stress and low grade inflammation are possible mechanisms involved in this impairment. Here, we highlighted amygdala as an important brain site of FI regulation in response to insulin. This regulation was dependent on PI3K/Akt pathway similarly what occurs in the hypothalamus. In addition, obese rats did not reduce FI in response to insulin and had decreased Akt phosphorylation in amygdala, suggesting insulin resistance. Insulin resistance was associated with ER stress and low grade inflammation in this brain region.

HIGHLIGHTS

- Lower food intake in response to insulin in amygdala dependents on PI3K/Akt pathway.
- Insulin receptor and Akt phosphorylation in amygdala are disrupted in obese rats.
- Insulin resistance, ER stress and inflammation are present in amygdala of obese rats.

1. Introduction

Insulin is an important signal to the brain controlling food intake and energy expenditure [1-3]. Deletion of the insulin receptor (IR) in the central nervous system (CNS) of mice induced obesity and altered metabolism *in vivo* [3,4]. Most studies consider the hypothalamus as the main region in the CNS which regulates energetic metabolism in response to insulin. However, insulin has effects in other brain regions, such as ventral tegmental area, substantia nigra, and amygdala [5-10].

In the hypothalamus, insulin acts through IR inducing insulin substrate 1 (IRS-1) tyrosine phosphorylation. IRS-1 tyrosine phosphorylation activates phosphoinositide 3- kinase (PI3K), which is required for the effects of insulin on feeding [11]. PI3K phosphorylates generates phosphatidylinositol-3,4,5-triphosphate [3-5], which activates protein kinase B (PKB or Akt). The effect of IR/PI3K/Akt pathway on food intake is well described in the hypothalamic nuclei [10-12]. However, whether insulin signaling in amygdala is important to control feeding is not known.

In obese animal models insulin-induced IR/PI3K/Akt pathway is impaired in the hypothalamus. Endoplasmic reticulum (ER) stress and low grade inflammation (LGI) are possible molecular mechanisms involved in this impairment. Both ER stress and LGI activate serine kinases such as c-Jun N-terminal kinase (JNK) and I kappa B kinase (IKKβ) which induce inhibitory IRS-1 serine 307 phosphorylation trigging hypothalamic insulin resistance in obese states [12,13]. However, whether a high fat diet induces LGI and/or ER stress in amygdala is not known.

Thus, the aim of the present study was to investigate whether insulin activates IR/PI3K/Akt pathway in the amygdala and whether this activation controls feeding. In addition, we aimed to investigate whether a high fat diet induces ER stress and LGI in parallel to insulin resistance in amygdala.

2. Materials and Methods

2.1. Materials

Eight week old male Wistar rats were obtained from Central Breeding Center of the State University of Campinas, São Paulo, Brazil. Human recombinant insulin was from Eli Lilly and Co. (Indianapolis, Indiana, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless specified elsewhere. Antibodies against beta-actin, phospho-IR, phospho-JNK, phospho-IKKα/β and phosphor-PERK were from Santa Cruz Biotechnology, Inc. Phospho-IRE1 alpha was from Novus Biologicals. Phospho-Akt, FKBP51 were from Cell Signaling Technology.

2.2. Animal Characterization

All experiments were approved by the Ethics Committee of the State University of Campinas. Eight week old male Wistar rats were maintained in cycles of 12h dark/light at 21 ºC. Animals were randomly divided into two groups with similar body weights (BW) (280±4g) according to the diet. Standard rodent chow (chow) and a highfat diet (HFD) were used for 2 months as previously described [13,14]. Food and water were available *ad libitum*.

2.3. ITT (Insulin Tolerance Test)

Awakened fasted rats were submitted to insulin tolerance test. Briefly, 1.5 IU/kg of insulin was injected intraperitoneally and glycemia was measured at 0, 5, 10, 15, 20, 25, and 30 min thereafter [14].

2.4. Food Intake Measurements

BW was measured 24h after insulin injection in amygdala. Food intake was recorded in a metabolic cage 4, 8, 12 and 24h after insulin injection in amygdala.

2.5. Cannula implantation

Rats were anesthetized with 1 mg/kg IP injections of a mixture of 70 mg/kg ketamine (Fort Dodge Animal Health, USA) and 2 mg/kg xylazine (Lloyd Laboratories, USA) and placed in a stereotaxic instrument (Ultra Precise - model 963 – Kopf). Briefly, rats were implanted with unilateral cannulas (26-gauge stainless-steel guide cannula (Plastics One, USA) aimed to the central nucleus of the amygdala (CeA): [coordinates (AP/L/DV to bregma) -2.16/-4.00/-7.18 mm] according to Paxinos and Watson and pilots experiments. Cannulas were fixed using two screws, special glue and acrylic cement. BW was monitored daily for 5-7 days following the surgery. A pilot experiment was performed to confirm the site of the cannulation. Briefly, rats received 2µl injection of methylene blue dye. Rats were killed immediately after the injection, and brains were collected on ice. Brains were sectioned 1mm in a coronal stainless steel matrix with razor blades to check the position of the cannula and the site of injection under microscopy. To further confirm the dissections the membranes were re-blotted from immunoblotting experiments with an antibody against to co-chaperone FK506

binding protein 51 (FKBP51). This protein is expressed in the CeA and is not expressed in the striatum which is a close region to amygdala.

2.6. Insulin injections

Insulin (2μg) or saline (2μL) injections were done on overnight fasted cannulated rats between 7-9h (AM) for tissues collections or food intake measurements. To inhibit PI3K, LY-294002 (50μMfrom Calbiochem) or its vehicle (5% DMSO in saline) was used [15,16].

2.7. Tissue Collection for Immunoblotting

Twelve hours-fasted rats received insulin or saline injection and after 15 min the amygdala was quickly dissected in a stainless-steel matrix with razor blades on ice [9,10]. A pool of 5 rats per sample and four samples (n=4) per group was used. Samples were immediately homogenized in a buffer and immunoblotting was performed as described before [13-16].

2.8. Statistical Analysis

Data are expressed as means ± SEM of the number of independent experiments indicated. For statistical analysis, groups were compared using a two tailed t-test. The level of significance adopted was $p<0.05$.

3. Results

3.1. Food intake is decreased and IR and Akt phosphorylation are increased in response to insulin in the amygdala of rats on chow

Insulin injection in the amygdala did not alter 24h-body weight of rats fed with standard chow (Fig 1A). To evaluate if insulin in the amygdala affects food intake, food intake in 4, 8, 12 and 24 h after the hormone administration were evaluated. Insulin injected in the amygdala did not decrease food ingestion after 4h. However, food intake was lower in response to insulin after 8, 12 and 24 h in rats on chow (Fig. 1B). IR tyrosine phosphorylation and Akt serine phosphorylation were increased in response to insulin injected in the amygdala compared to saline injected rats (Fig. 1C, D). In order to confirm whether the dissections of amygdala were corrected, the membranes with anti-co-chaperone FK506 binding protein 51 (FKBP5) antibody were re-blotted. It was observed the presence of FKBP5 in their membranes, indicating that the dissections of amygdala were appropriate (data not shown). FKBP5 is expressed in the amygdala but not in striatum (Fig. 1E).

3.2. The ability of insulin to decrease food intake and increase Akt phosphorylation in the amygdala is dependent on PI3K

Insulin plus LY or vehicle injections in the amygdala did not alter 24h-body weight of rats fed with standard chow (Fig 2A). To evaluate if insulin in the amygdala affects food intake is dependent of PI3K, food intake 8 h after the hormone administration with prior LY or vehicle treatment was evaluated. As expected, food intake was lower in response to insulin after 8 h in rats on chow. However, the administration of LY prior to insulin in the amygdala abolished this effect (Fig. 2B). Akt serine phosphorylation was increased in response to insulin in the amygdala compared to saline injected rats. The administration of LY prior to insulin in the amygdala impaired Akt phosphorylation in response to insulin (Fig. 2C, D).

3.3. Insulin action and signaling in the amygdala are impaired in rats fed with HFD

Body weight was increased in rats fed with HFD compared to control rats (Fig. 3A). Insulin tolerance test showed that obese rats were insulin resistant compared to control rats (Fig. 3B). To evaluate whether HFD induces insulin resistance in the amygdala, food intake 4, 8, 12 and 24 h in response to insulin or saline were evaluated. Insulin injected in the amygdala did not decrease food intake after 4, 8, 12, 24 h in obese rats (Fig. 3C). IR tyrosine phosphorylation and Akt serine phosphorylation were increased in response to insulin in the amygdala in control rats compared to saline injected rats. However, this effect was blunted in rats on HFD (Fig. 3D, E).

3.4. HFD induces ER stress and low grade inflammation in amygdala

These results were obtained from rats without cannulas to reduce a possible interference of the surgery and chronic cannulas implantation on inflammatory and ER stress conditions. To investigate if high fat feeding alters ER stress, the phosphorylation of RNA-activated protein kinase-like ER resident kinase (PERK) and inositol-requiring kinase alpha (IRE1α) were evaluated. PERK and IRE1α phosphorylation were increased in the amygdala of rats fed a HFD compared to control rats, suggesting an increased ER stress in the amygdala of obese rats (Fig.4A and B). JNK phosphorylation was increased in the amygdala of rats fed with a HFD compared to control animals (Fig.4C). Similarly, IKKα/β phosphorylation was increased in the amygdala of obese rats (Fig. 4D).

4. Discussion

Our data indicate that insulin signaling in amygdala may have an important role in the control of food intake, and this effect is mediated by PI3K pathway. It also shown that in high fat feeding rats there is an increase in inflammatory pathways and ER stress in the amygdala, and in parallel, insulin signaling is reduced in this brain region.

The anorexigenic effects of insulin are well described in the hypothalamus. In the hypothalamus, insulin decreases food ingestion by signaling through IR/PI3K/Akt pathway [1,16]. Besides insulin receptors were abundant in the amygdala [8,7], more recently, amygdala was highlighted as an important site to regulate food intake.

Amygdala is involved in the control of emotion and cognitive functions as memory, learning, fear, anxiety, aversion and food preferences [16-21]. Inhibition of melanocortin or an injection of neuropeptide Y into amygdala increased food intake. In contrast, injections of melanocortin agonist or enterostatin in the amygdala reduced food ingestion [10, 22-24].

It was shown that insulin in the amygdala diminishes food intake in rats on chow. Similar result was obtained by Boghossian et al. (2009) [9]. However, in their study they did not investigate which pathway may account for the effect of insulin in amygdala [8,9]. Data were shown that LY injection abolished the anorexigenic effect of insulin, suggesting that the effect of insulin in the amygdala is mediated by PI3K/Akt pathway.

It is well known that HF feeding induces insulin resistance in the CNS of rodents [25]. Indeed, it was observed that insulin in the amygdala failed to reduce food intake in rats fed with HFD for 2 months. In parallel, we observed that Akt phosphorylation was faint in response to insulin in the amygdala of obese animals, suggesting that insulin resistance in this region of CNS triggered by obesity.

Several mechanisms may contribute to the dysregulation of the insulin signaling pathway in the CNS of obese rodents [25-28].

It is well known that obesity may induce ER stress in peripheral tissues and also in the hypothalamus [13, 28, 29]. Elevated caloric intake stresses the ER due to na increase in protein synthesis resulting in the accumulation of synthesized unfolded proteins [30].

Increased PERK and IRE1α phosphorylation are marks of ER stress. Dietinduced obesity and ob/ob mice have higher levels of PERK and IRE1α phosphorylation in multiple tissues [29, 31]. Enhanced PERK and IRE1α phosphorylation are also seen in the hypothalamus of obese mice [13, 28, 32]. Herein, it was observed that PERK and IRE1α phosphorylation were increased in the amygdala of rats on HFD. This suggests that in addition to the hypothalamus [12,13] high fat feeding increases ER stress also in the amygdala.

ER stress and inflammatory pathways have many links [33-35]. IRE1α induces JNK activation in many tissues which triggers a modulation of several inflammatory genes [36]. In addition, both IRE1α and PERK activate IKKβ/nuclear factor kappa B (NF-κB) pathway driving inflammatory response [35].

The activation of JNK and/or IKKβ induce inhibitory IRS-1 serine phosphorylation leading to insulin resistance in peripheral tissues and also in the hypothalamus [25, 37, 38]. Genetic disruption of IKKβ in AgRP neurons protects mice from diet induced obesity [39]. Conditional deletion of JNK1 in the CNS of mice improved insulin signaling and action in the hypothalamus upon high fat diet [40, 41].

It was demonstrated an increase in JNK and IKKβ phosphorylation, in agreement with reduced Akt phosphorylation in response to insulin in the amygdala of obese rats, suggesting

that these serine kinases may have important role downregulating insulin signaling in this brain region.

Summing up, this results suggested that amygdala is an important region for food intake regulation in response to insulin and this regulation is disrupted in obese rats. It also shown that food intake is regulated in a PI3K/Akt manner in the amygdala similarly to that occurs in the hypothalamus. Besides, data were provided suggesting that obese rats may have low grade inflammation and ER stress in parallel to insulin resistance in the amygdala.

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References

[1] Belgardt, B.F., and Bruning, J.C. 2010. CNS leptin and insulin action in the control of energy homeostasis. Ann N Y Acad Sci 1212, 97-113.

[2] Benoit, S.C., Clegg, D.J., Seeley, R.J., and Woods, S.C. 2004. Insulin and leptin as adiposity signals. Recent Prog Horm Res 59, 267-285.

[3] Berthoud, H.R. 2007. Interactions between the "cognitive" and "metabolic" brain in the control of food intake. Physiol Behav 9, 486-498.

[4] Bruning, J.C., Gautam, D., Burks, D.J., Gillette, J., Schubert, M., Orban, P.C., Klein, R., Krone, W., Muller-Wieland, D., and Kahn, C.R. 2000. Role of brain insulin receptor in control of body weight and reproduction. Science 289, 2122- 2125.

[5] Elmquist, J.K. 1998. CNS regulation of energy balance and body weight: insights from rodent models. Lab Anim Sci 48., 630-637.

[6] Figlewicz, D.P., Evans, S.B., Murphy, J., Hoen, M., and Baskin, D.G. 2003. Expression of receptors for insulin and leptin in the ventral tegmental area/substantia nigra (VTA/SN) of the rat. Brain Res 964, 107-115.

[7] Havrankova, J., Schmechel, D., Roth, J., and Brownstein, M. 1978. Identification of insulin in rat brain. Proc Natl Acad Sci U S A 75, 5737-5741.

[8] Havrankova, J., Roth, J., and Brownstein, M. 1978. Insulin receptors are widely distributed in the central nervous system of the rat. Nature 272, 827-829.

[9] Boghossian, S., Lemmon, K., Park, M., and York, D.A. 2009. High-fat dietsinduce a rapid loss of the insulin anorectic response in the amygdala. Am J Physiol Regul Integr Comp Physiol 297, R1302-1311.

[10] Boghossian, S., Park, M., and York, D.A. 2010. Melanocortin activity in the amygdala controls appetite for dietary fat. Am J Physiol Regul Integr Comp Physiol 298, R385-393.

[11] Niswender, K.D., Morrison, C.D., Clegg, D.J., Olson, R., Baskin, D.G., Myers, M.G., Jr., Seeley, R.J., and Schwartz, M.W. 2003. Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. Diabetes 52, 227-231.

[12] Belgardt, B.F., and Bruning, J.C. 2010. CNS leptin and insulin action in the control of energy homeostasis. Ann N Y Acad Sci 1212, 97-113.

[13] Ropelle, E.R., Flores, M.B., Cintra, D.E., Rocha, G.Z., Pauli, J.R., Morari, J., de Souza, C.T., Moraes, J.C., Prada, P.O., Guadagnini, D., et al. 2010. IL-6 and IL- 10 anti-inflammatory activity links exercise to hypothalamic insulin and leptin sensitivity through IKKbeta and ER stress inhibition. PLoS Biol 8.

[14] Tsukumo, D.M., Carvalho-Filho, M.A., Carvalheira, J.B., Prada, P.O., Hirabara, S.M., Schenka, A.A., Araujo, E.P., Vassallo, J., Curi, R., Velloso, L.A., et al. 2007. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes 56, 1986-1998.

[15] Prada, P.O., Ropelle, E.R., Mourao, R.H., de Souza, C.T., Pauli, J.R., Cintra, D.E., Schenka, A., Rocco, S.A., Rittner, R., Franchini, K.G., et al. 2009. EGFR tyrosine kinase inhibitor (PD153035) improves glucose tolerance and insulin action in high-fat diet-fed mice. Diabetes 58, 2910-2919.

[16] De Souza, C.T., Frederico, M.J., da Luz, G., Cintra, D.E., Ropelle, E.R., Pauli, J.R., and Velloso, L.A. 2010. Acute exercise reduces hepatic glucose production through inhibition of the Foxo1/HNF-4alpha pathway in insulin resistant mice. JPhysiol 588, 2239-2253.

[17] Figlewicz, D.P., MacDonald Naleid, A., and Sipols, A.J. 2007. Modulation of food reward by adiposity signals. Physiol Behav 91, 473-478.

[18] Glass, M.J., Billington, C.J., and Levine, A.S. 2000. Naltrexone administered to central nucleus of amygdala or PVN: neural dissociation of diet and energy. Am J Physiol Regul Integr Comp Physiol 279, R86-92.

[19] Glass, M.J., Billington, C.J., and Levine, A.S. 1999. Opioids and food intake: distributed functional neural pathways? Neuropeptides 33, 360-368.

[20] Kelley, A.E. 2004. Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. Neurosci Biobehav Rev 27, 765-776.

[21] Yamamoto, T. 2007. Brain regions responsible for the expression of conditioned taste aversion in rats. Chem Senses 32, 105-109.

[22] Primeaux, S.D., York, D.A., and Bray, G.A. 2006. Neuropeptide Y administration into the amygdala alters high fat food intake. Peptides 27,1644-1651.

[23] Lin, L., Park, M., Hulver, M., and York, D.A. 2006. Different metabolic responses to central and peripheral injection of enterostatin. Am J Physiol Regul Integr Comp Physiol 290, R909-915.

[24] Lin, L., Park, M., and York, D.A. 2007. Enterostatin inhibition of dietary fat intake is modulated through the melanocortin system. Peptides 28, 643-649.

[25] Prada, P.O., Zecchin, H.G., Gasparetti, A.L., Torsoni, M.A., Ueno, M., Hirata, A.E., Corezola do Amaral, M.E., Hoer, N.F., Boschero, A.C., and Saad, M.J. 2005. Western diet modulates insulin signaling, c-Jun N-terminal kinase activity, and insulin receptor substrate-1ser307 phosphorylation in a tissuespecific fashion. Endocrinology 146, 1576-1587.

[26] Benoit, S.C., Kemp, C.J., Elias, C.F., Abplanalp, W., Herman, J.P., Migrenne, S., Lefevre, A.L., Cruciani-Guglielmacci, C., Magnan, C., Yu, F., et al. 2009. Palmitic acid mediates hypothalamic insulin resistance by altering PKC-theta subcellular localization in rodents. J Clin Invest 119, 2577-2589.

[27] Park-York, M., Boghossian, S., Oh, H., and York, D.A. 2012. PKCtheta Expression in the Amygdala Regulates Insulin Signaling, Food Intake and Body Weight. Obesity (Silver Spring).

[28] Ozcan, L., Ergin, A.S., Lu, A., Chung, J., Sarkar, S., Nie, D., Myers, M.G., Jr., and Ozcan, U. 2009. Endoplasmic reticulum stress plays a central role in development of leptin resistance. Cell Metab 9, 35-51.

[29] Ozcan, U., Cao, Q., Yilmaz, E., Lee, A.H., Iwakoshi, N.N., Ozdelen, E., Tuncman, G., Gorgun, C., Glimcher, L.H., and Hotamisligil, G.S. 2004. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 306, 457-461.

[30] Hotamisligil, G.S. 2010. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell 140, 900-917.

[31] Tsutsumi, A., Motoshima, H., Kondo, T., Kawasaki, S., Matsumura, T., Hanatani, S., Igata, M., Ishii, N., Kinoshita, H., Kawashima, J., et al. 2011. Caloric restriction decreases ER stress in liver and adipose tissue in ob/ob mice. Biochem Biophys Res Commun 404, 339-344.

[32] Won, J.C., Jang, P.G., Namkoong, C., Koh, E.H., Kim, S.K., Park, J.Y., Lee, K.U., and Kim, M.S. 2009. Central administration of an endoplasmic reticulum stress inducer inhibits the anorexigenic effects of leptin and insulin. Obesity (Silver Spring) 17, 1861-1865.

[33] Deng, J., Lu, P.D., Zhang, Y., Scheuner, D., Kaufman, R.J., Sonenberg, N., Harding, H.P., and Ron, D. 2004. Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. Mol Cell Biol 24,10161-10168.

[34] Hu, P., Han, Z., Couvillon, A.D., Kaufman, R.J., and Exton, J.H. 2006. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. Mol Cell Biol 26, 3071-3084.

[35] Hummasti, S., and Hotamisligil, G.S. 2010. Endoplasmic reticulum stress and inflammation in obesity and diabetes. Circ Res 107, 579-591.

[36] Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H.P., and Ron, D. 2000. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 287, 664-666.

[37] Lee, Y.H., Giraud, J., Davis, R.J., and White, M.F. 2003. c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. J Biol Chem 278, 2896-2902.

[38] Aguirre, V., Werner, E.D., Giraud, J., Lee, Y.H., Shoelson, S.E., and White, M.F. 2002. Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J Biol Chem 277, 1531-1537.

[39] Zhang, X., Zhang, G., Zhang, H., Karin, M., Bai, H., and Cai, D. 2008. Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. Cell 135, 61-73.

[40] Belgardt, B.F., Mauer, J., Wunderlich, F.T., Ernst, M.B., Pal, M., Spohn, G., Bronneke, H.S., Brodesser, S., Hampel, B., Schauss, A.C., et al. 2010. Hypothalamic and pituitary c-Jun N-terminal kinase 1 signaling coordinately regulates glucose metabolism. Proc Natl Acad Sci U S A 107, 6028-6033.

[41] Unger, E.K., Piper, M.L., Olofsson, L.E., and Xu, A.W. 2010. Functional role of c-Jun-N-terminal kinase in feeding regulation. Endocrinology 151, 671-682.

Figure Legends

Fig. 1. ***Food intake and insulin signaling in amygdala of rats on chow***. (A) Body weight (BW) after insulin injections; (B) Food intake in g/g of BW in response to insulin (2μg) injected in amygdala; (C) Insulin receptor (IR) and (D) Protein kinase B (PKB or Akt) phosphorylation in response to insulin; (E) protein expression of cochaperone FK506 binding protein 51 (FKBP51) in amygdala (AMY) and striatum (STR). Data are means +/- SD from 10 rats per group. To performed immunoblotting (IB) of amygdala (C and D) it was used a pool of 5 rats per sample and four samples (n=4) per group. It was used β-actin as a loading control. Two tailed T Test was used. *P<0.05 versus saline injected rat.

Fig. 2. ***Insulin decreases food intake via PI3K in the amygdala***. (A) Body weight (BW) after insulin (2μg), and LY (294002 -50 μM) injections; (B) Food intake in g/g of BW in response to insulin (2μg) or saline with prior injection of LY or vehicle (5% DMSO in saline) in amygdala; (C) Protein kinase B (PKB or Akt) phosphorylation in response to insulin with prior injection of LY or vehicle in amygdala. Data are presented as means +/- SD from 10 rats per group. To performed immunoblotting (IB) of amygdala (C) it was used a pool of 5 rats per sample and four samples (n=4) per group. It was used β-actin as a loading control. Two tailed T Test was used. *P<0.05 versus other groups; #P<0.05 versus saline injected rats. Veh: vehicle.

Fig. 3. ***High fat diet (HFD) impairs insulin action and signaling in amygdala***. (A) Body weight (BW) of rats on chow or HFD; (B) Blood glucose during insulin tolerance test (ITT) of awake rats on chow or HFD; (C) Food intake in g/g of BW in response to insulin (2μg) injected in amygdala of HFD animals; (D) Insulin receptor (IR) and (E) Protein kinase B (PKB or Akt) phosphorylation in response to insulin (2μg) of rats on chow or HFD. Data are presented as means +/- SD from 10 rats. To performed immunoblotting (IB) of amygdala (D and E) it was used a pool of 5 rats per sample and four samples (n=4) per group. It was used β-actin as a loading control. Two tailed T Test was used. *P<0.05 versus chow; #P<0.05 versus other groups.

Fig. 4. ***HFD induces ER stress and low grade inflammation in amygdala***. (A) PERK and (B) IRE1α phosphorylation in amygdala of rats on chow or HFD. (C) JNK and (D) IKK α/β phosphorylation in amygdala of rats on chow or HFD; Data are presented as means +/- SD. To performed immunoblotting (IB) of amygdala it was used a pool of 5 non-cannulated rats per sample and three samples per group. Two tailed T Test was used. *P<0.05 versus control.

Figure 1.

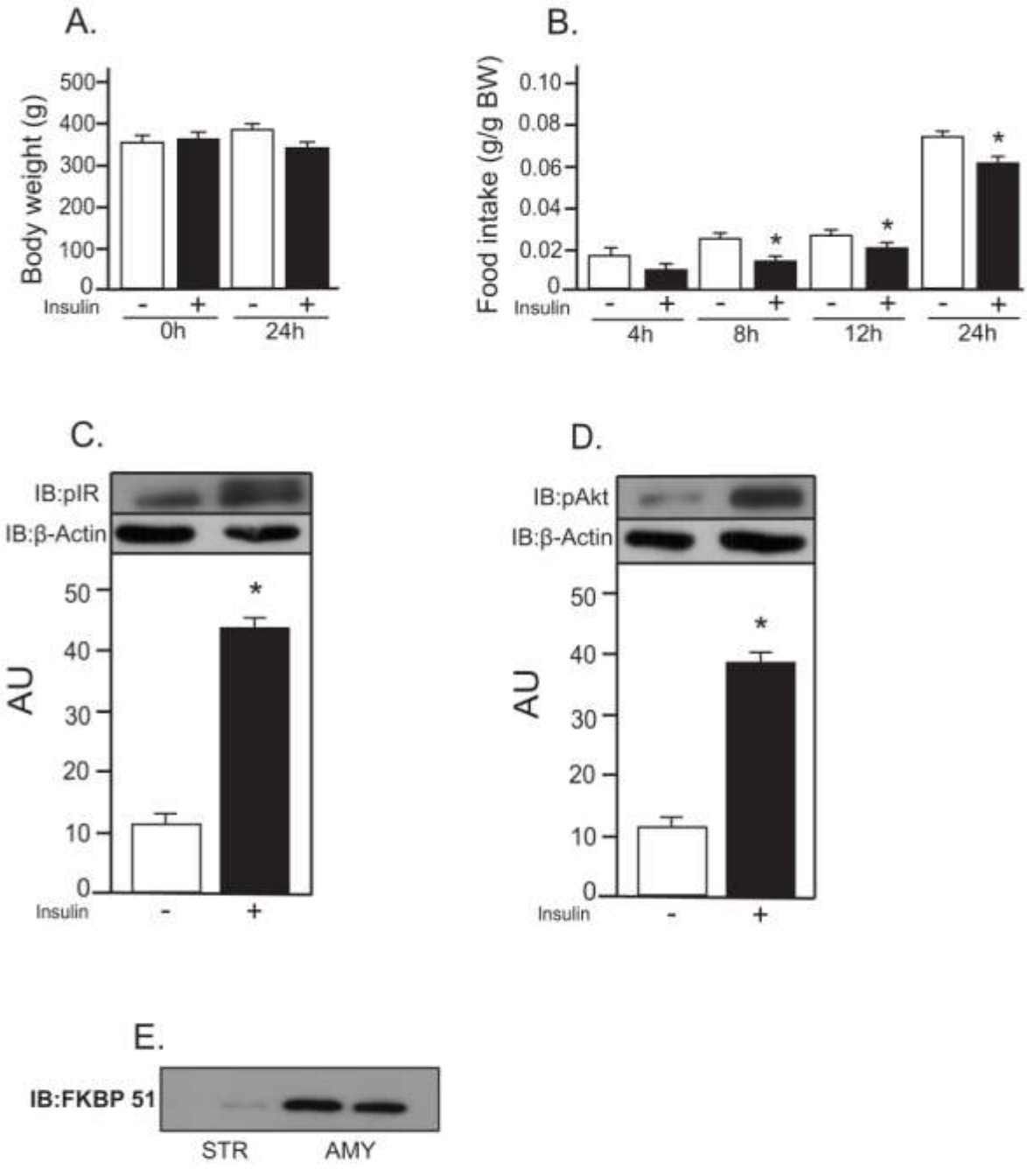
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Figure 2.

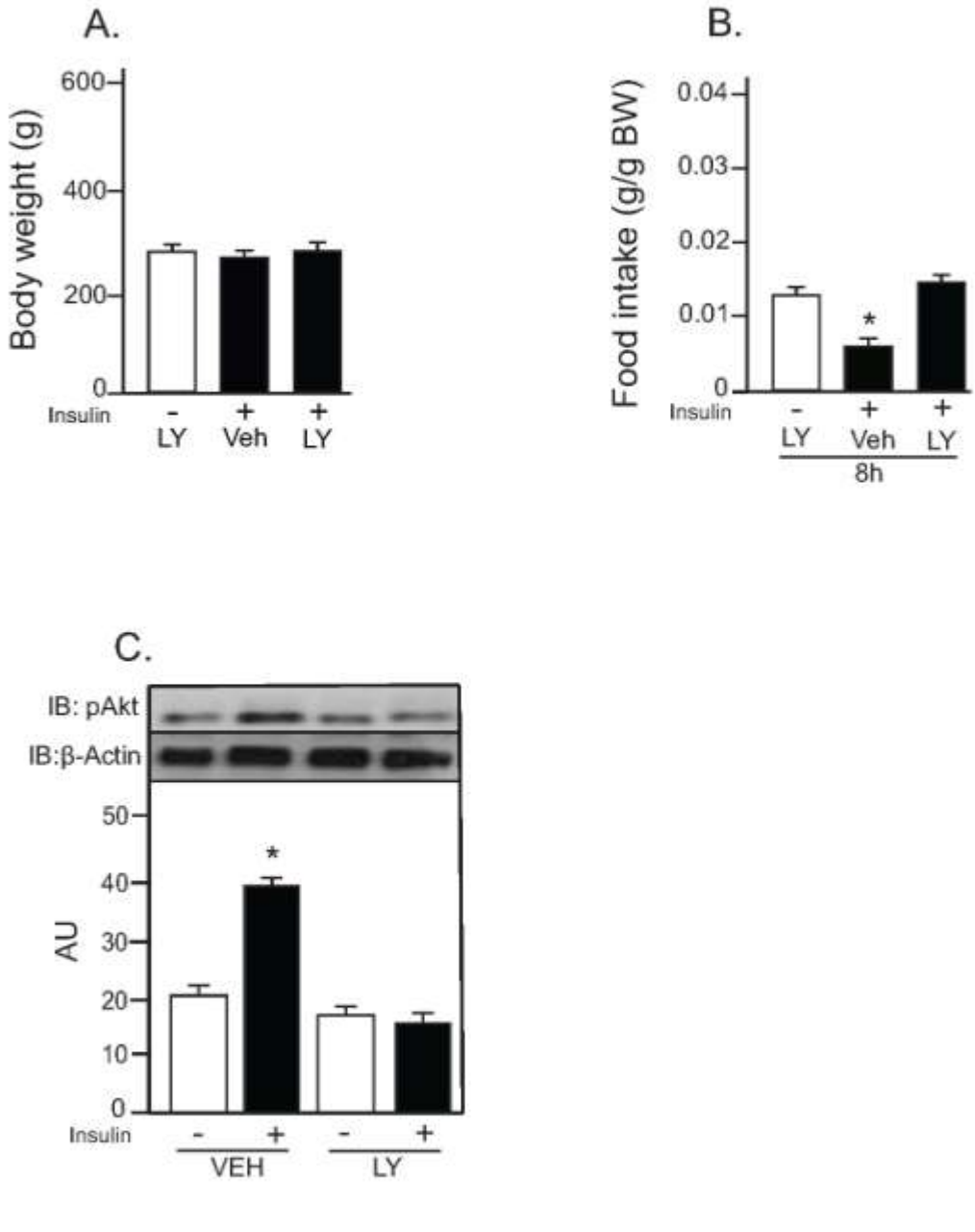
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Figure 3.

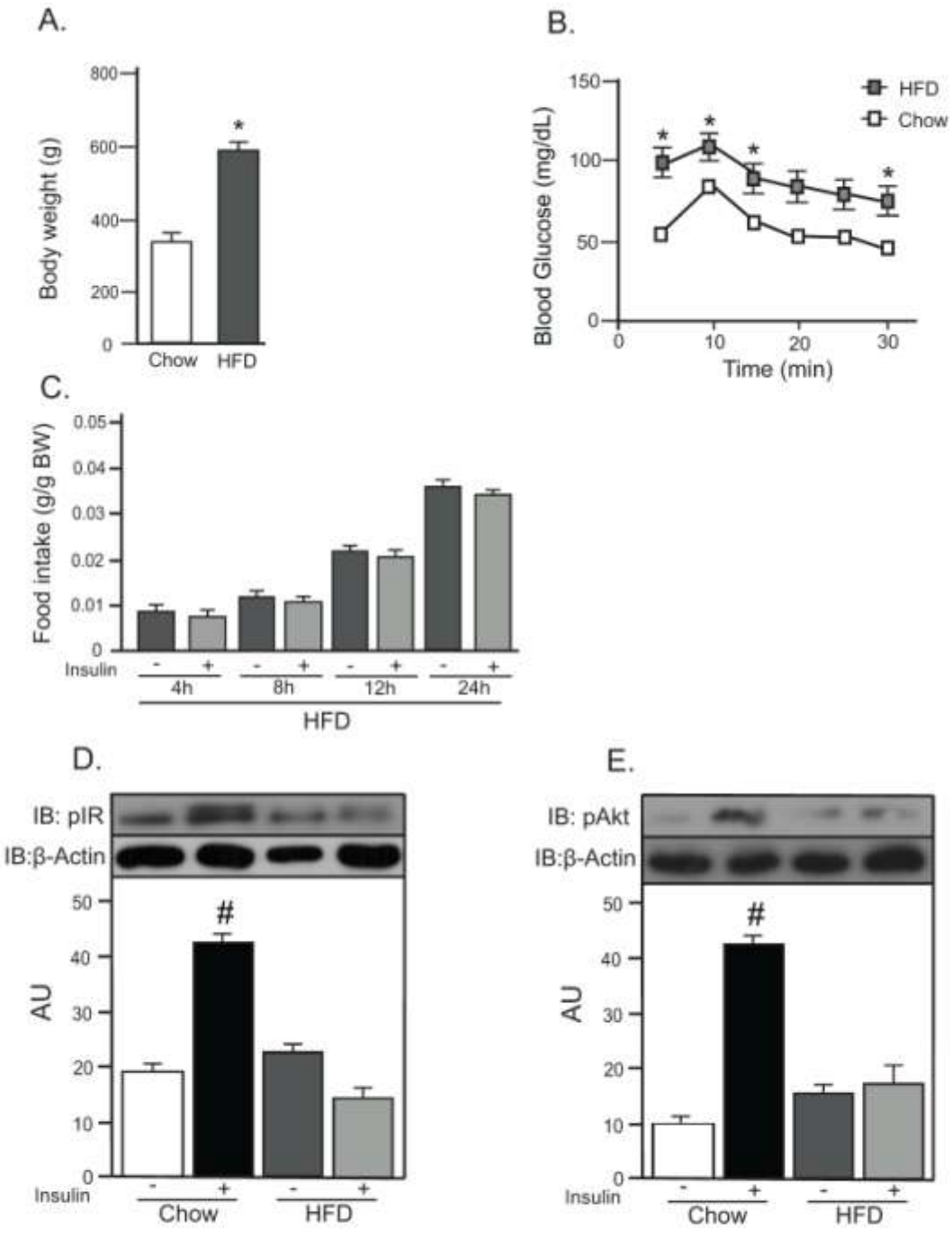
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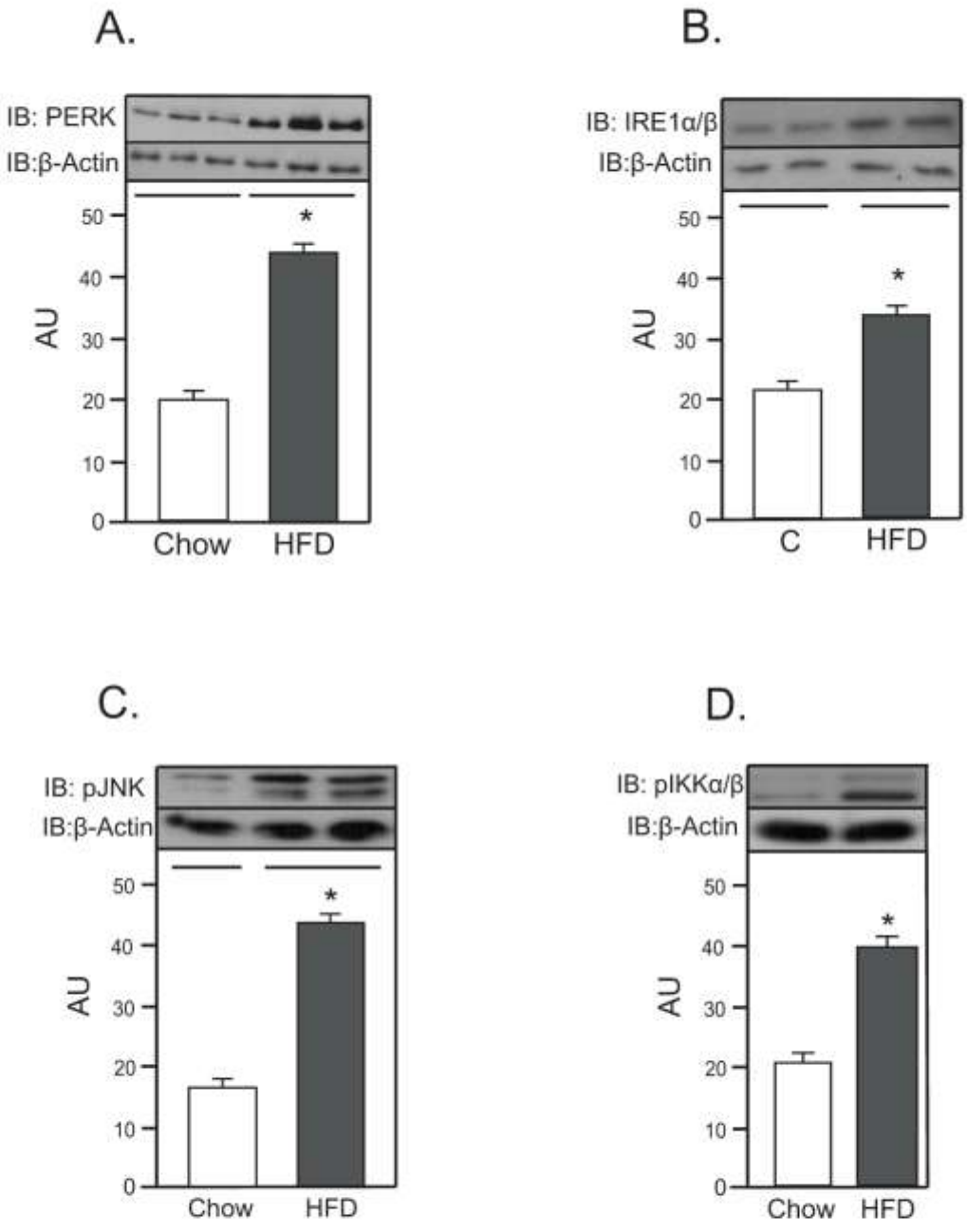
Figure 4.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HFD
B.
IB: IRE1α/β
IB:β-Actin
AU
50
40
30
20
10
0
*
C
HFD
C.
IB: pJNK
IB:β-Actin
AU
50
40
30
20
10
0
*
Chow
HFD
D.
IB: pIKKα/β
IB:β-Actin
AU
50
40
30
20
10
0
*
Chow
HFD

4. CONCLUSÃO

1. O controle da ingestão alimentar é realizado, pelo menos em parte, pela ação da insulina na amígdala.

2. O efeito da insulina na amígdala é via IR/PI3q/Akt.

3. Os animais obesos apresentam resistência à ação da insulina em amígdala e não reduzem a ingestão em resposta a este hormônio.

4. A resistência à insulina na amígdala de obesos é associada à redução da ativação da via IR/Akt nesta região.

5. A dieta hiperlipídica aumenta a ativação, em amígdala, de serinas quinases que participam da via inflamatória, o que pode justificar a resistência à insulina.

6. A dieta hiperlipídica aumenta o stress de retículo endoplasmático em amígdala, podendo ser mais um elemento indutor de resistência à insulina nesta região do sistema nervoso central.

5. ANEXOS

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **2279-1**, sobre **"Estudo do Efeito da insulina e da leptina em striatum, área tegmental ventral e núcleo accumbens em roedores"**, sob a responsabilidade de **Profa. Dra. Patrícia de Oliveira Prada / Maria Fernanda Condes Areias**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais – CEUA/Unicamp em **18 de novembro de 2010**

CERTIFICATE

We certify that the protocol nº **2279-1**, entitled "_____", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **November 18, 2010**.

Campinas, 18 de novembro de 2010.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
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Para: pprada@fcm.unicamp.br

Ms. Ref. No.: FEBSOPENBIO-D-12-00011
Title: DIET-INDUCED OBESITY INDUCES ENDOPLASMIC RETICULUM STRESS AND INSULIN RESISTANCE IN AMYGDALA OF RATS
FEBS Open Bio

Dear Prof. Patricia O Prada,

We are pleased to inform you that your manuscript entitled 'DIET-INDUCED OBESITY INDUCES ENDOPLASMIC RETICULUM STRESS AND INSULIN RESISTENCE IN AMYGDALA OF RATS' has been accepted for publication in FEBS Open Bio.

Kind regards,

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5. REFERÊNCIAS BIBLIOGRÁFICAS

Ahima, RS. Digging deeper into obesity. The journal of Clinical Investigation. 2011; 121 (6) 2076-79.

Belgardt, BF; Husch, A; Rother, E; Ernst, MB; Wunderlich, FT; Hampel, B; Klöckener, T; Alessi, D; Kloppenburg, P; Brüning, JC. PDK1 deficiency in POMC-expressing cells reveals FOXO1-dependent and -independent pathways in control of energy homeostasis and stress response. Cell Metab. 2008; 7(4):291-301.

Boghossian, S; Lemmon, K; Park, M; York, DA. High-fat diets induce a rapid loss of the insulin anorectic response in the amygdala. Am J Physiol Regul Integr Comp Physiol. 2009; 297: 302-311.

Bruning, JC; Gautam, D; Burks, DJ; Schubert, M; Orban, PC; Klein, R; Krone, W; Muller-Wieland, D; Kand, CR. Role of brain insulin receptor in control of body weight and reproduction. Science. 2000;289(5487):2122-5.

Eizirik, DL; Cardozo, AK; Cnop, M.The role for endoplasmic reticulum stress in diabetes mellitus. Endocr Rev. 2008 ; 29(1):42-61.

Farooqi, IS; Bullmore, E; Keogh, J; Gillard, J; O`Rahilly, S; Fletcher, PC. Lepitin regulates striatal regions and human eating behavior. Science. 2007:317-1355.

Fletcher, PC; Napolitano, A; Skeggs, A; Miller, SR; Delafont, B; Cambridge, VC de Wit S; Nathan, PJ; Brooke, A; O'Rahilly, S; Farooqi, IS; Bullmore, ET. Distinct modulatory effects of satiety and sibutramine on brain responses to food images in humans: a double dissociation across hypothalamus, amygdala, and ventral striatum. J Neurosci. 2010;30(43):14346-55.

Guimarães, M. As mutações da fome. Pesquisa FAPESP. [periódico online] edição impressa 138 agosto 2007. Disponível em: http://www.revistapesquisa.fapesp.br/?art=3310&bd=1&pg=1 [15 mar 2008]

Hajnal, A; Pothos, EN; Lénárd, L; Hoebel, BG. Effects of feeding and insulin on extracellular acetylcholine in the amygdala of freely moving rats. Brain Res. 1998; 785(1):41-8.

Holsen, LM; Zarcone, JR; Brooks, WM; Butler, MG; Thompson, TI; Ahluwalia, JS; Nollen, NL; Savage CR. Neural mechanisms underlying hyperphagia in Prader-Willi syndrome. Obesity (Silver Spring). 2006;14(6):1028-37.

Hotamisligil, GS. Inflammatory pathways and insulin action. J Obes Relat Metab Disord. 2006; (3):53-5.

King, BM; Sam , H; Arceneaux, ER; Kass, JM.Effect on food intake and body weight of lesion in and adjacent to posterodorsal amydala in rats. Physiology & Behavior. 1994; 55 (5): 963-966.

King, BM; Cook, JT; Dallman, MF. Hyperinsulinemia in rats with obesity-inducing amydaloid lesions. Am J Physiol. 1996; 271: 1156-9.

Klöckener, T; Hess, S; Belgardt, BF; Paeger, L; Verhagen, LA; Husch, A; Sohn, JW; Hampel, B; Dhillon,H;Zigman, JM; Lowell, BB; Williams,KW; Elmquist, JK; Horvath, TL;Kloppenburg, P; Brüning JC. High-fat feeding promotes obesity via insulin receptor/PI3K-dependent inhibition of SF-1 VMH neurons. Nat Neurosci. 2011; 14(7):911-8.

Könner, AC; Klöckener, T; Brüning JC. Control of energy homeostasis by insulin and leptin: targeting the arcuate nucleus and beyond. Physiol Behav. 2009; 97(5):632-8.

Könner, AC; Hess, S; Tovar, S; Mesaros, A; Sánchez-Lasheras, C; Evers, N; Verhagen, LA; Brönneke, HS; Kleinridders, A; Hampel, B; Kloppenburg, P; Brüning JC. Role for insulin signaling in catecholaminergic neurons in control of energy homeostasis. Cell Metab. 2011; 13(6):720-8.

Malik, S; McGlone, F; Bedrossian, D; Dagher A. Ghrelin modulates brain activity in areas that control appetitive behavior. Cell Metab. 2008; 7(5):400-9.

Morton, GJ; Schwartz, MW. Leptin and the central nervous system control of glucose metabolism. Physiol Rev. 2011; 91(2):389-411.

Obici, S; Feng, Z; Karkanias, G; Baskin, DG,;Rossetti L.Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats. Nat Neurosci. 2002; 5(6):566-72.

Picardi, PK; Calegari, C; Prada, PO; Moraes, JC; Araújo, E; Marcondes, MCCG; Ueno, M; Carvalheira, JBC; Velloso, LA; Saad, MJA. Reduction of hypothalamic protein tyrosine phosphatase improves insulin and leptin resistance in diet induce obese rats. Endocrinology. 2009; 149(8):3870-3880.

Prada, PO; Zecchin, HG; Gasparetti, AL; Torsoni, MA; Ueno, M; Hirata, AE; Amaral, MEC; Hoer, NF; Boschero, AC; Saad, MJA. Western diet modulates insulin signaling c-Jun n-terminal kinase activity and insulin receptor substrate-1 phosphorylation in tissue specific fashion Endocrinology. 2005;146(3):1576-1587.

Plum, L; Schubert, M; Bruning, JC. The role of insulin receptor signaling in the brain. Trends Endocrinol Metab 2005; 16:59-65.

Ropelle, ER; Pauli, JR; Prada, P; Cintra, DE; Rocha, GZ; Moraes, JC; Frederico, MJ; Da Luz, G; Pinho, RA; Carvalheira, JB; Velloso, LA; Saad, MA;De Souza, CT. Inhibition of hypothalamic Foxo1 expression reduced food intake in diet-induced obesity rats. J Physiol. 2009; 587(10):2341-51.

Rother, E; Könner, AC; Brüning, JC. Neurocircuits integrating hormone and nutrient signaling in control of glucose metabolism. Am J Physiol Endocrinol Metab. 2008; 294(5):810-6.

Sánchez-Lasheras, C; Könne, AC; Brüning, JC. Integrative neurobiology of energy homeostasis-neurocircuits, signals and mediators. Front Neuroendocrinol. 2010; 31(1):4-15.

Stoeckel, LE; Weller, RE; Giddings, M; Cox, JE. Peptide YY levels are associated with appetite suppression in response to long-chain fatty acids. Physiol Behav. 2008; 93:(1-2):289-95.

Tye, KM; Prakash, R; Kim, S; Fenno, LE; Grosenick, L; Zarabi, H; Thompson, K; Grandinaru, V; Ramakrishnan, C; Deisseroth, K. Amydala circuitry mediating reversible and bidirectional controlo f anxiety. Nature. 2011; 471:358-362.

Xu, AW; Kaelin, CB; Takeda, K; Akira, S; Schwartz, MW; Barsh, GS. PI3K integrates the action of insulin and leptin on hypothalamic neurons. J Clin Invest 2005; 115:951-8.

White, MF. The insulin signaling system and the IRS proteins. Diabetologia 1997; 40:S2-17.

Zhang, X; Zhang, G; Zhang, H; Karin, M; Bai, H; Cai, D. Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesety. Cell. 2009;135(1):61-73.