PAULA GABRIELE FERNANDES QUARESMA

# ESTUDO DA REGULAÇÃO DA PROTEÍNA CDC2-LIKE KINASE (Clk2) EM HIPOTÁLAMO E FÍGADO DE CAMUNDONGOS CONTROLES E OBESOS

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UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Ciências Médicas

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**Orientadora: Profa. Dra. Patrícia de Oliveira Prada**

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Clínica Médica da Faculdade de Ciências Médicas da Universidade de Campinas para obtenção de título de Mestra em Clínica Médica, área de concentração Clínica Médica

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*Dedicatória*

Dedico esta dissertação aos que me deram o dom da vida

e confiaram em mim desde o início,

meus pais.

Dedico ainda esta dissertação aos que não tiveram

a oportunidade de serem universitários,

mas são doutores na escola da vida,

meus avós.

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# *Epígrafe*

*"Nunca andes pelo caminho traçado, pois ele conduz somente aonde outros já foram"*

**(Alexander Graham Bell)**

# *Resumo*

O hipotálamo é um órgão crucial na regulação do balanço energético por integrar sinais hormonais e nutricionais de órgão periféricos. O hormônio produzido pelo pâncreas – insulina – e o hormônio derivado de células adiposas – leptina- reconhecidamente, agem no SNC controlando a ingestão alimentar e o gasto energético. Recentemente foi demonstrado que a fosforilação em treonina 343 da proteina Cdc2-like kinase 2 (Clk2) é induzida pela sinalização de PI3-q/Akt no fígado. Esta regulação envolve a repressão de genes que controlam a gliconeogênese e produção de glicose hepática, levando a hipoglicemia. Porém, não há informações de que a insulina ou a leptina podem regular a Clk2 no hipotálamo *in vivo*. Camundongos das linhagens Swiss, db/db e C57/BL6J com oito semanas de idade foram utilizados nos experimentos. Nossos resultados mostraram que a Clk2 é expressa e regulada por insulina e leptina em hipotálamo e também que a inibição da Clk2 causou aumento da adiposidade e ingestão alimentar, diminuição do gasto energético e alterações na expressão de neuropeptídeos e do metabolismo de glicose. Além disso, a fosforilação no sítio treonina 343 da Clk2 está diminuída em animais com obesidade induzida por dieta e geneticamente obesos (db/db). A avaliação da gliconeogênese hepática em animais com a proteína Clk2 inibida via ICV mostrou uma tendência ao aumento da produção hepática de glicose, revelando uma possível participação da proteína Clk2 no controle hipotalâmico da gliconeogênese hepática. Sendo assim, podemos sugerir que a Clk2 hipotalâmica é importante no controle do balanço energético pois sua inibição acarreta obesidade acompanhada por aumento da ingestão alimentar e diminuição do gasto energético, e também podemos sugerir um papel no controle hipotalâmico da produção hepática de glicose.

# *Abstract*

The hypothalamus plays an important role in the regulation of whole-body energy balance by integrating nutrients and hormones signals from peripheral inputs. The pancreatic hormone - insulin - and the adipocyte hormone - leptin – are known to act in the CNS controlling food intake and energy expenditure. Leptin and insulin signaling regulate anorexigenic neuropeptide expression. Recently, it was shown that Cdc2-like kinase 2 (Clk2) threonine 343 phosphorylation is induced by PI3K/Akt signaling in the liver. This regulation is involved in the repression of gluconeogenic gene expression and hepatic glucose output leading to hypoglycemia. Thus, it was not shown if insulin or leptin are able to regulate Clk2 threonine 343 phosphorylation in the hypothalamus *in vivo*. Swiss, db/db and C57/BL6J mice eight-weeks-old were used to proceed the experiments. Our data show that Clk2 is expressed and regulated by insulin and leptin in hypothalamus and hypothalamic Clk2 inhibition increased adiposity and food intake, decreased energy expenditure and disrupted neuropeptides expressions and glucose metabolism. Indeed, Clk2 threonine 343 phosphorylation is impaired in the hypothalamus of DIO and db/db mice. Hepatic gluconeogenesis was evaluated and showed increase in ICV inhibited Clk2 mice, it is plausible that Clk2 participates of hypothalamic control of hepatic gluconeogenesis. We suggest that hypothalamic Clk2 is crucial to control energy balance because its inhibition triggers obesity accompanied by increased food intake, decreased energy expenditure and increased hepatic gluconeogenesis.

# *Abreviaturas*

AgRP – *Agouti-related Protein*

ARC – *Arcuate nucleus*

ASO – *Antisense oligonucleotide*

CART – *Cocaine-and-amphetamine-regulated transcript*

CCK – *Cholecystokinin*

CDK – *Cyclin-dependent quinase*

CRH – *Corticotrophin-releasing hormone*

DMH – *Dorsomedial hypothalamus*

FoxO1 – *Forkhead transcription factor O1*

GIP – *Gastric inhibitory polypeptide*

GLP-1 – *Glucagon-like peptide 1*

GSK – *Glycogen synthase kinase*

ICV – *Intracerebroventricular*

IR – *Insulin Receptor*

IRS – *Insulin receptor substrates*

JAK2 – *Janus-kinase 2*

LEPR – *Leptin receptor*

LH – *Lateral hypothalamus*

MAPK – *Mitogen-activated protein kinase*

MCH - *Melanin-concentrating hormone*

NPY – *Neuropeptide Y*

NTS – *Nucleus tractus solitarius*

PDK-1 – *Phosphoinositide-dependent kinase 1*

PGC-1α – *Peroxisome proliferator-activated receptor-γ coactivator*

PI3-q – *Phosphoinositide-3-Phosphate*

POMC – *Proopiomelanocortin*

PVN – *Paraventricular nucleus*

siRNA – *small interference RNA*

SNC – *Central Nervous System*

STAT3 - *Signal transducer and activator of transcription 3*

TRH – *Thyrotropin-releasing hormone*

VMH – *Ventromedial hypothalamus*

DIO – *Diet-induced obesity*

# *Sumário*

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# *Introdução*

## Obesidade

A Organização Mundial de Saúde estimou em 2008 que 1,5 bilhão de adultos maiores de 20 anos e mais de 200 milhões de homens e 300 milhões de mulheres – aproximadamente 10% dos adultos – eram obesos. Em 2010, aproximadamente 43 milhões de crianças abaixo dos cinco anos apresentavam sobrepeso. A obesidade não é um problema apenas de países desenvolvidos. Houve um grande aumento da obesidade em países em desenvolvimento a partir da década de 80, particularmente na Oceania, América Latina e norte da África [1].

No Brasil, a atual prevalência do sobrepeso é de 30% em crianças e 50% em adultos. A obesidade atinge 16% da população adulta brasileira, com a possibilidade de chegar a 30% em 2025, de acordo com a Organização Mundial de Saúde. [2]

O desenvolvimento da obesidade é indubitavelmente relacionado a fatores genéticos, maternos, perinatais e sedentarismo, porém o recente aumento mundial da prevalência da obesidade é devido ao aumento significativo do consumo de calorias, principalmente de dieta rica em lipídios e carboidratos e deficiente em vitaminas e outros micronutrientes [3-4].

Conjuntamente, estes fatores acarretam no surgimento de outras doenças como Diabetes Mellitus tipo 2, doenças cardiovasculares, osteoartrite, apneia do sono e alguns tipos de câncer [3].

Modificações no estilo de vida, dieta adequada e exercício físico têm sido recomendados com efetividade, entretanto, a falta de regularidade em seguir as recomendações alimentares e de atividade física resulta em re-ganho de peso. Além disso, os medicamentos disponíveis para tratar a obesidade vêm apresentando baixa eficácia e efeitos colaterais indesejáveis [3].

Portanto, tornou-se imperativo buscar um entendimento dos mecanismos moleculares que contribuem para a instalação da obesidade e de suas doenças associadas [5].

## Balanço energético

A manutenção da homeostase energética requer um balanço entre a ingestão alimentar e o gasto energético, e a ingestão alimentar deve ser adequada à necessidade energética (atividade física, metabolismo basal e termogênese) do organismo [6].

O hipotálamo é conhecido como o centro de controle do balanço energético, sendo responsável por funções fisiológicas importantes como funções motoras, emoções, funções endócrinas, ingestão alimentar e hídrica. É um órgão essencial para a homeostase corporal por receber informações neurais, hormonais e nutricionais e fazer regulações compensatórias através da liberação de neuropeptídeos. Está localizado centralmente na base do cérebro, intimamente relacionado com a glândula hipófise.

O hipotálamo também recebe inervação de várias áreas, como do núcleo do trato solitário (NTS) e área postrema, que enviam sinais do trato gastrointestinal. Outros sinais a respeito de cheiro, sabor, memória, visão e contexto social são enviados para o hipotálamo e podem influenciar a ingestão alimentar [7].

O estômago foi o órgão primeiramente apontado como responsável pelo controle da ingestão alimentar em 1927. Porém, Stellar, em 1954, identificou o hipotálamo como fonte de comportamentos motivacionais como o apetite, demonstrando que um órgão central participava do controle do apetite [8].

Uma série de experimentos baseados em lesões hipotalâmicas foram executados para demonstrar o papel crucial deste órgão no apetite e no controle da ingestão alimentar. Nesta época já se definia fome como um forte desejo ou necessidade por alimentos e saciedade como a condição de estar satisfeito após a refeição. O hipotálamo lateral era considerado o "centro da fome", pois lesões neste local causavam hipofagia; o hipotálamo medial foi descrito como "centro da saciedade", pois lesões nesta região acarretavam em hiperfagia [9-10].

**Ação da insulina e da leptina no hipotálamo**

A descoberta dos camundongos com mutações espontâneas no gene "ob" e "db" resultou na descoberta de um fator que poderia controlar o apetite, a leptina. A leptina é produzida pelos adipócitos, atravessa a barreira hemato-encefálica e atua em estruturas cerebrais como o hipotálamo, onde sua ação é crucial para a regulação do metabolismo energético [6, 11-12]. Camundongos ob/ob que têm deficiência na produção de leptina ou camundongos db/db que apresentam mutação dos receptores de leptina (LEPR) são

obesos e hiperfágicos [13-14]. A deleção do LEPR em neurônios do núcleo ventromedial induz obesidade grave com redução de gasto energético em camundongos [15-16].

Da mesma forma que a leptina, a insulina é implicada no controle da homeostase energética. A inibição da expressão dos receptores de insulina (IR) no hipotálamo, com oligonucleotídeo antisense (ASO), aumenta a ingestão alimentar e a adiposidade em ratos [17]. Animais *knockouts* para IR em neurônios do sistema nervoso central (SNC) são hiperfágicos e apresentam aumento da adiposidade, hipertrigliceridemia, hiperinsulinemia e hiperleptinemia. Injeções ICV ou aplicações intranasais de insulina, que mimetizam o aumento das concentrações de insulina no SNC, diminuem a ingestão alimentar e o peso corpóreo de camundongos, ratos, babuínos e homens [17-19]. Camundongos *knockouts* para IRS-2 são hiperfágicos e obesos, assim como a inibição da expressão da PI3-q ou FoxO1 em hipotálamo altera a ingestão alimentar de formas variadas [17-22].

Os hormônios leptina e insulina são ativamente transportados ao líquido cefalorraquidiano e agem em seus receptores específicos, principalmente no núcleo ARC.A leptina se liga ao seu receptor (LEPR), levando ao recrutamento da Janus quinase 2 (JAK2), que fosforila o LEPR. O transdutor-de-sinal-e-ativador-de-transcrição 3 (STAT3) se liga ao LEPR ativado e é fosforilado pela JAK2. A STAT3 fosforilada transloca para o núcleo, onde aumenta a transcrição de genes que reduzem a ingestão alimentar e aumentam o gasto energético. É descrito que a leptina também induz a ativação da enzima fosfatidilinositol-3-quinase (PI3-q), através de um *crosstalk* com a via da insulina [23]. A inibição da PI3-q, por meio da administração de LY294002 ou *wortmannin* por injeções intracerebroventriculares (ICV), reduz o efeito anorexigênico da leptina em ratos, sugerindo que a via PI3-q é central no controle da ingestão alimentar mediado por este hormônio [23-24].

A insulina sinaliza através do seu receptor que sofre uma modificação conformacional, ativando sua subunidade β [25]. 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 PI3q. Uma vez ativa, a PI3-q catalisa a fosforilação dos fosfoinositídeos na posição 3 do anel de inositol produzindo fosfatidilinositol-3-fosfato, fosfatidilinositol-3,4-difosfato e fosfatidilinositol-3,4,5-

trifosfato. O produto fosfatidilinositol-3,4,5-trifosfato gerado pela PI3-q pode regular a PDK-1 (*phosphoinositide-dependent kinase 1*), uma serina/treonina quinase que fosforila e ativa a Akt. A Akt fosforilada migra para o núcleo celular, onde fosforila a FoxO1 (*forkhead transcription factor O1*) que é excluída do núcleo. Este efeito altera a expressão de neuropeptídeos ligados ao balanço energético nos núcleos hipotalâmicos.

**Neuropeptídeos que controlam o balanço energético**

Nos últimos anos alguns estudos demonstraram que a administração de neuropeptídeos hipotalâmicos afetam a ingestão alimentar e o ganho de peso em animais. Além disso, modelos genéticos com mutações ou deleções de genes hipotalâmicos ligados ao metabolismo energético demonstraram a importância do hipotálamo no comportamento alimentar. Sinais periféricos que transmitem informações sobre as condições nutricionais, hormonais e metabólicas são integradas pelos circuitos hipotalâmicos a fim de regular ingestão alimentar e gasto energético [6].

O hipotálamo consiste em vários núcleos que comunicam entre si e com outras áreas do SNC. São os núcleos: arqueado (ARC), paraventricular (PVN), ventromedial (VMH), dorsomedial (DMH) e lateral (LH).

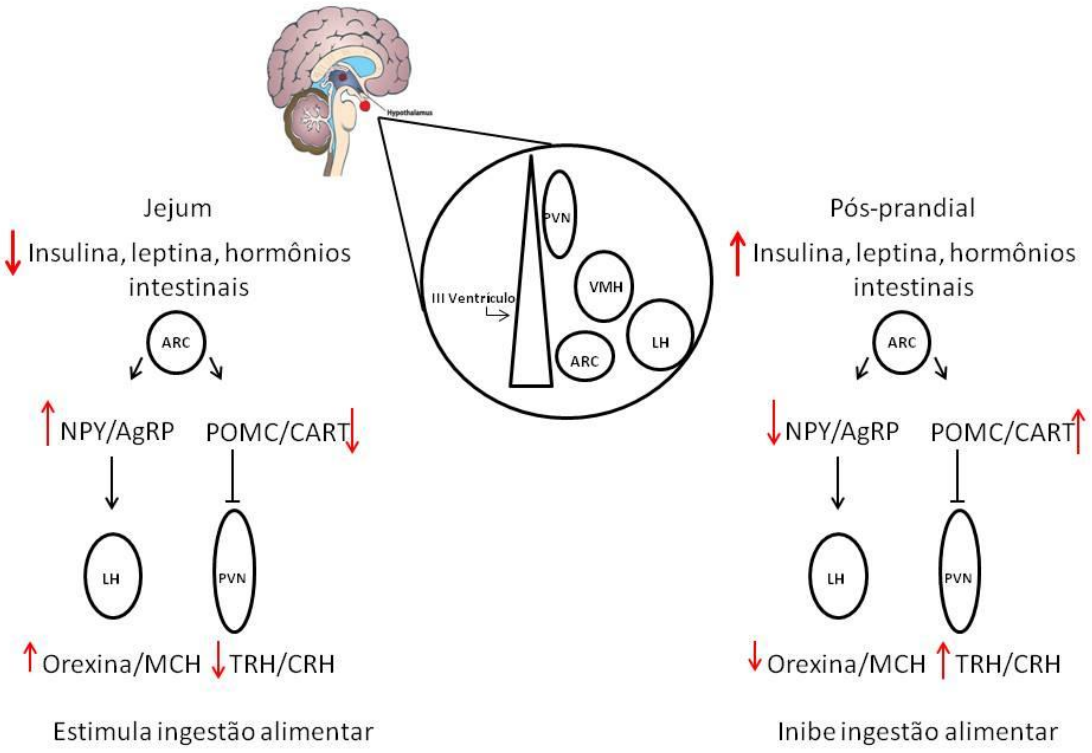
Duas populações distintas de neurônios do núcleo ARC agem como sensores do estoque de energia corporal e coordenam uma complexa rede de neurônios que, de acordo com a necessidade, controlam a ingestão alimentar e termogênese. Esses neurônios de primeira ordem são equipados com receptores e sistemas moleculares intracelulares capazes de detectar mudanças agudas e crônicas nos níveis de hormônios e nutrientes da circulação. A resposta a essas mudanças se baseia na modulação da produção de determinados neuropeptídeos que são liberados por neurônios específicos [26].

As subpopulações dos neurônios do núcleo ARC são caracterizadas pelos neuropeptídeos que cada uma produz e libera. Uma das subpopulações expressa os peptídeos orexigênicos NPY e AgRP, enquanto a outra subpopulação expressa POMC e CART. Ambas as subpopulações se projetam aos núcleos LH e PVN do hipotálamo, onde eles controlam as funções dos neurônios de segunda ordem. No núcleo PVN, duas subpopulações distintas de neurônios produzem os neuropeptídeos anorexigênicos e pró-termogênicos, TRH e CRH. No LH, outras duas subpopulações de neurônios

produzem os neuropeptídeos orexigênicos: orexina e o neuropeptídeo anti-termogênico MCH [26].

Durante o jejum ou quando os estoques de energia estão baixos, a expressão de NPY e AgRP são aumentadas, enquanto a expressão de POMC e CART está reduzida. Esta resposta coordenada é dependente da simultânea detecção de diminuição da disponibilidade de nutrientes, redução dos níveis dos hormônios leptina e insulina, redução dos níveis de hormônios do intestino, CCK, GLP-1 e GIP e aumento nos níveis do hormônio gástrico grelina. Os neurônios do tipo NPY/AgRP enviam sinais inibitórios para o PVN, reduzindo a expressão de TRH e CRH, e projeções estimulantes para o LH, impulsionando a expressão de orexina e MCH [26].

No período pós-prandial ou quando os estoques de energia estão repletos, os neurônios NPY/AgRP são inibidos e os neurônios POMC/CART são ativados. Neste contexto, a disponibilidade de nutrientes e os níveis de insulina e leptina estão aumentados, assim como os de CCK, GLP-1 e GIP. Contrariamente, o nível de grelina e ativação da expressão de CRH e TRH no PVN [26].




**Proteína Cdc2-Like Kinase (Clk2)**

As Clks (*Cdc2-like kinases*), também denominadas quinases LAMMER, são proteínas conservadas durante a evolução da família das quinases CMGC [*CDK (cyclin-dependent kinase), MAPK (mitogen-activated protein kinase), GSK (glycogen synthase kinase), e CDK-like*] encontradas na maioria dos eucariotos. Embora haja pouco entendimento do alvo, função e regulação biológica das Clks, um estudo demonstrou que um membro dessas quinases, a Clk2 é regulada em condições de jejum e realimentação no fígado. Neste mesmo estudo também foi demonstrado que a ativação da via PI3-q/Akt induzida por insulina aumenta a fosforilação da Clk2 no resíduo treonina 343 em tecido hepático e, este evento está relacionado ao aumento da sua atividade quinase. Uma vez ativa, a Clk2 torna-se mais estável, podendo fosforilar o domínio SR da PGC-1α, causando repressão da atividade transcricional dos genes responsáveis pela gliconeogênese, reduzindo assim, a produção hepática de glicose. Adicionalmente, a super-expressão de Clk2 em fígado de animais db/db reduziu a liberação de glicose pelo fígado, diminuindo, assim, a glicemia de jejum destes animais [27-28].

Em outro estudo, utilizando células HeLa foi demonstrado que a Clk2 é diretamente fosforilada em treonina pela Akt. Quando estas células foram submetidas à siRNA (*small interference RNA*) para reduzir a expressão de Akt houve uma menor fosforilação da Clk2 após estímulo com insulina, sugerindo que a fosforilação da Clk2 induzida pela insulina é dependente da Akt [29].

Em conjunto estes dados sugerem que a Clk2 hepática é regulada por realimentação após jejum, é fosforilada em resposta à insulina, e esta fosforilação depende da Akt.

Entretanto, ainda não foi demonstrado se a Clk2 é também expressa e regulada por leptina e insulina em hipotálamo e se esta proteína pode exercer algum efeito no balanço energético controlado pelo hipotálamo.

# *Objetivos*

Os objetivos deste estudo foram:

1) investigar a expressão e regulação da proteína Clk2 no hipotálamo de camundongos controles.

2) investigar se a Clk2 hipotalâmica participa do controle da ingestão alimentar, expressão de neuropeptídeos e do gasto energético.

3) investigar se a obesidade altera a regulação da Clk2 pela Akt em hipotálamo.

4) investigar se a Clk2 do hipotálamo participa do controle da gliconeogênese hepática.

# *Capítulo*

**Title: Clk2 has a Key Role in Insulin and Leptin Signaling/Action in Hypothalamus *in vivo***

**Paula G.F. Quaresma, Andressa C. Santos, Laís Weissmann, Alexandre Hilário Berenguer de Matos, Andrea M. Caricilli, Joseane Morari, Licio Augusto Velloso, Iscia Lopes Cendes, José Barreto C. Carvalheira, Mario J. Saad, Patrícia O. Prada**

Short running tittle: Clk2 is key for insulin/leptin action in hypothalamus

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**Abstract**

The hypothalamus plays an important role in the regulation of whole-body energy balance by integrating nutrients and hormones signals from peripheral inputs. The pancreatic hormone - insulin - and the adipocyte hormone - leptin – are known to act in the CNS controlling food intake and energy expenditure. Leptin and insulin signaling regulate anorexigenic neuropeptide expression. Recently, it was shown that Cdc2-like kinase 2 (Clk2) threonine 343 phosphorylation is induced by PI3K/Akt signaling in the liver. This regulation is involved in the repression of gluconeogenic gene expression and hepatic glucose output leading to hypoglycemia. Thus, it was not shown if insulin or leptin are able to regulate Clk2 threonine 343 phosphorylation in the hypothalamus *in vivo*. Our data show that Clk2 is expressed and regulated by insulin and leptin in hypothalamus and hypothalamic Clk2 inhibition increased adiposity and food intake, decreased energy expenditure and disrupted neuropeptides expressions and glucose metabolism. Indeed, Clk2 threonine 343 phosphorylation is impaired in the hypothalamus of DIO and db/db mice. We suggest that hypothalamic Clk2 is crucial to control energy balance because its inhibition triggers obesity accompanied by increased food intake and decreased energy expenditure.

KEY WORDS: Clk2, hypothalamus, insulin, leptin, food intake, body weight, obesity.

## Introduction

Obesity is characterized by excessive lipid accumulation in adipose tissue and other tissues. The World Health Organization estimated that in 2008, approximately 10% of adults were obese. In 2010, about 43 million children under five years of age were overweight [30-33]. Obese individuals have a greater risk of developing type 2 diabetes, cardiovascular diseases and cancer compared with those of normal weight [32-34].

The hypothalamus plays an important role in the regulation of whole-body energy balance by integrating nutrients and hormones signals from peripheral inputs [35-39].

The pancreatic hormone - insulin - and the adipocyte hormone - leptin – are known to act in the CNS controlling food intake and energy expenditure [35-39].

The canonical leptin signaling involves the activation of Janus kinase 2 (Jak2) which recruitto the leptin receptor (LEPR) where it phosphorylates several tyrosine residues on the LEPR. Phosphorylated LEPR binds to and phosphorylates signal transducer and activator of transcription (STAT) 3 proteins which translocates to the nucleus where it is thought to bind to specific DNA sequences to regulate anorexigenic neuropeptide expression.

Insulin acts through the insulin receptor (IR), which is a protein with endogenous tyrosine kinase activity, and in the hypothalamus insulin signals through IRS/PI3k/Akt/FoxO1 to control food intake [18, 36-37, 40-45]. Recently, it was shown that Cdc2-like kinase 2 (Clk2) threonine 343 phosphorylation is induced by PI3K/Akt

signaling in the liver. This regulation is involved in the repression of gluconeogenic gene expression and hepatic glucose output leading to hypoglycemia [27].

The Cdc2-like kinases (Clk), also termed LAMMER kinases, are an evolutionary conserved family of dual-specificity CMGC kinases ubiquitously expressed in eukaryotes. They are putative high-level regulators of alternative splicing through phosphorylation of SR domains on splicing factors [27]. However, there is an extremely limited understanding of Clk targets, function, and regulation in a biological context. Also, it was not shown if insulin or leptin are able to regulate Clk2 threonine 343 phosphorylation in the hypothalamus *in vivo*.

Thus, in the present study, we investigated whether insulin, leptin and obesity may alter Clk2 threonine 343 phosphorylation ($Clk2^{Thr343}$)*in vivo* in the hypothalamus, and the role of hypothalamic Clk2 in the regulation of energy balance and neuropeptide expression.

**Research design and methods**

All experiments were approved by the Ethics Committee of the State University of Campinas. Eight-week-old male Swiss, db/db e C57/BL6J mice obtained from the University of Campinas, São Paulo, were assigned to receive a standard rodent chow or a high-fat diet (HFD) as previously described [46-47], and water *ad libitum*. For fasting and refeeding experiments, fasted (24h) mice were allowed to refeed for 1, 2, 4 or 8h and hypothalami were dissected for protein studies, as described below. All feeding tests were conducted between 0800 and 1000 A.M.

**Intracerebroventricular (ICV) cannulation**. Anesthetized mice were stereotaxically instrumented (Ultra Precise - model 963 - Kopf) to implant stainless steel cannulas (26-gauge, Plastics One) in the right lateral ventricle. The coordinates used from the bregma were: anterior/posterior: -0.34 mm, lateral: -1.0 mm, dorso/ventral: -2.2 mm. Mice were single-housed following surgery and were allowed to recover for 5-7 days. The correct implantation of cannulas was checked by 10ng angiotensin II ICV injection, which elicits an intake of water [48]. Animals that did not reach this criterion were excluded from the experiments.

**Hypothalamic nuclei dissection.** Arc, medial hypothalamus (ventromedial and dorsomedial; MH), PVN, and LH were quickly dissected in a stainless-steel matrix with razor blades as described previously [49], and frozen in liquid nitrogen for further protein studies.

**ICV injections**. To determine if insulin or leptin induce Clk2 threonine 343 phosphorylation *in vivo*, overnight fasted mice on chow or HFD received an ICV injection of insulin (human recombinant insulin, Eli Lilly and Co. Indianapolis, IN, USA) or recombinant leptin (Calbiochem, San Diego, CA, USA), and hypothalami were quickly dissected and frozen in liquid nitrogen for further protein studies. To investigate whether PI3K/Akt and PI3K/Jak2 pathways were involved in Clk2 regulation, PI3K inhibitor (LY294002), (2µl/40µM) and/or Akt inhibitor (AktVIII) (2µl/40µM) and Jak2 inhibitor (AG490) (2µl/50µM) and/or LY294002 (2µl/40µM) were injected ICV 1 hour prior to insulin (2µL) or leptin (2µL) injections respectively in control mice. After 15 min hypothalami were dissected to investigate Clk2 phosphorylation by immunoblotting. To inhibit Clk2 expression in the hypothalamus, we used TG003 inhibitor (2µL/60 µM) injected ICV twice a daily (0800 and 0500 PM) for seven days in control mice. In another group of mice we inhibited the expression of Clk2 by small

interference RNA (siRNA) which was infused continuously by ICV micro-osmotic pump Alzet 1007D (DURECT Corporation, Cupertino, CA, USA). Body weight, fat mass, 4h and 8h food intake in response to ICV insulin and leptin, leptin and insulin signaling in hypothalamus, O2 consumption, CO2 production, RQ, locomotor activity, UCP-1 protein expression in BAT, PEPCK protein expression in liver and the pyruvate test were measured.

**Oxygen consumption/carbon dioxide production, and respiratory exchange ratio determination**. Oxygen (O2) consumption, carbon dioxide (CO2) production, and respiratory exchange ratio were measured in fed mice through an indirect open circuit calorimeter (Oxymax Deluxe System; Columbus Instruments, Columbus, OH), as described previously [47]. Mice were allowed to adapt 2 days before. Measurements were done on the last day of TG003 or siRNA treatment.

**Pyruvate test.** Mice deprived of food for 12 h were injected intraperitoneally with sodium pyruvate (2g/kg). Blood samples were collected from the tail vein immediately before and at various time points (0–180 min) after the pyruvate load.

**Immunoprecipitation (IP) and immunoblotting (IB).** Hypothalami were homogenized in extraction buffer. For IP experiments, the whole lysates were incubated with Clk2 specific antibody and protein A-sepharose and the beads were washed three times. Precipitated proteins were analyzed by SDS-PAGE. For IB, whole lysates were directly analyzed by SDS-PAGE. The separated proteins were visualized by autoradiography using specific antibodies after transfer to nitrocellulose membranes [48]. Clk2, PCG-1α, UCP-1, Pepck, PHLPP1 and pJak2 antibodies were from Santa Cruz Technology (Santa Cruz, CA, USA). pAkt, Jak2, Akt e PP2A from Cell Signaling (Boston, MA, USA) and pThr343 from New England Peptide (Gardner, MA, USA).

**RNA Extraction and Real-Time PCR.** Random fed or fasted (24h) mice treated for seven days with siRNA, TG003 or vehicle received ICV insulin or leptin injection, and the hypothalami or hypothalamic nuclei were harvested after 6h, quickly frozen in liquid nitrogen and stored at -80°C for processing. Total RNA was obtained using RNeasy® Mini Kit (Cat. 74106, QiagenInc.CA, USA). Real-time PCR was performed using TaqMan RT-PCR Master Mix (Applied Biosystems) in an Mx3000P thermocycler (Stratagene). The Mx3000P software was used to calculate the cycle threshold for each reaction. Relative expression levels were determined using the comparative Ct method with normalization of target gene expression levels to *18s*. Primers and probes sequences were purchased from Applied Biosystems and were: Clk2, Mm00432578_m1; NPY, Mm00445771_m1; POMC, Mm00435874_m1; MCH, Mm01242886_g1 for mouse. The PCR conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Real time data were analyzed using the engine provided by Applied Biosystems (Carlsbad, CA, USA).

**Statistical analysis**. Results are expressed as means ± SD. Significance was determined using two-tailed Student's t test or one-way analysis of variance (ANOVA) with Bonferroni post-test, as appropriate, and differences were considered significant if $P< 0.05$. We used GraphPad Prism (GraphPad Software, San Diego, CA, USA).

## Results

**Clk2 is expressed and regulated in the hypothalamus** Previous study showed that Clk2 was expressed in liver [27]. Here, we showed that Clk2 was expressed in multiple hypothalamic nuclei as well as in the hippocampus and cortex of control mice (Fig. 1A). We identified Clk2 as being regulated by nutritional status in the hypothalamus. Clk2 threonine 343 phosphorylation were strongly induced after 1h of

refeeding in the hypothalamus of control mice (Fig. 1B). Clk2 threonine 343 phosphorylation was induced in response to ICV insulin after 15 min in the hypothalamus of control mice (Fig. 1C). Insulin ICV injection was able to induce Clk2 threonine 343 phosphorylation in ARC, LH, PVN, MH of control mice (Fig 1D). Clk2 threonine 343 phosphorylation was dependent of PI3K/Akt in Hypothalamus because the inhibition of PI3K by LY294002 or Akt by Akt VIII was able to abolish $Clk2^{Thr343}$(Fig. 1E).

Similarly to insulin, leptin ICV injection was able to induce Clk2 threonine 343 phosphorylation in the hypothalamus of control mice after 15 minutes (Fig. 1F). The inhibition of PI3K/Jak2 pathway resulted in decrease of leptin-induced Clk2 threonine 343 phosphorylation (Fig. 1G). Thus, the leptin-induced Clk2 threonine 343 phosphorylation was dependent of PI3K/Jak2 pathway.

**Hypothalamic Clk2 inhibition alters adiposity and energy expenditure.** siRNA treatment reduced the expression of Clk2 in neuro-2A cells culture (Fig. 2A). $Clk2^{Thr343}$ in response to insulin was blunted in the hypothalamus of siRNA or TG003 treated mice (Fig. 2B). Mice treated with TG003 or siRNA for seven days had higher body weight compared to vehicle-treated mice (Fig. 2C). Epididymal, retroperitoneal and mesenteric fat pads were greater in TG003 or siRNA treated mice in comparison to vehicle-treated mice (Fig. 2D).

The inhibition of Clk2 in the hypothalamus with TG003 or siRNA decreased O2 consumption, CO2 production and RER (Fig. 3 E-G). This result was consistently with the decrease of UCP-1 and PGC-1alpha protein expression in brown adipose tissue in TG003 or siRNA treated mice (Fig. 3H and I).

**The inhibition of Clk2 in the hypothalamus alters food intake and neuropeptides expressions**

The treatment with siRNA and TG003 increased food intake (Fig. 3A). Food intake was lower in response to insulin or leptin in mice treated with vehicle. However, TG003 or siRNA treatment blunted the anorexigenic effect of insulin and leptin (Fig. 3B). Next we investigated whether TG003 and siRNA treatment for seven days affects neuropeptides expressions in the hypothalamus. As expected, insulin decreased NPY, orexin and MCH expression in the hypothalamus of vehicle-treated mice. However, TG003 treatment blunted the effect of insulin (Fig. 3C-E). On the other hand, insulin was able to increase POMC expression in the hypothalamus of vehicle-treated mice. The inhibition of Clk2 with TG003 abolished the effect of insulin (Fig. 3F).siRNA or TG003 treatment did not impair insulin or leptin signaling, because Akt and Jak2 phosphorylation were intact in response to insulin and leptin respectively in hypothalamus of mice(Fig. 3 G, H).

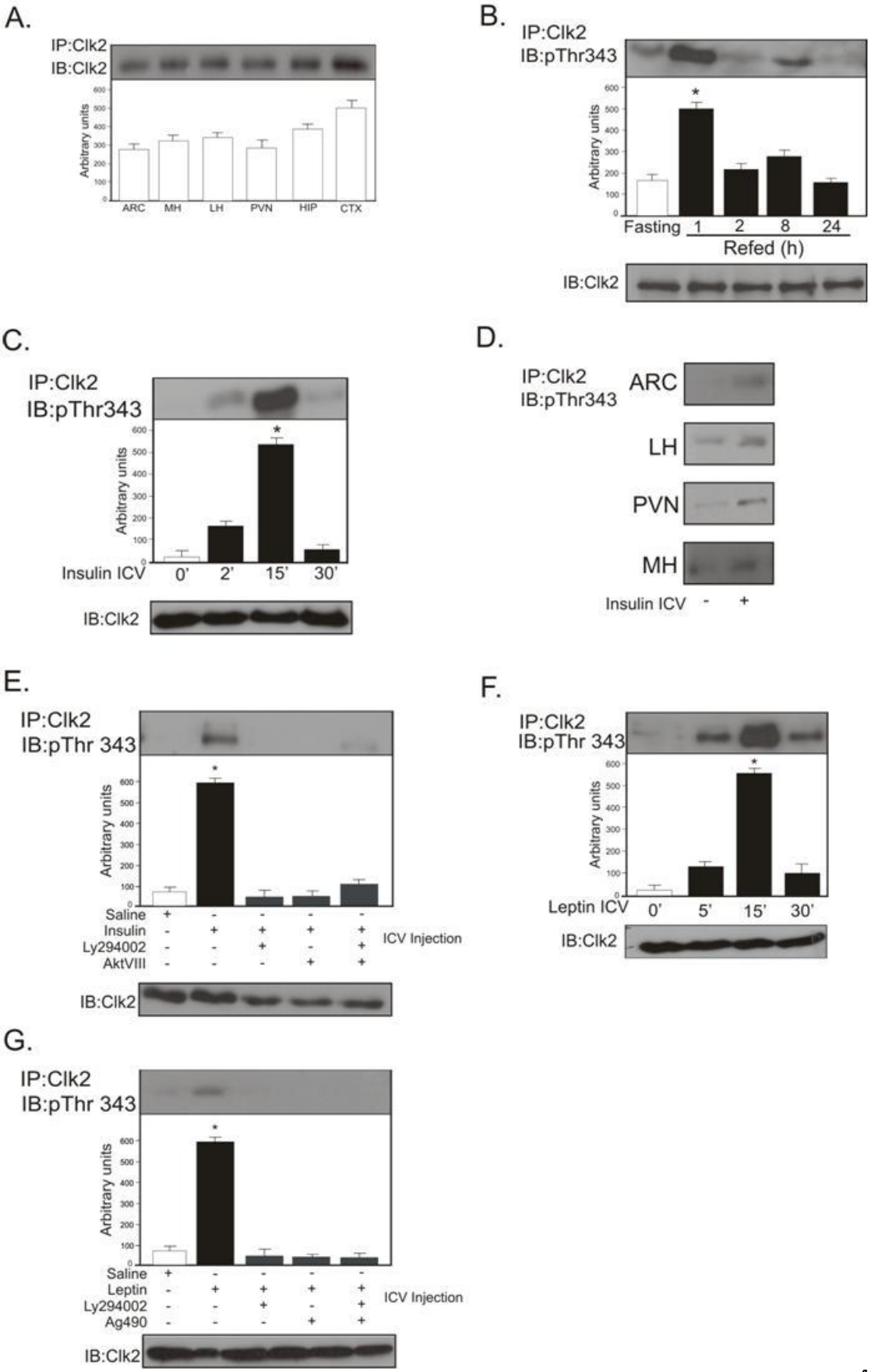
**Clk2 threonine 343 phosphorylation is impaired in the hypothalamus of DIO and db/db mice.**

We investigated whether a high fat diet or a genetic obesity could alter Clk2 threonine 343 phosphorylation in response to insulin. As expected, acute ICV treatment with insulin or leptin increased Clk2 threonine 343 phosphorylation in the hypothalamus of control mice. This effect was reduced in mice on HFD (Fig. 4A, B). In agreement we observed that refeeding increased Clk2 threonine 343 phosphorylation in the hypothalamus of control mice and this effect was blunted in db/db mice (Fig. 4C). It is known that Akt can phosphorylate Clk2 [27], so, we investigated whether ICV insulin increase Clk2 and Akt association. Clk2/Akt association in the hypothalamus was higher in response to insulin in lean mice,, but this phenomenon did not occur in mice fed with high-fat diet (Fig. 4D). Since PP2A and PHLPP1 dephosphorylates Akt in threonine, we next investigated if insulin and leptin can induce an increase in

Clk2/PHLPP1 and Clk2/PP2A association in the hypothalamus, using co-immunoprecipitation. Our data showed that there was an increase in Clk2/PHLPP1 and Clk2/PP2A interactions in response to insulin and leptin in the hypothalamus of obese mice (Fig. 4E,F).

**siRNA or TG003 treatment impairs glucose metabolism.** The inhibition of Clk2 increased fasting glycemia and glucose production measured by pyruvate test (Fig. 4A, B). PEPCK and PGC-1 alpha expression were higher in the liver of mice treated with TG003 or siRNA (Fig. 4C, D). These results suggest that the inhibition of Clk2 impaired glucose metabolism.

# Figure 01


A.
IP:Clk2
IB:Clk2
Arbitrary units
ARC
MH
LH
PVN
HIP
CTX
B.
IP:Clk2
IB:pThr343
Arbitrary units
Fasting
1
2
8
24
Refed (h)
IB:Clk2
C.
IP:Clk2
IB:pThr343
Arbitrary units
Insulin ICV
0'
2'
15'
30'
IB:Clk2
D.
IP:Clk2
IB:pThr343
ARC
LH
PVN
MH
Insulin ICV
-
+
E.
IP:Clk2
IB:pThr 343
Arbitrary units
Saline
Insulin
Ly294002
AktVIII
ICV Injection
IB:Clk2
F.
IP:Clk2
IB:pThr 343
Arbitrary units
Leptin ICV
0'
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IB:Clk2
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IP:Clk2
IB:pThr 343
Arbitrary units
Saline
Leptin
Ly294002
Ag490
ICV Injection
IB:Clk2

**Figure 1.** (A) Blots of Clk2 protein expression in hypothalamic nuclei, including Arc (arcuate nucleus), MH (medial hypothalamus), PVN (paraventricular nucleus), LH (lateral hypothalamus), hippocampus (Hip) and cortex (CTX). (B) Effect of fasting (24h) and refeeding on Clk2 threonine 343 phosphorylation in hypothalamus. Mice were sacrificed at 1, 2, 8, 24h after refeeding. (C) Clk2 threonine 343 phosphorylation in response to insulin in whole hypothalamus. (D) Clk2 threonine 343 phosphorylation in response to insulin in hypothalamic nuclei after 15 minutes. (E) Clk2 threonine 343 phosphorylation in response to insulin with or without LY249003 or Akt VIII inhibitors. (F) Clk2 threonine 343 phosphorylation in response to leptin in the hypothalamus. (G) Clk2 threonine 343 phosphorylation in response to leptin with or without LY249003 and AG490 inhibitors. Data are presented as means +/- SD from 5 mice. One -way analysis of variance (ANOVA) with Bonferroni post-test was used. *P<0.05 versus other groups.

# Figure 02

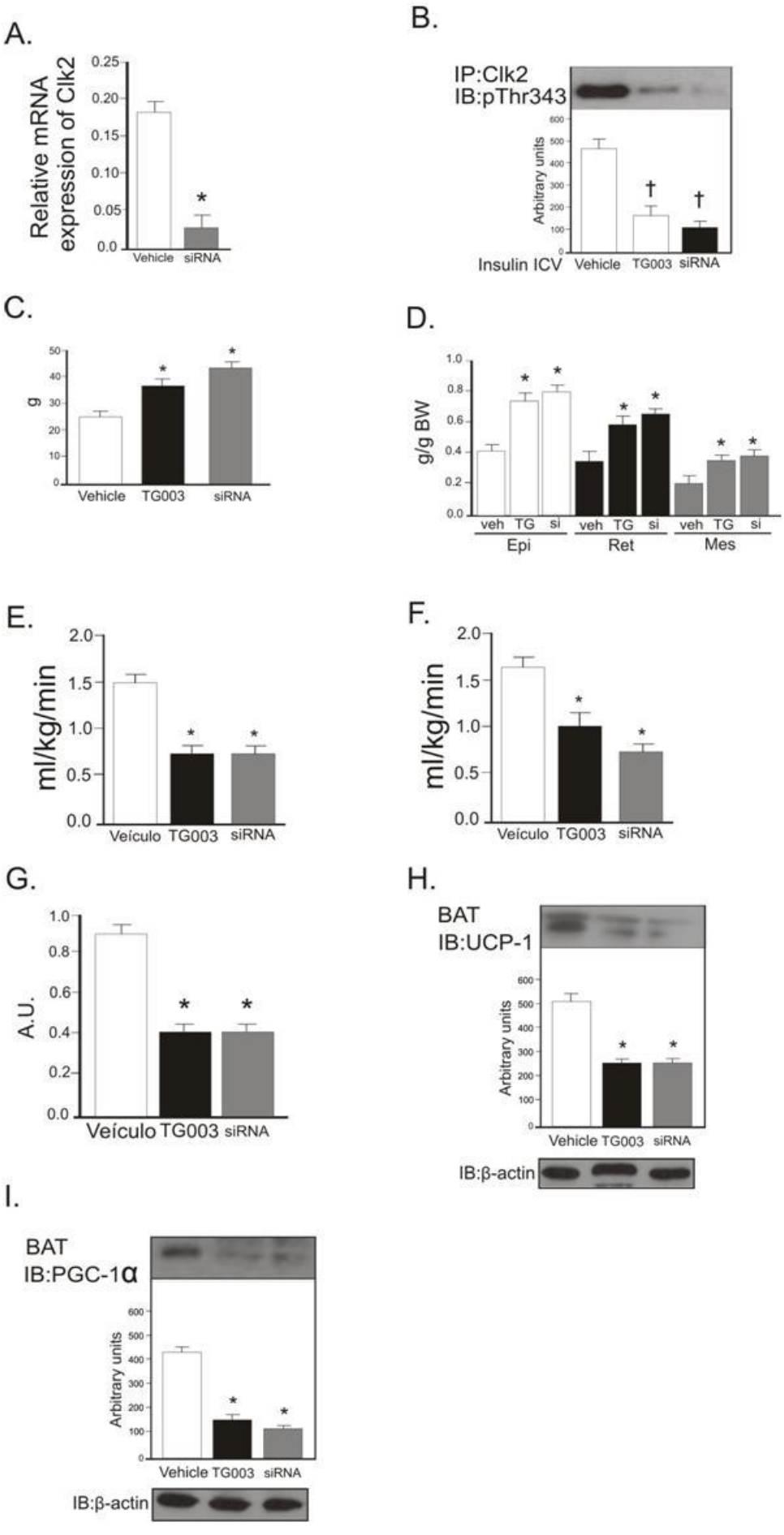
A.
Relative mRNA expression of Clk2
0.25
0.20
0.15
0.10
0.05
0.0
*
Vehicle siRNA
B.
IP:Clk2
IB:pThr343
Arbitrary units
600
500
400
300
200
100
0
†
†
Insulin ICV Vehicle TG003 siRNA
C.
g
50
40
30
20
10
0
*
*
Vehicle TG003 siRNA
D.
g/g BW
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
*
*
veh TG si veh TG si veh TG si
Epi Ret Mes
E.
ml/kg/min
2.0
1.5
1.0
0.5
0.0
*
*
Veículo TG003 siRNA
F.
ml/kg/min
2.0
1.5
1.0
0.5
0.0
*
*
Veículo TG003 siRNA
G.
A.U.
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Veículo TG003 siRNA
H.
BAT
IB:UCP-1
Arbitrary units
600
500
400
300
200
100
0
*
*
Vehicle TG003 siRNA
IB:β-actin
I.
BAT
IB:PGC-1α
Arbitrary units
600
500
400
300
200
100
0
*
*
Vehicle TG003 siRNA
IB:β-actin

**Figure 2.** (A) Relative mRNA expression of Clk2 in neuro-2A cells induced Clk2 inhibition with siRNA. (B) ICV injection of TG003 or siRNA for 7 days decreases Clk2 phosphorylation in hypothalamus. ICV injection of TG003 or siRNA for 7 days increased body weight (C) and epididimal, retroperitoneal and mesenteric relative fat mass (D). The TG003 or siRNA treatment decreased O2 consumption (E), CO2 production (F) and RER (G). UCP-1(H) and PGC-1 (I) expression in BAT is decreased in treated animals. Data are presented as means **+/-** SD from 5-7 mice. Two-tailed Student's t test or one-way analysis of variance (ANOVA) with Bonferroni post-test were used. *P<0.05 versus Vehicle or Vehicle-saline. †P<0.05 versus Vehicle.

# Figure 03

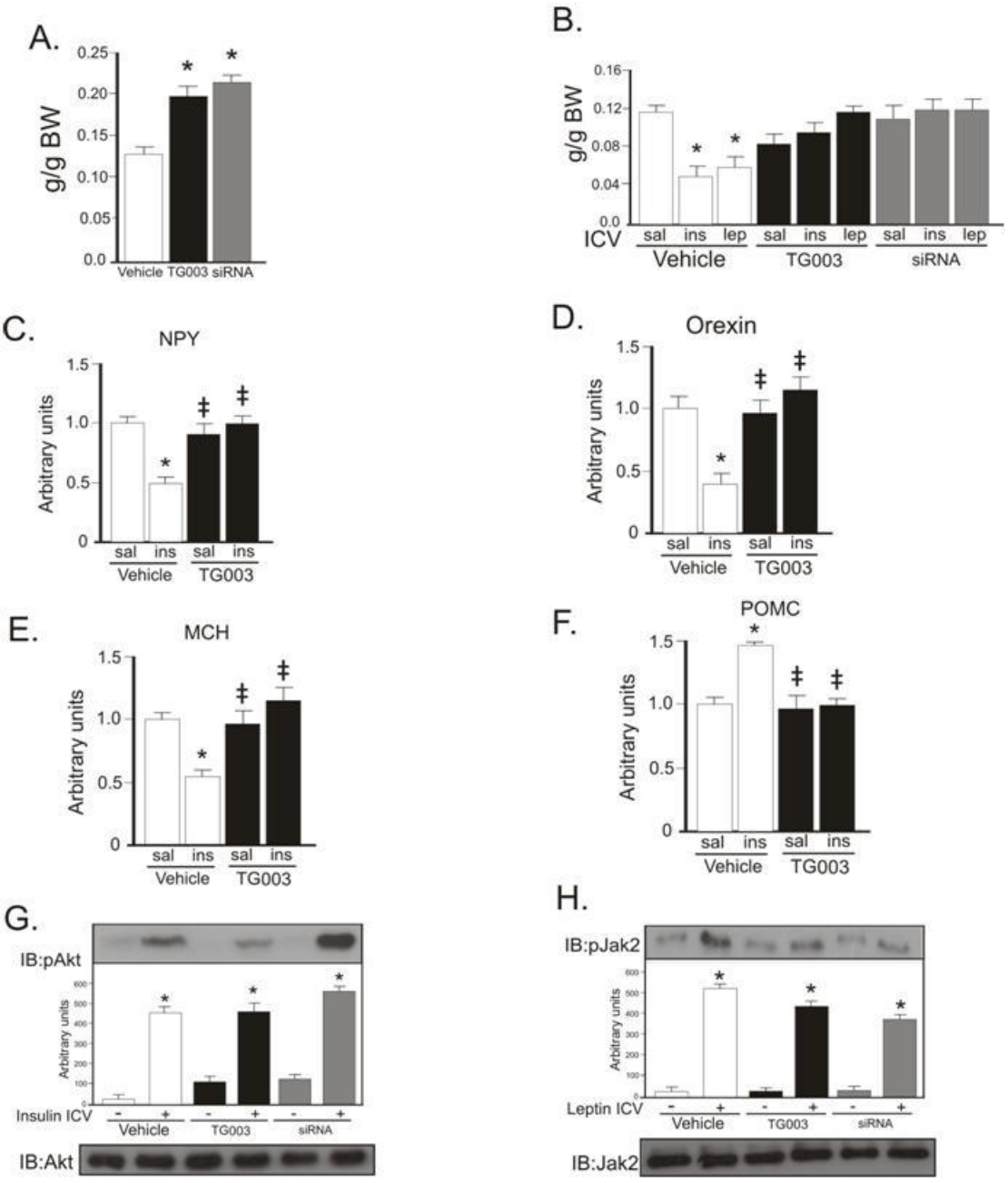


**Figure 3.** (A) ICV injection of TG003 or siRNA increases food intake in 24 hours. (B)The TG003 or siRNA treatment blunts insulin and leptin induced anorexia. NPY (C), orexin (D), MCH (E) and POMC (F) expression is disrupted in treated mice. (G)(H) Insulin and leptin signaling after TG003 or siRNA. Data are presented as means **+/-** SD from 5-7 mice. One-way analysis of variance (ANOVA) with Bonferroni post-

test were used. *P<0.05 versus Vehicle or Vehicle-Saline.‡P<0.05 versus Vehicle-Insulin.

Figure 04

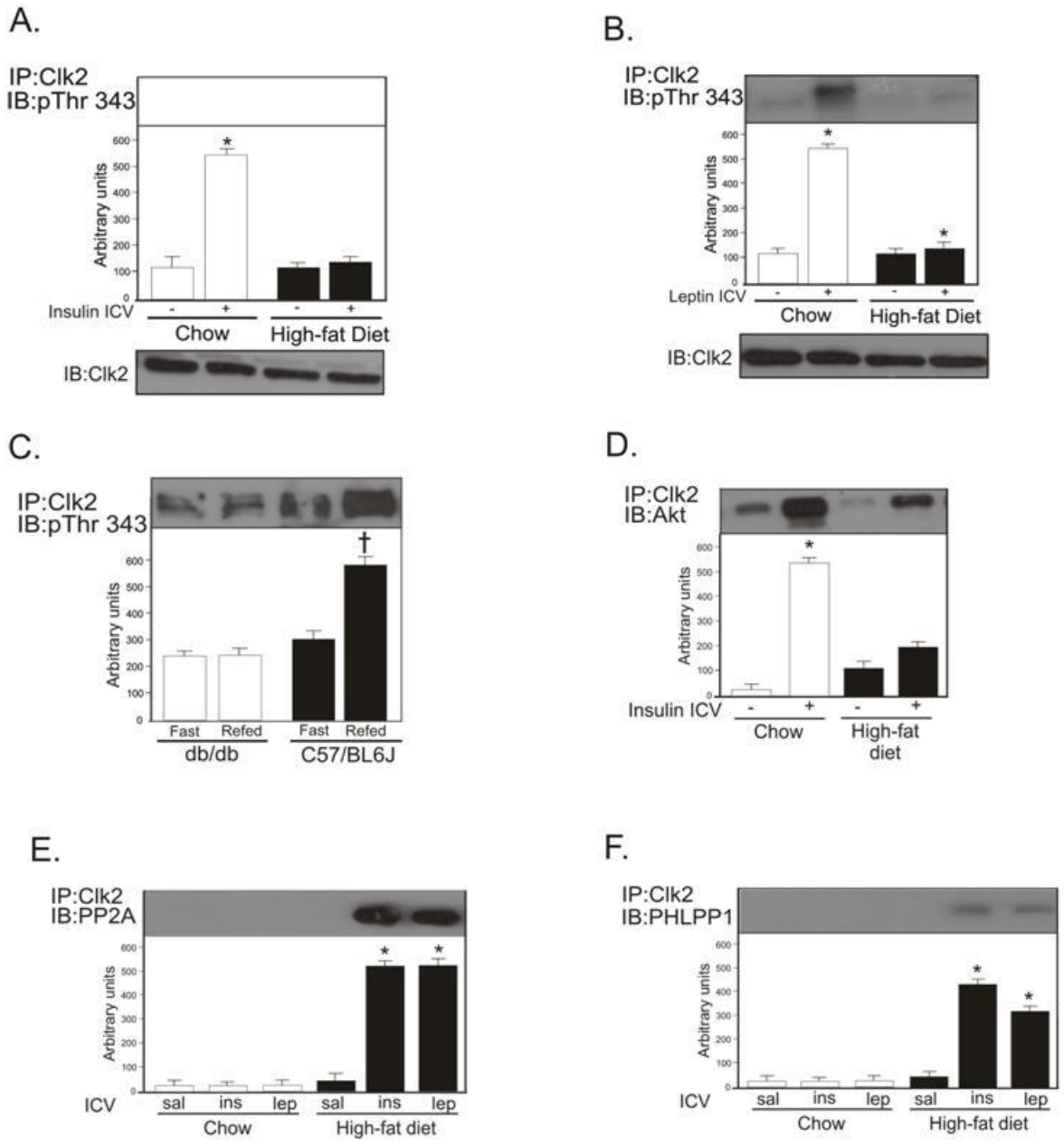


**Figure 4.** (A) Chow and high-fat diet fed mice which received or not ICV insulin. (B) Chow and high-fat diet fed mice which received or not ICV leptin. (C) db/db mice on fast or refed state. (D) Chow, high-fat diet and TG003 treated mice association of

Clk2/Akt. (E) High-fat diet and chow fed animals association of Clk2/PP2A in hypothalamus. (F) High-fat diet and chow fed animals association of Clk2/PHLPP1 in hypothalamus. Data are presented as means +/- SD from 6 mice. One-way analysis of variance (ANOVA) with Bonferroni post-test was used. *$P$<0.05 versus Chow-saline or Fast .

Figure 05

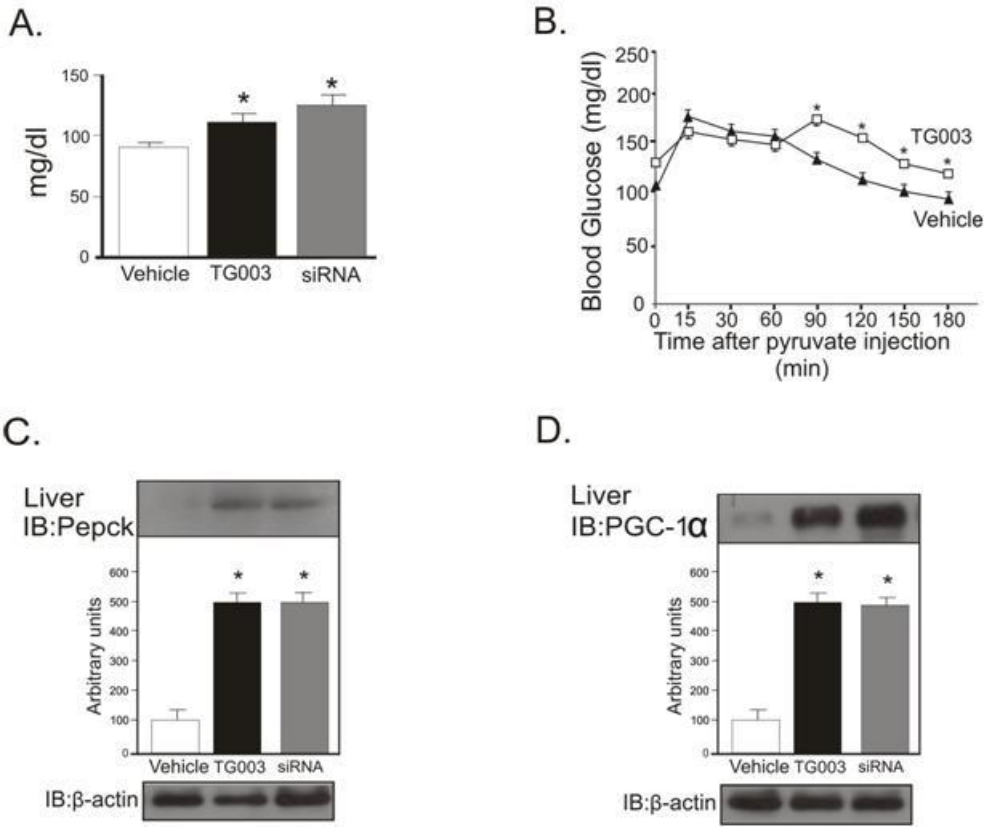


**Figure 5.** (A)Glycemia after 7 days of TG003 or siRNA is higher compared to non-treated animals. (B) Pyruvate tolerance test. PEPCK (C) and PGC-1(D) expression in liver of treated animals. Data are presented as means +/- SD from 5 mice. One-way analysis of variance (ANOVA) with Bonferroni post-test was used. *$P$<0.05 versus Vehicle.

**Discussion**

The results of the present study demonstrate that Clk2 is expressed and regulated in the hypothalamus in response to refeeding, suggesting that Clk2 is regulated by nutritional status.

Rodgers, et al (2010) demonstrated that hepatic Clk2 protein expression is upregulated by refeeding [18]. Herein, we showed that the same result in the hypothalamus.

Our data also shows that hypothalamic Clk2 is important to maintain energy balance because its reduction triggers obesity in control mice. Higher adiposity induced by Clk2 reduction was a reflex of enhanced food intake and reduced energy expenditure in control mice.

Insulin and leptin receptors are broadly expressed in the central nervous system (CNS), including in all hypothalamic nuclei. The signaling of these hormones in the hypothalamus is key to induce anorexia and weight loss [21, 50-53].

The anorexigenic effects of insulin are, at least in part, due to its effects on the PI3K/Akt pathway in the hypothalamus [35]. Here we observed that Clk2 threonine 343 phosphosphorylation was enhanced by insulin injection in a time depend manner. We also observed that LY294002 or Akt VIII or both together blunted Clk2 phosphorylation in response to insulin in the hypothalamus.

Besides the effects of insulin in the hypothalamus, leptin acts through the JAK2 and PI3K to decrease food intake and induce weight loss [35-39]. Here we observed an increase in Clk2 threonine 343 phosphorylation in the hypothalamus in response to leptin and the injection of LY prior leptin abolished this effect.

Taking these results together it seems that Clk2 is regulated by insulin and leptin and is downstream to PI3K. Rodgers, et al (2010) also found that Clk2 phosphorylation in liver was dependent of PI3K [27].

High fat diet is known to impair insulin and leptin in the hypothalamus [54]. It is demonstrated that obese db/db mice ($Lepr^{db}/Lepr^{db}$) had a striking downregulation of hepatic Clk2 protein expression and overexpression of Clk2 in db/db mice dramatically reduced glucose production [27] . By analogy, we investigate whether Clk2 was downregulated in obese mice. We found that in mice fed with a high fat diet and in db/db mice Clk2Th343 phosphorylation was reduced in response to refeeding or ICV insulin in the hypothalamus. This reduction may contribute to hyperphagia observed in those mice.

The impairment of Clk2 Thr343 phosphorylation may be due to negative modulators such as threonine phosphatases as PP2A and PHLPP1 [55-60]. We observed an increase in Clk2/PP2A and Clk2/PHLPP1 association in animals fed with high fat diet which may suggest an interaction of Clk2 with those phosphatases. This phenomenon deserves more studies.

PHLPP1 is a Ser/Thr protein phosphatase that has been implicated in negatively regulate Akt by selectively dephosphorylating its Ser473 (pAKT473) site. In human subcutaneous fat and skeletal muscle it was shown increased abundance of PHLPP-1 in obese patients with insulin resistance. In the brain PHLPP1 has been linked to the control of neuronal Akt and apoptosis [61-64].

PP2A plays important roles in regulation of cell cycle, signal transduction, cell differentiation, and transformation. Its activity is related to several diseases, including

neurodegenerative diseases, obesity and cancer. PP2A is another Ser/Thr protein phosphatase that can dephosphorylate T308 and S473 sites on Akt [65-66]

Insulin and leptin mediate their effects on body weight and fuel metabolism by acting on specific neurons [35]. POMC-expressing neurons in the ARC is increased by insulin and induce anorexia, and increase energy expenditure [35]. We observed that the inhibition of Clk2 with TG003 blocked the effect of insulin in enhance POMC mRNA expression. NPY is also expressed in ARC, however, it induces hyperphagia. In this study, insulin was not able to decrease NPY mRNA expression after chronic TG003 treatment. Similarly, insulin did not reduce orexin and MCH mRNA expression in the hypothalamus of mice treated with TG003. Together, these results suggest that the inhibition of Clk2 in the hypothalamus impaired insulin to induce increase in anorexigenic and decrease in orexigenic signals.

POMC mRNA levels are critical to regulate energy expenditure via α-MSH [67]. Herein, we showed impairment in insulin increased POMC mRNA levels which may contribute to lower energy expenditure observed in mice treated with TG003 or siRNA. In accordance to reduced energy expenditure it was a decreased in UCP-1 and PGC-1 alpha protein expression in BAT.

Beyond that, hypothalamus plays a role regulating hepatic gluconeogenesis [68]. During Clk2 inhibition we observed an increased in fasting glycemia and glucose production. Furthermore, PEPCK and PGC-1 alpha protein expression were higher in the liver of mice treated with TG003 or siRNA. These results suggest that hypothalamic Clk2 may has a role regulating glucose metabolism in liver.

In summary, our data provide evidence that hypothalamic Clk2 protein is expressed and regulated in the hypothalamus. Refeeding, insulin and leptin induced

Clk2 Threonine 343 phosphorylation which is dependent of PI3K pathway. Obesity states impair insulin or leptin induced Clk2 phosphorylation. We also suggest that hypothalamic Clk2 is crucial to control energy balance because its inhibition triggers obesity accompanied by increased food intake and decreased energy expenditure.

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## References


1. Allison DB, Fontaine KR, Manson JE, Stevens J, VanItallie TB. Annual deaths attributable to obesity in the United States. JAMA. 1999;282(16):1530-8.
2. Hu FB, Willett WC, Li T, Stampfer MJ, Colditz GA, Manson JE. Adiposity as compared with physical activity in predicting mortality among women. N Engl J Med. 2004;351(26):2694-703.
3. Olshansky SJ, Passaro DJ, Hershow RC, Layden J, Carnes BA, Brody J, et al. A potential decline in life expectancy in the United States in the 21st century. N Engl J Med. 2005;352(11):1138-45.
4. Pischon T, Boeing H, Hoffmann K, Bergmann M, Schulze MB, Overvad K, et al. General and abdominal adiposity and risk of death in Europe. N Engl J Med. 2008;359(20):2105-20.
5. Stamler R, Stamler J, Riedlinger WF, Algera G, Roberts RH. Weight and blood pressure. Findings in hypertension screening of 1 million Americans. JAMA. 1978;240(15):1607-10.
6. Belgardt BF, Bruning JC. CNS leptin and insulin action in the control of energy homeostasis. Ann N Y Acad Sci.1212:97-113.
7. Carvalheira JB, Torsoni MA, Ueno M, Amaral ME, Araujo EP, Velloso LA, et al. Cross-talk between the insulin and leptin signaling systems in rat hypothalamus. Obes Res. 2005;13(1):48-57.
8. Morton GJ, Schwartz MW. The NPY/AgRP neuron and energy homeostasis. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2001;25 Suppl 5:S56-62.
9. Woods SC, Schwartz MW, Baskin DG, Seeley RJ. Food intake and the regulation of body weight. Annu Rev Psychol. 2000;51:255-77.
10. Belgardt BF, Okamura T, Bruning JC. Hormone and glucose signalling in POMC and AgRP neurons. J Physiol. 2009;587(Pt 22):5305-14.
11. Belgardt BF, Bruning JC. CNS leptin and insulin action in the control of energy homeostasis. Annals of the New York Academy of Sciences. 2010;1212:97-113.
12. Saad MJ, Folli F, Kahn JA, Kahn CR. Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. J Clin Invest. 1993;92(4):2065-72. PMCID: 288376.

13. Pardini AW, Nguyen HT, Figlewicz DP, Baskin DG, Williams DL, Kim F, et al. Distribution of insulin receptor substrate-2 in brain areas involved in energy homeostasis. Brain research. 2006;1112(1):169-78.

14. Belgardt BF, Husch A, Rother E, Ernst MB, Wunderlich FT, Hampel B, et al. PDK1 deficiency in POMC-expressing cells reveals FOXO1-dependent and -independent pathways in control of energy homeostasis and stress response. Cell metabolism. 2008;7(4):291-301.

15. Fukuda M, Jones JE, Olson D, Hill J, Lee CE, Gautron L, et al. Monitoring FoxO1 localization in chemically identified neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008;28(50):13640-8. PMCID: 2615536.

16. Kitamura T, Feng Y, Kitamura YI, Chua SC, Jr., Xu AW, Barsh GS, et al. Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. Nature medicine. 2006;12(5):534-40.

17. Kim MS, Pak YK, Jang PG, Namkoong C, Choi YS, Won JC, et al. Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. Nature neuroscience. 2006;9(7):901-6.

18. Rodgers JT, Haas W, Gygi SP, Puigserver P. Cdc2-like kinase 2 is an insulin-regulated suppressor of hepatic gluconeogenesis. Cell metabolism. 2010;11(1):23-34. PMCID: 2807620.

19. Prada PO, Ropelle ER, Mourao RH, de Souza CT, Pauli JR, Cintra DE, et al. EGFR tyrosine kinase inhibitor (PD153035) improves glucose tolerance and insulin action in high-fat diet-fed mice. Diabetes. 2009;58(12):2910-9. PMCID: 2780887.

20. Prada PO, Hirabara SM, de Souza CT, Schenka AA, Zecchin HG, Vassallo J, et al. L-glutamine supplementation induces insulin resistance in adipose tissue and improves insulin signalling in liver and muscle of rats with diet-induced obesity. Diabetologia. 2007;50(9):1949-59.

21. Prada PO, Zecchin HG, Gasparetti AL, Torsoni MA, Ueno M, Hirata AE, et al. Western diet modulates insulin signaling, c-Jun N-terminal kinase activity, and insulin receptor substrate-1ser307 phosphorylation in a tissue-specific fashion. Endocrinology. 2005;146(3):1576-87.

22. Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, Xue B, et al. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature. 2004;428(6982):569-74.

23. Air EL, Benoit SC, Clegg DJ, Seeley RJ, Woods SC. Insulin and leptin combine additively to reduce food intake and body weight in rats. Endocrinology. 2002;143(6):2449-52.

24. Brown LM, Clegg DJ, Benoit SC, Woods SC. Intraventricular insulin and leptin reduce food intake and body weight in C57BL/6J mice. Physiol Behav. 2006;89(5):687-91.

25. Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, et al. Role of brain insulin receptor in control of body weight and reproduction. Science. 2000;289(5487):2122-5.

26. Myers MG, Jr., Leibel RL, Seeley RJ, Schwartz MW. Obesity and leptin resistance: distinguishing cause from effect. Trends Endocrinol Metab.21(11):643-51.

27. Leinninger GM, Jo YH, Leshan RL, Louis GW, Yang H, Barrera JG, et al. Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding. Cell Metab. 2009;10(2):89-98.

28. Velloso LA, Schwartz MW. Altered hypothalamic function in diet-induced obesity. Int J Obes (Lond). 2011;35(12):1455-65. PMCID: 3383790.

29. Andreozzi F, Procopio C, Greco A, Mannino GC, Miele C, Raciti GA, et al. Increased levels of the Akt-specific phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP)-1 in obese participants are associated with insulin resistance. Diabetologia.54(7):1879-87.

30. Ahmad F, Considine RV, Goldstein BJ. Increased abundance of the receptor-type protein-tyrosine phosphatase LAR accounts for the elevated insulin receptor dephosphorylating activity in adipose tissue of obese human subjects. J Clin Invest. 1995;95(6):2806-12.

31. Ahmad F, Goldstein BJ. Functional association between the insulin receptor and the transmembrane protein-tyrosine phosphatase LAR in intact cells. J Biol Chem. 1997;272(1):448-57.

32. Kulas DT, Zhang WR, Goldstein BJ, Furlanetto RW, Mooney RA. Insulin receptor signaling is augmented by antisense inhibition of the protein tyrosine phosphatase LAR. J Biol Chem. 1995;270(6):2435-8.

33. Bence KK, Delibegovic M, Xue B, Gorgun CZ, Hotamisligil GS, Neel BG, et al. Neuronal PTP1B regulates body weight, adiposity and leptin action. Nat Med. 2006;12(8):917-24.

34. Picardi PK, Calegari VC, Prada Pde O, Moraes JC, Araujo E, Marcondes MC, et al. Reduction of hypothalamic protein tyrosine phosphatase improves insulin and leptin resistance in diet-induced obese rats. Endocrinology. 2008;149(8):3870-80.

35. O'Neill AK, Niederst MJ, Newton AC. Suppression of survival signalling pathways by the phosphatase PHLPP. FEBS J. 2012.

36. Andreozzi F, Procopio C, Greco A, Mannino GC, Miele C, Raciti GA, et al. Increased levels of the Akt-specific phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP)-1 in obese participants are associated with insulin resistance. Diabetologia. 2011;54(7):1879-87.

37. Gao T, Furnari F, Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol Cell. 2005;18(1):13-24.

38. Hirano I, Nakamura S, Yokota D, Ono T, Shigeno K, Fujisawa S, et al. Depletion of Pleckstrin homology domain leucine-rich repeat protein phosphatases 1 and 2 by Bcr-Abl promotes chronic myelogenous leukemia cell proliferation through continuous phosphorylation of Akt isoforms. The Journal of biological chemistry. 2009;284(33):22155-65. PMCID: 2755940.

39. Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG. An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. Cell. 2005;122(2):261-73.

40. Bertoli C, Copetti T, Lam EW, Demarchi F, Schneider C. Calpain small-1 modulates Akt/FoxO3A signaling and apoptosis through PP2A. Oncogene. 2009;28(5):721-33.

41. Coll AP, Loraine Tung YC. Pro-opiomelanocortin (POMC)-derived peptides and the regulation of energy homeostasis. Mol Cell Endocrinol. 2009;300(1-2):147-51.

42. Morton GJ. Hypothalamic leptin regulation of energy homeostasis and glucose metabolism. The Journal of physiology. 2007;583(Pt 2):437-43. PMCID: 2277030.

# *Conclusão*

A proteína Clk2 é expressa em hipotálamo de camundongos Swiss e que a realimentação, injeção ICV de insulina ou leptina induzem a fosforilação da Clk2 em treonina 343, sítio importante para atividade desta proteína. Esta modulação da fosforilação da Clk2 por insulina acontece de maneira dependente da via PI3-q/Akt e a leptina age na fosforilação da Clk2 através do *crosstalk* entre Jak2 e PI3-q. A inibição crônica da Clk2 via ICV por TG003 ou siRNA acarretou em aumento de peso corporal, adiposidade, ingestão alimentar, redução dos efeitos anorexigênicos de insulina e leptina, desregulação da expressão de neuropeptídeos, menor gasto energético e menor termogênese. Em animais obesos (DIO ou db/db) a fosforilação de Clk2 após realimentação e injeção ICV de insulina e leptina está diminuída, levando assim a sugerir o papel da Clk2 para a manutenção do balanço energético.

# *Referências Bibliográficas*

1. Finucane, M.M., et al., *National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9.1 million participants.* Lancet, 2011. **377**(9765): p. 557-67.
2. Kopelman, P.G., *Obesity as a medical problem.* Nature, 2000. **404**(6778): p. 635-43.
3. Ahima, R.S., *Digging deeper into obesity.* J Clin Invest, 2011. **121**(6): p. 2076-9.
4. *Obesity: preventing and managing the global epidemic. Report of a WHO consultation.* World Health Organ Tech Rep Ser, 2000. **894**: p. i-xii, 1-253.
5. Baker, J.L. and T.I. Sorensen, *Obesity research based on the Copenhagen School Health Records Register.* Scand J Public Health, 2011. **39**(7 Suppl): p. 196-200.
6. Smith, P.M. and A.V. Ferguson, *Neurophysiology of hunger and satiety.* Dev Disabil Res Rev, 2008. **14**(2): p. 96-104.
7. Wilding, J.P., *Neuropeptides and appetite control.* Diabet Med, 2002. **19**(8): p. 619-27.
8. Stellar, E., *The physiology of motivation.* Psychol Rev, 1954. **61**(1): p. 5-22.
9. Brobeck, J.R., *Mechanism of the development of obesity in animals with hypothalamic lesions.* Physiol Rev, 1946. **26**(4): p. 541-59.
10. Stellar, E., *Long-term perspectives on the study of eating behavior.* Ann N Y Acad Sci, 1989. **575**: p. 478-86.
11. Coleman, D.L. and K.P. Hummel, *The influence of genetic background on the expression of the obese (Ob) gene in the mouse.* Diabetologia, 1973. **9**(4): p. 287-93.
12. Green, E.D., et al., *The human obese (OB) gene: RNA expression pattern and mapping on the physical, cytogenetic, and genetic maps of chromosome 7.* Genome Res, 1995. **5**(1): p. 5-12.
13. Clement, K., et al., *A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction.* Nature, 1998. **392**(6674): p. 398-401.
14. Ingalls, A.M., M.M. Dickie, and G.D. Snell, *Obese, a new mutation in the house mouse.* J Hered, 1950. **41**(12): p. 317-8.

15. Cohen, P., et al., *Selective deletion of leptin receptor in neurons leads to obesity.* J Clin Invest, 2001. **108**(8): p. 1113-21.
16. Bjorbaek, C. and B.B. Kahn, *Leptin signaling in the central nervous system and the periphery.* Recent Prog Horm Res, 2004. **59**: p. 305-31.
17. Obici, S., et al., *Decreasing hypothalamic insulin receptors causes hyperphagia and insulin resistance in rats.* Nat Neurosci, 2002. **5**(6): p. 566-72.
18. Belgardt, B.F. and J.C. Bruning, *CNS leptin and insulin action in the control of energy homeostasis.* Ann N Y Acad Sci, 2010. **1212**: p. 97-113.
19. Woods, S.C. and R.J. Seeley, *Adiposity signals and the control of energy homeostasis.* Nutrition, 2000. **16**(10): p. 894-902.
20. Ropelle, E.R., et al., *Inhibition of hypothalamic Foxo1 expression reduced food intake in diet-induced obesity rats.* J Physiol, 2009. **587**(Pt 10): p. 2341-51.
21. Bruning, J.C., et al., *Role of brain insulin receptor in control of body weight and reproduction.* Science, 2000. **289**(5487): p. 2122-5.
22. Klockener, T., et al., *High-fat feeding promotes obesity via insulin receptor/PI3K-dependent inhibition of SF-1 VMH neurons.* Nat Neurosci, 2011. **14**(7): p. 911-8.
23. Niswender, K.D., et al., *Intracellular signalling. Key enzyme in leptin-induced anorexia.* Nature, 2001. **413**(6858): p. 794-5.
24. Niswender, K.D., et al., *Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia.* Diabetes, 2003. **52**(2): p. 227-31.
25. White, M.F., *The insulin signalling system and the IRS proteins.* Diabetologia, 1997. **40 Suppl 2**: p. S2-17.
26. Velloso, L.A., *[New model, old medicine].* Arq Bras Endocrinol Metabol, 2009. **53**(4): p. 389-90.
27. Rodgers, J.T., et al., *Cdc2-like kinase 2 is an insulin-regulated suppressor of hepatic gluconeogenesis.* Cell Metab, 2010. **11**(1): p. 23-34.
28. Lee, K., et al., *Activity and autophosphorylation of LAMMER protein kinases.* J Biol Chem, 1996. **271**(44): p. 27299-303.
29. Nam, S.Y., et al., *Phosphorylation of CLK2 at serine 34 and threonine 127 by AKT controls cell survival after ionizing radiation.* J Biol Chem, 2010. **285**(41): p. 31157-63.

30. Allison, D.B., et al., *Annual deaths attributable to obesity in the United States.* JAMA, 1999. **282**(16): p. 1530-8.
31. Hu, F.B., et al., *Adiposity as compared with physical activity in predicting mortality among women.* N Engl J Med, 2004. **351**(26): p. 2694-703.
32. Olshansky, S.J., et al., *A potential decline in life expectancy in the United States in the 21st century.* N Engl J Med, 2005. **352**(11): p. 1138-45.
33. Pischon, T., et al., *General and abdominal adiposity and risk of death in Europe.* N Engl J Med, 2008. **359**(20): p. 2105-20.
34. Stamler, R., et al., *Weight and blood pressure. Findings in hypertension screening of 1 million Americans.* JAMA, 1978. **240**(15): p. 1607-10.
35. Belgardt, B.F. and J.C. Bruning, *CNS leptin and insulin action in the control of energy homeostasis.* Ann N Y Acad Sci. **1212**: p. 97-113.
36. Carvalheira, J.B., et al., *Cross-talk between the insulin and leptin signaling systems in rat hypothalamus.* Obes Res, 2005. **13**(1): p. 48-57.
37. Morton, G.J. and M.W. Schwartz, *The NPY/AgRP neuron and energy homeostasis.* Int J Obes Relat Metab Disord, 2001. **25 Suppl 5**: p. S56-62.
38. Woods, S.C., et al., *Food intake and the regulation of body weight.* Annu Rev Psychol, 2000. **51**: p. 255-77.
39. Belgardt, B.F., T. Okamura, and J.C. Bruning, *Hormone and glucose signalling in POMC and AgRP neurons.* J Physiol, 2009. **587**(Pt 22): p. 5305-14.
40. Saad, M.J., et al., *Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats.* J Clin Invest, 1993. **92**(4): p. 2065-72.
41. Pardini, A.W., et al., *Distribution of insulin receptor substrate-2 in brain areas involved in energy homeostasis.* Brain Res, 2006. **1112**(1): p. 169-78.
42. Belgardt, B.F., et al., *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): p. 291-301.
43. Fukuda, M., et al., *Monitoring FoxO1 localization in chemically identified neurons.* J Neurosci, 2008. **28**(50): p. 13640-8.
44. Kitamura, T., et al., *Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake.* Nat Med, 2006. **12**(5): p. 534-40.
45. Kim, M.S., et al., *Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis.* Nat Neurosci, 2006. **9**(7): p. 901-6.

46. Prada, P.O., et al., *EGFR tyrosine kinase inhibitor (PD153035) improves glucose tolerance and insulin action in high-fat diet-fed mice.* Diabetes, 2009. **58**(12): p. 2910-9.
47. Prada, P.O., et al., *L-glutamine supplementation induces insulin resistance in adipose tissue and improves insulin signalling in liver and muscle of rats with diet-induced obesity.* Diabetologia, 2007. **50**(9): p. 1949-59.
48. Prada, P.O., et al., *Western diet modulates insulin signaling, c-Jun N-terminal kinase activity, and insulin receptor substrate-1ser307 phosphorylation in a tissue-specific fashion.* Endocrinology, 2005. **146**(3): p. 1576-87.
49. Minokoshi, Y., et al., *AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus.* Nature, 2004. **428**(6982): p. 569-74.
50. Air, E.L., et al., *Insulin and leptin combine additively to reduce food intake and body weight in rats.* Endocrinology, 2002. **143**(6): p. 2449-52.
51. Brown, L.M., et al., *Intraventricular insulin and leptin reduce food intake and body weight in C57BL/6J mice.* Physiol Behav, 2006. **89**(5): p. 687-91.
52. Myers, M.G., Jr., et al., *Obesity and leptin resistance: distinguishing cause from effect.* Trends Endocrinol Metab. **21**(11): p. 643-51.
53. Leinninger, G.M., et al., *Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding.* Cell Metab, 2009. **10**(2): p. 89-98.
54. Velloso, L.A. and M.W. Schwartz, *Altered hypothalamic function in diet-induced obesity.* Int J Obes (Lond), 2011. **35**(12): p. 1455-65.
55. Andreozzi, F., et al., *Increased levels of the Akt-specific phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP)-1 in obese participants are associated with insulin resistance.* Diabetologia. **54**(7): p. 1879-87.
56. Ahmad, F., R.V. Considine, and B.J. Goldstein, *Increased abundance of the receptor-type protein-tyrosine phosphatase LAR accounts for the elevated insulin receptor dephosphorylating activity in adipose tissue of obese human subjects.* J Clin Invest, 1995. **95**(6): p. 2806-12.
57. Ahmad, F. and B.J. Goldstein, *Functional association between the insulin receptor and the transmembrane protein-tyrosine phosphatase LAR in intact cells.* J Biol Chem, 1997. **272**(1): p. 448-57.

58. Kulas, D.T., et al., *Insulin receptor signaling is augmented by antisense inhibition of the protein tyrosine phosphatase LAR.* J Biol Chem, 1995. **270**(6): p. 2435-8.
59. Bence, K.K., et al., *Neuronal PTP1B regulates body weight, adiposity and leptin action.* Nat Med, 2006. **12**(8): p. 917-24.
60. Picardi, P.K., et al., *Reduction of hypothalamic protein tyrosine phosphatase improves insulin and leptin resistance in diet-induced obese rats.* Endocrinology, 2008. **149**(8): p. 3870-80.
61. O'Neill, A.K., M.J. Niederst, and A.C. Newton, *Suppression of survival signalling pathways by the phosphatase PHLPP.* FEBS J, 2012.
62. Andreozzi, F., et al., *Increased levels of the Akt-specific phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP)-1 in obese participants are associated with insulin resistance.* Diabetologia, 2011. **54**(7): p. 1879-87.
63. Gao, T., F. Furnari, and A.C. Newton, *PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth.* Mol Cell, 2005. **18**(1): p. 13-24.
64. Hirano, I., et al., *Depletion of Pleckstrin homology domain leucine-rich repeat protein phosphatases 1 and 2 by Bcr-Abl promotes chronic myelogenous leukemia cell proliferation through continuous phosphorylation of Akt isoforms.* J Biol Chem, 2009. **284**(33): p. 22155-65.
65. Beaulieu, J.M., et al., *An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior.* Cell, 2005. **122**(2): p. 261-73.
66. Bertoli, C., et al., *Calpain small-1 modulates Akt/FoxO3A signaling and apoptosis through PP2A.* Oncogene, 2009. **28**(5): p. 721-33.
67. Coll, A.P. and Y.C. Loraine Tung, *Pro-opiomelanocortin (POMC)-derived peptides and the regulation of energy homeostasis.* Mol Cell Endocrinol, 2009. **300**(1-2): p. 147-51.
68. Morton, G.J., *Hypothalamic leptin regulation of energy homeostasis and glucose metabolism.* J Physiol, 2007. **583**(Pt 2): p. 437-43.