Vagner Ramon Rodrigues Silva

EFEITOS DO EXERCÍCIO FÍSICO SOBRE A SINALIZAÇÃO DA LEPTINA NO HIPOTÁLAMO DE RATOS: O PAPEL DA S1PR1 NEURONAL.

Limeira / SP

2013

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS APLICADAS

Vagner Ramon Rodrigues Silva

EFEITOS DO EXERCÍCIO FÍSICO SOBRE A SINALIZAÇÃO DA LEPTINA NO HIPOTÁLAMO DE RATOS: O PAPEL DA S1PR1 NEURONAL.

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências da Nutrição e do Esporte e Metabolismo da Faculdade de Ciências Aplicadas da Universidade Estadual de Campinas para obtenção de título de Mestre em Ciências da Nutrição e do Esporte e Metabolismo, área de concentração Metabolismo e Biologia molecular.

Orientador: Prof. Dr. Eduardo Rochete Ropelle

ESTE EXEMPLAR CORRESPONDE A VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELO ALUNO VAGNER RAMON RODRIGUES SILVA E ORIENTADO PELO PROF. DR. EDUARDO ROCHETE ROPELLE.

Assinatura do Orientador

Limeira / SP
2013

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Ciências Aplicadas
Sueli Ferreira Júlio de Oliveira - CRB 8/2380

Si38e

Silva, Vagner Ramon Rodrigues, 1985-
Efeitos do exercício físico sobre a sinalização da leptina no hipotálamo de ratos : o papel da S1PR1 neuronal / Vagner Ramon Rodrigues Silva. – Campinas, SP : [s.n.], 2013.

Orientador: Eduardo Rochete Ropelle.
Dissertação (mestrado) – Universidade Estadual de Campinas, Faculdade de Ciências Aplicadas.

1. Exercício físico. 2. Obesidade. 3. Leptina. 4. Hipotálamo. 5. Proteína S1PR1. I. Ropelle, Eduardo Rochete. II. Universidade Estadual de Campinas. Faculdade de Ciências Aplicadas. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Effects of physical exercise on leptina signaling in the hypothalamus of rats : the role of neuronal S1PR1
Palavras-chave em inglês:
Physical exercise
Obesity
Leptin
Hypothalamus
S1PR1 Protein
Área de concentração: Metabolismo e Biologia Molecular
Titulação: Mestre em Ciências da Nutrição e do Esporte e Metabolismo
Banca examinadora:
Eduardo Rochete Ropelle [Orientador]
Bruno de Melo Carvalho
Marciane Milanski
Data de defesa: 11-11-2013
Programa de Pós-Graduação: Ciências da Nutrição e do Esporte e Metabolismo

COMISSÃO JULGADORA – DISSERTAÇÃO DE MESTRADO

Candidato: Vagner Ramon Rodrigues Silva

Data de Defesa: 11 de novembro de 2013

Título da Dissertação: "Efeitos do Exercício Físico sobre a Sensibilidade a Leptina em Hipotálamo de Ratos: O Papel da S1PR1 Neuronal.

Profº Dr. Eduardo Rochete Ropelle (Presidente): _____

Profª Drª Marciane Milanski: _____

Profº Dr. Bruno de Melo Carvalho: _____

Resumo

A ingestão alimentar e o gasto energético são minuciosamente regulados por neurônios específicos localizados no hipotálamo. Durante as duas últimas décadas, a localização dos receptores da leptina em núcleos hipotalâmicos, bem como a descrição da via de transmissão intracelular disparado por este hormônio em neurônios hipotalâmicos, foi determinante para o entendimento do controle da ingestão alimentar e do gasto energético. Cada vez mais os distúrbios alimentares associados a doenças como obesidade são relacionados à disfunções na transmissão do sinal da leptina no hipotálamo. O processo inflamatório subclínica frequentemente observado em modelos experimentais de obesidade estão diretamente associados à distintos mecanismos de resistência à leptina no hipotálamo e resultam em aumento da ingestão alimentar e ganho de peso corporal. Por outro lado, estudos demonstram que o exercício físico é capaz de aumentar a sensibilidade da leptina no hipotálamo de animas obesos através de citocinas anti-imflamatórias, contudo, esses mecanismos permanecem apenas parcialmente conhecidos. Recentemente, a proteína S1PR1 (sphingosine-1-phosphate receptor-1) foi descrita como uma molécula com alta capacidade de exercer potentes efeitos sinérgicos sobre a via de sinalização da leptina, sustentando a ativação da via Jak2/STAT3 em algumas linhagens celulares. Assim, o presente estudo tem por objetivo investigar o os efeitos do exercício físico sobre a atividade da SIPR1 e a sensibilidade à leptina em hipotálamo de roedores obesos. Acreditamos que a realização do presente estudo contribuirá para caracterizar a participação da S1PR1 na sinalização da leptina no hipotálamo, bem como determinar os efeitos do exercício físico sobre a atividade da S1PR1 neuronal.

ABSTRACT

The food intake and energy expenditure are closely regulated by specific neurons in the hypothalamus. During the last two decades, the location of the leptin receptor in hypothalamic nuclei as well as the description of the route of transmission Intracellular triggered by this hormone in hypothalamic neurons, were crucial to the understanding of the control of food intake and energy expenditure.Increasingly, eating disorders, diseases associated with obesity are related to signal transmission malfunction of leptin in the hypothalamus. The subclinical inflammatory process frequently observed in experimental models of obesity are directly associated with distinct mechanisms of leptin resistance in the hypothalamus and result in increased food intake and body weight gain. Furthermore, studies have shown that physical exercise can increase the sensitivity of leptin in the hypothalamus of obese animals, through of antiinflammatory cytokines, however, these mechanisms remain only partially understood. Recently, the protein S1PR1 (sphingosine-1-phosphate receptor-1) was described as a molecule with high ability to exert potent synergistic effects on the signaling pathway of leptin, supporting the activation of Jak2/STAT3 in some cell lines. Thus, this project aims to investigate the effects of exercise on the activity of SIPR1 and leptin sensitivity in hypothalamus of obese rodents. We believe that the completion of this project will contribute to characterize the involvement of S1PR1 in leptin signaling in the hypothalamus, and to determine the effects of exercise on the activity of neuronal S1PR1.

Sumário

Dedicatória

Dedico essa dissertação totalmente à Maria Dalva Rodrigues Silva. Mãe, sem você nada do que tenho e do que sou hoje seria possível. Sou grato pela sua amizade e pelo seu cuidado comigo em todos os momentos de minha vida.

Amor eterno à você.

Agradecimentos

Agradeço primeiramente à Deus pelo dom da vida e por me dar forças e coragem para continuar seguindo com os meus sonhos.

A minha família, base de toda uma vida: Maria Dalva Rodrigues Silva (Mãe), João Pereira da Silva (pai), Deibson Rodrigues da Silva (irmão), Fabio Rodrigues da Silva (irmão), Maiara de Paula (cunhada), Cristiane Estefen (cunhada) e meus sobrinhos maravilhosos (João Gabriel, Felipe, Luiz Fernando e Maria Clara), que me proporcionam eternas alegrias.

Ao meu orientador, Prof. Dr. Eduardo Rochete Ropelle por acreditar em minha capacidade e pela paciência durante os anos juntos, pelas dicas e por ter ensinado o que sei até o momento. Deixo aqui meu sincero obrigado e sentimento de orgulho de ser seu primeiro orientando, com muita honra.

Ao meu amigo, Felipe Lovaglio Belozo (Professor), parceiro em todas as horas, que com certeza contribuiu muito para este mestrado professor, e da maneira mais importante, pois muitas vezes a vontade de desistir de tudo foi grande, mas você jamais me deixaria desistir. Obrigado!

Ao meu amigo, Felipe Diogenes de Sousa (oreinha), pelos 20 anos de amizade sincera e por estar na luta comigo.

Aos meus amigos Luciene Lenhare (Lú), Carlos K. Katashima (Carlota), Gustavo Duarte Pimentel (Gustin), Thayana O. Micheletti (Thay), pelos dias de luta no laboratório, madrugadas, finais de semanas, feriados, congressos, fazendo experimentos para que tudo saísse nos prazos pré-dispostos. Com certeza nesses dois anos vocês foram minha segunda família (saudável ou não , passamos mais tempo juntos do que com a nossa família). Obrigado!

Aos amigos do laboratório de investigação clínica em resistência à insulina (LICRI), Todos que me ajudaram e me ensinaram a crescer como pesquisador e como ser

humano. Pelas diversas risadas, brincadeiras e contribuições intelectuais na construção de minha tese de mestrado.

Aos professores Mário Abdalla Saad ,José Barreto Carvalheira e Lício Augusto Veloso, por auxiliar nas ideias, na elaboração dos artigos científicos, e por ceder espaços em seus laboratórios para que nosso trabalho fosse feito com qualidade e seriedade.

Aos Professores Dennys Cintra, José Rodrigo Pauli e Patrícia Prada pela contribuição tanto experimental quanto intelectual na elaboração dos artigos científicos.

Á Sandra e Dioze, pela total atenção dada a mim e por estarem sempre dispostas a amparar no que for necessário.

Aos técnicos, Senhores Luís e Jósimo pelas risadas e brincadeiras e por estarem sempre dispostos a nos ajudar.

Aos técnicos do núcleo, Marino, seu Zé, seu António (pastor) pelos dois anos de dedicação, cuidando dos animais para que tudo desse certo. Com certeza sem vocês esse trabalho ficaria mais difícil para ser desenvolvido. Muito obrigado!

Ás técnicas do Cemib, Erica e Regina pelo ótimo trabalho realizado no biotério, pelas disponibilidades dos animais e total agilidade.

Á professora Cristiane Pavioti, pelas correções ortográficas de minha dissertação, pelas dicas e colaboração.

Ás agências de fomento: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes).

E a todos que de maneira indireta contribuíram para que esse sonho se tornasse realidade. Com toda sinceridade, obrigado!

Lista de Figuras

INTRODUÇÃO

O hipotálamo é reconhecido como a principal estrutura anatômica do sistema nervoso central (SNC), envolvida no controle da ingestão alimentar e do gasto energético. Os núcleos hipotalâmicos arqueado e paraventricular possuem como função integrar as informações periféricas por intermédio de hormônios e nutrientes para o controle da ingestão alimentar e do gasto energético (Spiegelman and Flier, 2001). Estudos realizados na década de 40 demonstraram que lesões no núcleo ventromedial do hipotálamo de roedores induziam à hiperfagia e à obesidade, enquanto estímulos no núcleo hipotalâmico lateral induziam à anorexia (Hervey, 1959). Esses achados foram determinantes na caracterização do hipotálamo como estrutura chave para o controle da homeostase energética em mamíferos.

A partir da identificação da descoberta do hormônio leptina em 1994 (Zhang, 1994), grandes avanços vem sendo obtidos na caracterização dos mecanismos neurais de controle da fome e do gasto energético mediado pela ação de hormônios no hipotálamo. Durante as duas últimas décadas, a localização dos receptores da leptina em núcleos hipotalâmicos de roedores, bem como a descrição da via de transmissão intracelular disparado por este hormônio em neurônios hipotalâmicos foram determinantes para o entendimento do controle da ingestão alimentar e do gasto energético (Schwartz et al., 2000; Spiegelman and Flier, 2001).

Transmissão do sinal da Leptina no Hipotálamo.

A leptina é expressa principalmente no tecido adiposo e em menores quantidades no epitélio gástrico e placenta (Bado et al., 1998; Maffei et al., 1995; Masuzaki et al., 1997). A proteína do gene *ob* que está presente no plasma de camundongos normais, como um monômero com peso molecular de 16 kda, não foi detectada em plasma de camundongos

ob/ob (camundongos com deficiência do gene da leptina), e foi observada em concentrações elevadas em camundongos *db/db* (camundongos com deficiência do gene do receptor da leptina) (Halaas et al., 1995). A administração de leptina a camundongos *ob/ob* resulta em diminuição da ingestão alimentar, perda de peso e redução dos níveis glicêmicos(Campfield et al., 1995), além de aumentar a atividade simpática em tecido adiposo marrom, com consequente aumento do gasto energético(Pelleymounter et al., 1995). Entretanto, o mesmo resultado não foi observado quando este hormônio foi injetado nos animais *db/db*.

Os níveis séricos de leptina correlacionam-se de forma positiva com o índice de massa corporal na grande maioria das populações estudadas (Considine et al., 1996; Frederich et al., 1995; Havel, 1998; Maffei et al., 1995). A secreção desse hormônio diminui com o jejum prolongado e estímulo β-adrenérgico (Ahima et al., 1996). e aumenta em resposta à administração de insulina e glicocorticoides (De Vos et al., 1995). A leptina é secretada de forma pulsátil e inversamente relacionada à atividade do eixo ACTH-Cortisol, ou seja, ocorre diminuição da secreção de leptina ao amanhecer e aumento no final da tarde (Licinio et al., 1997).

O receptor de leptina (OBR) é membro da família gp130 da classe I dos receptores de citoquinas (Tartaglia, 1997), é encontrado em muitos tecidos com várias formas de *splicing*, sendo duas as mais encontradas: a forma curta (OBRs), expressa em vários tecidos, que apresenta domínios intracelulares truncados, e a forma longa (OBRl), que apresenta domínios intracelulares longos e é expressa principalmente no hipotálamo (núcleos paraventricular, arqueado, ventromedial e dorsomedial) (Mercer et al., 1996; Woods et al., 1996). O OBRs não tem sua função bem definida, mas parece influir no transporte da leptina através da barreira hematoencefálica e talvez contribua para a depuração da leptina atuando como uma fonte de receptor solúvel.

A homologia do receptor de leptina a classe I dos receptores de citoquinas forneceu informações importantes para a descoberta dos possíveis mediadores intracelulares da ação da leptina. Os receptores da classe I das citoquinas agem através das famílias das proteínas Jak (*Janus Kinase*) e STAT (*Signal Transducers Activators of Transcription*) (Heldin,

1995). Tipicamente, as proteínas Jak estão constitutivamente associadas com sequências de aminoácidos dos receptores, e adquirem sua atividade tirosina quinase após a ligação do hormônio a seu receptor. Uma vez ativada, a proteína Jak fosforila o receptor induzindo a formação de um sítio de ligação para as proteínas STAT, as quais são ativadas após terem se associado ao receptor e serem fosforiladas pela Jak. As proteínas STAT ativadas são translocadas para o núcleo e estimulam a transcrição (Schwartz et al., 2000). No entanto, a homologia do receptor de leptina à classe I dos receptores de citoquinas, permite que várias outras citoquinas amplifiquem a transmissão do sinal da leptina. Assim as proteínas subsequentes ao receptor de leptina (Jak e STAT) podem exercer uma interface no controle da ingestão alimentar, regulando fatores de saciedade e adiposidade a longo prazo (através da própria leptina) ou desenvolvendo sinais anorexigênicos patológicos (através de citoquinas)(Plata-Salaman, 1996). O receptor de leptina é capaz de estimular outras vias de sinalização além da Jak/STAT, tais como a via da proteína quinase ativadora de mitose (MAPK) e a via de fosfatidilinositol 3-quinase (PI 3-quinase), e é possível que a capacidade do OBR controlar o peso dependa também destas vias de sinalização (Schwartz et al., 2000).

Após a ativação dos receptores de leptina no cérebro e das proteínas envolvidas na transmissão do sinal desse hormônio, respostas neuronais integradas são necessárias para modular a ingestão alimentar e o gasto energético. Alguns neurotransmissores importantes para o funcionamento dessa rede neuronal estimulam a ingestão alimentar como o neuropeptídeo Y (NPY)(Stephens et al., 1995) e o *Agouti related peptide* (AGRP)(Shutter et al., 1997), enquanto outros provocam a redução da ingestão alimentar como o *cocaine-and anphetamine–regulated transcription* (CART)(Kristensen et al., 1998), *proopiomelanocortin* (POMC) (Schwartz et al., 2000; Spiegelman and Flier, 2001) e o *melanocyte stimulating hormone* (α-MSH)(Fan et al., 1997). A leptina regula o balanço energético diminuindo os níveis de neuropeptídios anabólicos NPY e AGRP e aumentando a concentração de neuropeptídios catabólicos CART, POMC e α-MSH.

Figura 1

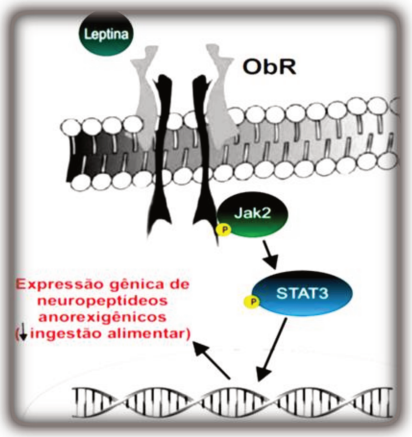


Figura 1- Via de transmissão do sinal da leptina em neurônios hipotalâmicos.

Durante as duas últimas décadas, as vias anorexigênicas controladas pela leptina no hipotálamo, vêm sendo intensamente investigadas no desenvolvimento de diversas doenças associadas à distúrbios alimentares, como obesidade e anorexia (Bence et al., 2006; Carvalheira et al., 2003; El-Haschimi et al., 2000; Elmquist and Flier, 2004; Grossberg et al., 2010a; Lee et al., 2010; Schwartz et al., 2000; Zhang et al., 2008). A seguir, serão descritos os achados mais relevantes que implicam a sinalização da leptina na fisiopatologia da obesidade.

Resistência à leptina no hipotálamo, hiperfagia e obesidade.

Estima-se que existam 300 milhões de obesos no mundo. Nos Estados Unidos, aproximadamente 32% da população é classificada como obesa e 34% com sobrepeso (Baskin et al., 2005; Ogden et al., 2006). Na região sudeste do Brasil, este índice é de 6,7%. Considerando que a obesidade é a principal responsável pelo estabelecimento da síndrome metabólica e está associada a doenças cardiovasculares, diabetes tipo 2, câncer, hipertensão, dislipidemias, esteatose hepática não alcoólica, entre outros (Bacha et al.,

2003; Carroll and Dudfield, 2004; Carvalheira and Saad, 2006). O aumento excessivo do peso corporal decorrente do acúmulo de tecido adiposo constitui uma das mais importantes questões de saúde pública e o desenvolvimento de diferentes abordagens para reduzir essa doença é um ponto de grande relevância atual(Abrantes et al., 2002).

Inicialmente, a descoberta da leptina passou a ser uma nova esperança para o tratamento da obesidade, entretanto, muito do entusiasmo com a leptina se desfez com a constatação de que indivíduos obesos respondem mal ao tratamento com leptina e que a administração desse hormônio em modelos experimentais de obesidade, demonstrou a existência de resistência central a esse hormônio(Carvalheira et al., 2003; El-Haschimi et al., 2000; Picardi et al., 2008; Zhang et al., 2008). A frequente associação clínica entre diabetes mellitus tipo 2 e obesidade, aliada ao fato de que pacientes obesos são em geral hiperleptinêmicos e hiperinsulinêmicos, fomentou a hipótese de que o controle inadequado da fome e da termogênese, que predispõem ao desenvolvimento de obesidade, deve-se a uma resistência hipotalâmica à ação da leptina. Tal suspeita foi confirmada por meio de estudos realizados em diferentes modelos animais com obesidade (El-Haschimi et al., 2000; Picardi et al., 2008; Zhang et al., 2008). Embora a leptina não seja a terapia anti-obesidade ideal, como esperado inicialmente, o desenvolvimento de estratégias para reduzir à resistência central a leptina pode ser um novo caminho para o tratamento da obesidade.

Na última década estudos passaram a identificar alguns dos possíveis mecanismos que induzem a resistência à leptina nos centros hipotalâmicos controladores do apetite que podem estar envolvidos com a hiperfagia e obesidade(Schwartz et al., 2000; Spiegelman and Flier, 2001). Diferentes grupos evidenciaram reduzida capacidade dos sinais da leptina em tecido hipotalâmico em diferentes modelos experimentais de obesidade(Bjorbaek et al., 1998; El-Haschimi et al., 2000; Picardi et al., 2008). A resistência à ação da leptina no sistema nervoso central bloqueia a ativação das vias anorexigênicas mediada por este hormônio e contribui diretamente para o desenvolvimento da obesidade (Schwartz et al., 2000; Spiegelman and Flier, 2001) . A hipótese de que possivelmente um processo inflamatório de baixa magnitude esteja envolvido com o descontrole dos sinais de saciedade, vem ganhando destaque. Ratos alimentados com dieta rica em gordura saturada apresentam discreto aumento da expressão de citoquinas inflamatórias como o Fator de

Necrose Tumoral alfa (TNFα) e Interleucina-1 beta (IL-1β) no hipotálamo (De Souza et al., 2005; Zhang et al., 2008). Neste cenário, algumas proteínas relacionadas à inflamação foram descritas como moduladores negativos da sinalização da leptina no hipotálamo, dentre elas destacam-se: a SOCS3 (*Supressor of Citokine Signaling 3*) (Bjorbaek et al., 1998), e a proteína tirosina fosfatase 1B, PTP1B (Bence et al., 2006; Picardi et al., 2008) e o IKK (Zhang et al., 2008).

Recentemente, Zhang e colaboradores demonstraram que animais obesos induzidos por dieta rica em gordura apresentam um aumento da atividade da via IKK/NF-kB e de proteínas envolvidas no estresse de retículo endoplasmático no tecido hipotalâmico, contribuindo diretamente com a redução da atividade da via PI-3K e da via Jak/STAT. Além disso, camundongos que expressam a proteína IKK constitutivamente ativada especificamente no hipotálamo, apresentam maior ingestão alimentar e maior ganho de peso quando comparado aos respectivos controles (Zhang et al., 2008). Por outro lado, camundongos que apresentam mutações que impedem a ativação da via IKK/NF-kB ficam protegidos do desenvolvimento de inflamação no sistema nervoso central, e de desenvolver resistência à insulina e à leptina, mesmo quando submetidos à dieta hiperlipídica (Milanski et al., 2009).

Efeitos do exercício físico sobre a resistência hipotalâmica à leptina.

A prática regular de exercício físico representa uma das melhores alternativas não invasivas para a prevenção e tratamento da obesidade e doenças associadas. Os efeitos decorrentes da prática de exercícios resultam em aumento do gasto energético, colaborando para a redução da adiposidade e, consequentemente, para a redução do peso corporal. Além destes efeitos, evidências acumuladas nos últimos anos apontam que a atividade física tem participação direta na sensibilidade à ação da insulina e da leptina em hipotálamo de ratos (Flores et al., 2006). Em modelo de obesidade genética BI e colaboradores evidenciaram que o exercício físico aumentou a sinalização da leptina, após administração exógena do

hormônio, prevenindo a hiperfagia (Bi et al., 2005). Adicionalmente, postula-se que a prática de atividade física seja capaz de reduzir os níveis teciduais e séricos de marcadores inflamatórios em modelos experimentais e também em humanos (Pedersen et al., 2001a, b). Nos últimos anos nosso laboratório se dedicou à avaliação dos efeitos do exercício físico sobre a sensibilidade à insulina e leptina no hipotálamo (Flores et al., 2006; Ropelle et al., 2010). Recentemente demonstramos que roedores obesos submetidos a uma única sessão de exercício em esteira ou natação, apresentaram redução significativa da ativação da via IKK/NF-kB em neurônios. Essa resposta anti-inflamatória deveu-se ao aumento da Interleucina-6 (IL-6) e da Interleucina-10 (IL-10) em alguns núcleos hipotalâmicos em resposta ao exercício, principalmente no núcleo arqueado. A atenuação da inflamação proporcionou melhora da sensibilidade à insulina e à leptina em hipotálamo dos animais obesos, contribuindo para redução da ingestão alimentar e do peso corporal (Ropelle et al., 2010). Esses resultados demonstram que o exercício físico, através da IL-6, pode ser uma forma eficaz para reduzir o processo inflamatório em células neuronais e recuperar as ações anorexigênicas, e, devido a isso, mudam completamente o entendimento do exercício como estratégia de combate à obesidade, conforme descrito em diferentes destaques editoriais (Martinez de Morentin et al., 2010) (Welberg, 2010).

Ativação persistente da via de sinalização Jak/STAT no hipotálamo: implicações fisiopatológicas para o desenvolvimento da anorexia do câncer.

A caquexia é um estado involuntário de perda de peso encontrado em doenças neoplásicas, infecciosas e inflamatórias que contribui marcadamente para a mortalidade dessas moléstias. A anorexia é um dos principais indutores de caquexia, embora o padrão de perda de peso observado na caquexia difira daquele observado na restrição alimentar (Tisdale, 1997).

A palavra caquexia deriva do grego “kakos” que significa “mal” e “hexis” que quer dizer “condição” (Tisdale, 1997). Aproximadamente metade dos pacientes com câncer

desenvolvem caquexia, caracterizada por anorexia e diminuição do tecido adiposo e massa muscular. Em geral, pacientes com tumores sólidos têm uma maior frequência de caquexia (Bruera, 1997). No momento do diagnóstico, aproximadamente 80% dos pacientes com câncer do trato gastrointestinal e 60% dos pacientes com câncer de pulmão têm perda de peso significativa. A caquexia é mais comum em crianças e idosos e se torna mais pronunciada com o evoluir da neoplasia.

A síndrome anorexia-caquexia é desencadeada por uma inter-relação complexa de variáveis metabólicas e comportamentais que se correlacionam com prognósticos ruins e comprometimento da qualidade de vida (Bruera, 1997; Larkin, 1998; Tisdale, 1997). Apesar da etiologia da caquexia não ser bem definida, várias hipóteses têm sido exploradas, incluindo a participação de citoquinas, hormônios circulantes, neuropeptídeos, neurotransmissores e fatores derivados dos tumores (Bruera, 1997; Plata-Salaman, 1996; Tisdale, 1997). Entretanto, a hipótese de que a síndrome anorexia-caquexia seja causada pela ação das citoquinas potencializando os efeitos anoréticos mediados pela leptina no sistema nervoso central (SNC) tem ganhado destaque nos últimos anos.

Postula-se que numerosas citoquinas (TNF-α, IL-1, IFN-γ) participem da síndrome anorexia-caquexia observada em pacientes com câncer. As citoquinas podem ser liberadas na circulação e transportadas para o cérebro através da barreira hematoencefálica e órgãos circunventriculares (áreas que permitem a passagem mais fácil de moléculas na barreira hematoencefálica). Por outro lado, as citoquinas também são produzidas por neurônios e células da glia do SNC, em resposta ao aumento das citoquinas periféricas (Haslett, 1998; Hopkins and Rothwell, 1995; Licinio and Wong, 1997; Mantovani et al., 1998; Rothwell and Hopkins, 1995; Sternberg, 1997). A administração crônica dessas citoquinas pró-inflamatórias, tanto isoladamente como em conjunto são capazes de reduzir a ingestão alimentar e reproduzir as diferentes características da síndrome anorexia-caquexia(Gelin et al., 1991; Mantovani et al., 1998; Matthys and Billiau, 1997; Moldawer et al., 1992; Tisdale, 1997). Recentemente, descrevemos que estratégias anti-inflamatórias dirigidas exclusivamente ao hipotálamo de roedores com anorexia induzida por câncer, são determinantes para o aumento da ingestão alimentar e da sobrevida (Ropelle et al., 2007). Outra citoquina frequentemente associada ao desenvolvimento da anorexia é o LIF

(*leukemia inhibitory factor*). Modelos experimentais de anorexia induzida por tumor identificaram elevados níveis circulantes de LIF (Metcalf and Gearing, 1989; Mori et al., 1991), sendo que a administração de LIF em hipotálamo de roedores normais, induz anorexia através da ativação da via de transmissão do sinal da leptina, com fosforilação robusta da proteína STAT3 em neurônios POMC (Grossberg et al., 2010b).

Outros fatores produzidos por tumores foram identificados como ativadores da sinalização da via Jak2/STAT3 no hipotálamo, sendo responsáveis pela indução de anorexia. Johnen e colegas demonstraram que a produção de um membro da família do TGF-β, o MIC-1 (*Macrophage inhibitory cytokine-1*) a partir de células de tumor de próstata, interagem com células neuronais no núcleo arqueado hipotalâmico, aumentando significativamente a fosforilação em tirosina da STAT3, induzindo anorexia em roedores (Johnen et al., 2007). Coletivamente, esses dados demonstram que a via de transmissão do sinal da leptina no hipotálamo é um atraente alvo terapêutico para o tratamento da anorexia do câncer.

A esfingosina 1 fosfato (S1P) e sua inter-relação com a via Jak/STAT

A S1P (*sphingosine-1-phosphate*) é uma molécula bioativa com uma ampla variedade de funções celulares em diversos organismos. Embora estruturalmente seja considerada uma molécula simples, as funções mediadas pela S1P são complexas. A formação de S1P pode ser determinada pela ativação de receptores de membrana acoplados à proteína G, receptores de citoquinas e receptores com atividade tirosina-quinase(Rivera et al., 2008). Uma vez ativado, esses receptores ativam proteínas chamadas esfingomielinases (SMase), que clivam as esfingomielinas, aumentando a produção de ceramidas. As ceramidas por sua vez também são clivadas pelas ceramidases (CERase) formando então a esfingosina. Por fim, a esfingosina é fosforilada pela esfingosina quinase (SPHK), formando assim a esfingosina-1-fosfato (S1P)(Hannun and Obeid, 2008; Rivera et al., 2008; Zhao et al., 2007). Em mamíferos a esfingosina quinase possui duas isoformas (SphK1 e SphK2); o nocaute de cada uma das isoformas causa redução dos níveis plasmáticos de S1P, por outro

lado o duplo nocaute não completa o desenvolvimento embrionário, portanto não é viável.(Rex et al., 2013). A ausência da esfingosina quinase SphK2 em modelos experimentais provoca redução de plaquetas no sangue e consequentemente redução intracelular de S1P (Maceyka et al., 2012; Zhang et al., 2013).

Os níveis circulantes de S1P parecem sofrer variações em condições fisiológicas ou fisiopatológicas distintas como obesidade, exercício físico e durante o desenvolvimento de alguns tipos de neoplasias. Estudos recentes observaram que em modelos experimentais de obesidade e câncer, os níveis circulantes de S1P apresentam aumento significativo(Liang et al., 2013). Independentemente da presença de doenças, os níveis plasmáticos de S1P também podem apresentar variações. Baranowski e colaboradores reportaram que agudamente, o exercício físico aumenta significativamente os níveis de S1P na corrente sanguínea em humanos saudáveis. O mesmo resultado também foi observado após múltiplas sessões de exercício (Baranowski et al., 2011). Coletivamente, esses resultados sugerem que a síntese de S1P é modulada em diferentes circunstâncias e que esse esfingolipídio poderia mediar suas funções biológicas de maneira parácrina. No entanto, os tipos celulares que promovem a síntese de S1P em cada uma dessas situações previamente mencionadas (obesidade, exercício e câncer) ainda não são conhecidos. Por outro lado, a identificação dos receptores de S1P em diferentes tecidos, está ajudando a elucidar os efeitos endógenos do S1P como veremos a seguir.

Uma vez sintetizada, o S1P pode exercer seus efeitos de duas maneiras. O S1P pode atuar através da sua ligação à uma família de receptores acoplados a proteína G (S1PRs), diferencialmente expressos em diferentes tipos celulares. Além de agir sobre os receptores localizados na membrana plasmática, S1P também pode funcionar no interior da célula, independentemente dos S1PRs (Rivera et al., 2008). Dentre os receptores responsivos ao S1P, o S1PR1 (*sphingosine-1-phosphate receptor-1*), também chamado de EDG1, do inglês *endothelial differentiation gene 1*, vêm ganhando destaque por sua participação em funções celulares relacionadas à inflamação (Camerer et al., 2009), migração celular (Konig et al., 2010) e principalmente sobre a carcinogênese (Lee et al., 2010; Schulte et al., 2001; Yamaguchi et al., 2003; Yoshida et al., 2010). O receptor S1PR1 foi clonado e sequenciado em 1990, a partir de células endoteliais humanas (Hla and Maciag, 1990). A proteína S1PR1 é composta de 380 aminoácidos e é estruturalmente similar aos receptores

acoplados a proteína G e possui sete domínios transmembrana (Rivera et al., 2008). A expressão de S1PR1 foi observada em vários órgãos de roedores incluindo cérebro, pulmão, coração, baço, placenta, músculo, fígado, útero e rins (Liu and Hla, 1997).

A relação da S1PR1 com a via Jak/STAT foi recentemente descrita em um interessante estudo conduzido por Lee e colaboradores (Lee et al., 2010). O aumento da fosforilação e da atividade nuclear da STAT3 está associado a ação transitória de fatores como a leptina e IL-6, neste estudo, os autores demonstraram que elevados níveis proteicos de S1PR1 encontrados em células tumorais foram associados à persistente ativação da proteína STAT3 (Lee et al., 2010), de tal forma que o aumento da fosforilação da STAT3 resultou em aumento da associação desta proteína com a região promotora que codifica o gene *S1PR1* em diferentes linhagens celulares. Adicionalmente, os autores demonstraram que o aumento da fosforilação da STAT3 ocorreu, pelo menos em parte, através do aumento da fosforilação da Jak2. Esses dados foram obtidos através de experimentos de imunoprecipitação, demonstrando a interação física existente entre a Jak2 e o receptor S1PR1 (Lee et al., 2010). O aumento da expressão da S1PR1 mediado pela STAT3 gera um mecanismo de retroalimentação positivo, gerando persistente ativação da STAT3, que pode ainda ser fosforilada pela leptina, IL-6 e S1P.

Liang e colaboradores demonstraram que o aumento da produção da esfingosina 1 fosfato (S1P), produzido pela esfingosina quinase 1(SphK1) promoveu aumento de inflamação crônica intestinal associado ao câncer de cólon. O aumento da produção de S1P promoveu aumento na produção do NF-kB e de IL-6 e ativação persistente da proteína STAT3 e do receptor S1PR1 de maneira recíproca. Por outro lado, o tratamento com inibidor farmacológico do receptor S1PR1, o FTY720, foi capaz de reduzir a expressão da quinase SphK1 e do receptor S1PR1, diminuindo a ativação da cascata NF-kB/IL-6/STAT3, proporcionando menor crescimento e proliferação do câncer de colón e reduzindo a atividade anorexigênica em roedores (Liang et al., 2013).

Figura 2.

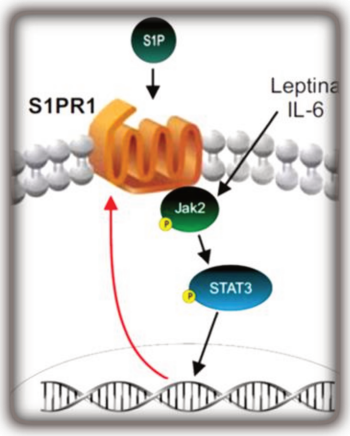


Figura 2 – O receptor S1PR1 aumenta a atividade da via Jak2/STAT3 através da interação com a proteína Jak2. A persistente fosforilação da STAT3 induz aumento da transcrição gênica do receptor S1PR1.

Em diversos tipos celulares, o aumento da via Jak2/STAT3 induz aumento da expressão de proteínas que funcionam como moduladores negativos desta via de sinalização, como por exemplo, a SOCS3 (*Supressor of Citokine Signaling 3*). Geralmente, o estímulo com leptina ou com IL-6 é acompanhado pelo aumento da expressão protéica de SOCS3 (Bjorbaek et al., 1998; Munzberg et al., 2005; Yang et al., 2005), no entanto, a estimulação da via Jak2/STAT3 induzida pelo S1PR1, não promove aumento da expressão de SOCS3, como demonstrado em células tumorais, favorecendo assim a ativação persistente da STAT3 (Lee et al., 2010) .

A participação do receptor S1PR1 no sistema nervoso central ainda é pouco conhecida. S1PR1 foi identificada em diferentes regiões do cérebro de *Zebrafish* durante a fase embrionária, sugerindo a importância do eixo S1P/S1PR1 durante a embriogênese em animais vertebrados (Im et al., 2000). Recentemente, o S1PR1 foi localizado em neurônios sensoriais de ratos, ao passo que a estimulação destes neurônios com S1P aumentou significativamente a excitabilidade destas células através do receptor S1PR1 (Chi and Nicol, 2010). Nishimura e colaboradores demonstraram elevados níveis proteicos de S1PR1 em algumas regiões do cérebro em humanos, no entanto suas funções permanecem

desconhecidas (Nishimura et al., 2010). Novos estudos são necessários para o delineamento das funções do eixo S1P/S1PR1 no sistema nervoso central.

JUSTIFICATIVA

A ingestão alimentar e o gasto energético são minuciosamente regulados por neurônios específicos localizados no hipotálamo. Durante as duas últimas décadas, a localização dos receptores da leptina em núcleos hipotalâmicos, bem como a descrição da via de transmissão intracelular disparado por este hormônio em neurônios hipotalâmicos, foram determinantes para o entendimento do controle da ingestão alimentar e do gasto energético. Cada vez mais os distúrbios alimentares associados a doenças como obesidade e câncer vêm sendo diretamente relacionados com a sinalização aberrante da via Jak2/STAT3 no hipotálamo. O processo inflamatório subclínico frequentemente observado em modelos experimentais de obesidade estão diretamente associados a distintos mecanismos de resistência à leptina no hipotálamo e resultam em aumento da ingestão alimentar e ganho de peso corporal. Por outro lado, a inflamação de grande magnitude, como observada em pacientes com câncer, é capaz de produzir potentes sinais anorexigênicos através da via Jak/STA3 no hipotálamo. No entanto, esses mecanismos moleculares que induzem hiperfagia ou anorexia são apenas parcialmente conhecidos. Desta forma, se faz necessário um entendimento mais amplo de como são desencadeados os sinais intracelulares envolvidos no controle da ingestão alimentar. Recentemente, a proteína S1PR1 foi descrita como uma molécula com alta capacidade de exercer potentes efeitos sinérgicos sobre a via de sinalização da leptina, sustentando a ativação da via Jak2/STAT3 em células tumorais, contudo, não existem informações à respeito de sua função biológica no tecido hipotalâmico relacionado ao controle da ingestão alimentar e do peso corporal. Esses achados poderão contribuir de maneira significativa para o entendimento de distúrbios associados à ingestão alimentar como na obesidade e anorexia induzida pelo câncer, abrindo novas perspectivas para o tratamento destas doenças.

OBJETIVOS

Objetivo geral

O objetivo principal do estudo foi caracterizar a participação da proteína S1PR1 hipotalâmica no controle de sinais anorexigênicos, ingestão alimentar e do peso corporal em roedores.

Objetivos específicos:

Parte 1- Caracterização do eixo S1P/S1PR1 hipotalâmico no controle da homeostase energética

1. Localizar a expressão da S1PR1 nos diferentes núcleos hipotalâmicos de ratos e a sua colocalização com as proteínas STAT3.

2. Avaliar o efeito da infusão intracerebroventricular de S1P sobre a ativação da via Jak/STAT e sobre a ingestão alimentar e o gasto energético em ratos.

3. Examinar o eixo S1P/S1PR1 em hipotálamo em modelo experimental de obesidade e anorexia induzida por tumor.

Parte 2- Determinar o efeito do exercício físico sobre o eixo S1P/S1PR1 em hipotálamo de ratos obesos

1. Investigar os efeitos do exercício físico agudo sobre os níveis séricos de S1P em ratos obesos induzidos por dieta hiperlipídica.

2. Avaliar o efeito do exercício físico agudo sobre a expressão da proteína S1PR1 e sobre a via de sinalização Jak2/STAT3 no hipotálamo de ratos obesos induzidos por dieta hiperlipídica.

ARTIGO 1

Hypothalamic S1P/S1PR1 axis controls energy homeostasis.

Vagner R. R. Silva[1], Thayana O. Micheletti[2], Gustavo D. Pimentel[2], Carlos K. Katashima[2], Luciene Lenhare[2], Joseane Morari[3], Maria Carolina S. Mendes[2], Daniela S. Razolli[3], Guilherme Z. Rocha[2], Claudio T. de Souza[4], Patrícia O. Prada[2], Lício A. Velloso[3], José B. C. Carvalheira[2], José Rodrigo Pauli[1], Dennys E. Cintra[1-3], Eduardo R. Ropelle[1-2].

1. Laboratory of Molecular Biology of Exercise (LaBMEx). School of Applied Science, University of Campinas (UNICAMP), Limeira, São Paulo, Brazil.
2. Department of Internal Medicine, Faculty of Medical Sciences, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil.
3. Laboratory of Cell Signalling. Faculty of Medical Sciences, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil.
4. Laboratory of Exercise Biochemistry and Physiology, Health Sciences Unit, University of Southern Santa Catarina, Criciúma, SC, Brazil.

Running head: Neuronal S1P/S1PR1 axis and energy homeostasis.

Key words: hypothalamus, obesity, anorexia, S1P, S1PR1.

Please address correspondence to:

Eduardo Rochete Ropelle, PhD.
School of Applied Sciences.
University of Campinas (UNICAMP),
Limeira, São Paulo, Brazil.
Phone: + 55 - 19 37016706
Email: eduardoropelle@gmail.com

ABSTRACT

Sphingosine 1-phosphate receptor 1, S1PR1 (also called EDG1), a G-protein-coupled receptor for sphingosine-1-phosphate (S1P), plays important roles in diverse cellular functions including cell migration, proliferation and differentiation in a variety of cell types. Here we report that neuronal S1P/S1PR1 axis coordinates the energy homeostasis in rodents. We detect that the S1PR1 protein is highly enriched in the mediobasal hypothalamus of rats and it is localized in neurons that possess proopiomelanocortin (POMC). Intracerebroventricular (ICV) injection of the endogenous circulating bioactive lipid sphingosine-1-phosphate (S1P), reduces the food consumption and increases the energy expenditure in rats through the persistent Signal Transducers and Activators of Transcription 3 (STAT3) activation. Notably, STAT3 activation plays a reciprocal role on hypothalamic S1PR1 expression, whereas leptin signaling deficient mice exhibit a strong reduction on hypothalamic S1PR1 protein levels. We identify that high-fat diet induces leptin resistance and down-regulation of hypothalamic S1PR1. However, central S1P administration recapitulated the anorexigenic signals and reduced the body weight in obese rats. In contrast, we found high levels of circulating S1P and aberrant S1PR1/STAT3 signaling activation in the hypothalamus during cancer-induced anorexia. Finally, the disruption of S1P/S1PR1 signaling, specifically in the hypothalamus, attenuated the cachexia/ anorexia syndrome and increased survival in tumor-bearing animals. Taken together, our data demonstrated that neuronal S1P/S1PR1/STAT3 axis plays a critical role in the control of energy homeostasis.

INTRODUCTION

The hypothalamus plays an important role in the regulation of energy homeostasis controlling feeding behavior and energy metabolism in mammals [1]. Body weight and appetite control are complex and incompletely characterized. It has been postulated that central mechanisms disturbance can lead to hyperfagia or anorexia. In this context, Jak/STAT signaling plays a fine tune in the control of anorexigenic and thermogenic signal upon hormonal [2-3] or inflammatory stimulus [4-6]. The disturbance in the hypothalamic Jak2/STAT3 signaling results in anomalous neurotransmitters production, generating abnormal anorexigenic and thermogenic response and favoring body mass gain or severe weight loss [5,7-10]. Thus, alternative strategies to control Jak/STAT cascade activation in the hypothalamus could be considered as potential therapeutic targets to maintain the energy homeostasis during abnormal feeding behavior, such as hyperphagia and anorexia.

Recently, a class of Sphingosine 1-phosphate receptors (S1PRs) has been implicated in the control of Jak2/STAT3 signaling [11-14]. S1PRs family is composed by five (1-5) specific G-protein-coupled receptors that activate diverse downstream signaling pathways in response to the common sphingosine-1-phosphate (S1P) [15]. The lysophospholipid, sphingosine-1-phosphate (S1P), is a circulating bioactive lipid metabolite formed by phosphorylation of sphingosine, in a reaction catalysed by sphingosine kinase, SPHK1 and 2 [16]. S1P/S1PRs axis plays a important role in the control of cardioprotection [11], intestinal inflammation [12], satellite cell activation [17] and tumor cells progression [13] through the persistent STAT3 activation. However the role of hypothalamic S1P/S1PR1/STAT3 axis in the control of the energy homeostasis was not reported.

Here we combined the physiological, pharmacological and genetic approaches to investigate the participation of hypothalamic S1P/S1PR1 axis in the control of anorexigenic signals, food consumption and energy expenditure in rodents. In addition, we examined the role of neuronal S1P/S1PR1/STAT3 signaling in distinct conditions of abnormal feeding behavior, including obesity and cancer-induced anorexia.

RESULTS

Characterization of hypothalamic S1PR1.

S1PR1 is involved in several intracellular pathways signaling activation in a variety of cell types. We sought to determine the role of S1PR1 in hypothalamic neurons in the control of the energy homeostasis. First, we evaluate the distribution of S1PR1 in different tissues of normal rats and observed that the S1PR1 protein was highly enriched in the hypothalamus, when compared with peripheral tissues, such as, heart, liver, skeletal muscle and adipose (Figure 1A). Thereafter, we examine the S1PR1 distribution pattern in the hypothalamus using the micro dissection of hypothalamic nuclei. This technique revealed that S1PR1 is expressed predominantly in the arcuate and ventromedial/dorsomedial nuclei in the hypothalamus and low protein levels were found in paraventricular nucleus and in lateral hypothalamus (Figure 1B). These data was confirmed by immunostaining assay (Figure 1C, left panel). We also employed the double-staining and observed that S1PR1 is stained predominantly in anorexigenic (POMC), but not in orexigenic (NPY) neurons in the arcuate nucleus (Figure 1C). We noted that S1PR1 is stained in the neurons but is barely stained in non-neuronal cells (such as astrocytes) in the arcuate nucleus (Supplemmentary figure S1).

Once S1PR1 is localized in hypothalamic nuclei and in neurons specialized in the control of energy homeostasis, we sought to determine whether the physiological nutritional status modulates the hypothalamic S1PR1 expression. We observed that 12-h of fasting was sufficient to reduce S1PR1 protein level in the hypothalamus of control rats by about 60%, whereas the refeeding period (6 hours) restored the protein levels of this receptor (Figure 1D). In addition, hypothalamic STAT3 tyrosine phosphorylation accompanied the pattern of S1PR1 protein levels in the fasting/refeeding experiment (Figure 1D – middle panel). After these results, we hypothesized that neuronal S1PR1 could be involved in the control of the energy homeostasis through the positive cross-talk mechanism, involving Jak/STAT3 cascade in neurons, as observed in other cell types [11-14,17].

Next, we examined the relationship between the S1PR1 and Jak/STAT signaling in the hypothalamus. First, we detected that most neurons expressing S1PR1 in the arcuate nucleus were shown to possess STAT3 in control rats, suggesting a possible interaction between these molecules (Figure 1C – lower panels). To determine whether S1PR1 activates hypothalamic STAT3, we next carried out a bilateral cannulation to access the VMH, and then used the endogenous S1PR1 activator, sphingosine-1-phosphate, S1P (50 ng) into one side of the VMH hypothalamus and vehicle into the other side of the VMH in the same rat (Figure 1E), as previously described [18]. Interestingly, S1P, but not vehicle, induced tyrosine phosphorylation of STAT3 in the hypothalamus of control rats (Figure 1F). Collectively, these data indicates that the hypothalamic S1PR1 is localized in the mediobasal of the hypothalamus in neurons specialized in the control of energy homeostasis and that S1PR1 activation increases the STAT3 activity in the hypothalamus of rats.

S1P modulates the energy homeostasis through hypothalamic S1PR1 activation.

Thereafter, we evaluate the effects of S1PR1 activator, S1P, in the control of food intake and energy expenditure in control rats. First, we performed an acute injection of S1P in the third ventricle of rats in different doses (2 and 50 ng) and monitored the food consumption during 4 and 12 hours. We observed that S1P reduced the food intake in a dose-dependent manner (Figure 2A). Western blotting analysis confirmed that S1P increased Jak2 and STAT3 tyrosine phosphorylation in the hypothalamus in a dose-dependent manner (Figure 2B). In order to investigate whether S1PR1 activates directly the leptin signal transduction, we carried out immunoprecipitation assay to evaluate the S1PR1/Jak2 association. Our experiments revealed that acute ICV injection of S1P promoted the S1PR1/Jak2 association in the hypothalamic tissue in a dose-dependent manner (Figure 2C). However, acute microinjection of S1P did not change the Insulin Receptor Substrate 1 (IRS1) and Akt phosphorylation in the hypothalamus (Supplemmentary figure S2).

We also monitored the energy expenditure after acute S1P injection. We note that acute ICV S1P injection increased the energy expenditure in rats, increasing the O_2 consumption and CO_2 production, in both, light and dark periods (Figure 2D), without any change in the physical activity pattern (Figure 2E).

Although S1P is considered the main endogenous S1PR1 activator, it has been demonstrated that this molecule is able to activate others Sphingosine- 1 phosphate receptors [19]. Thus, we performed an acute microinjection of specific S1PR1 activator (SEW2871) into the third ventricle of rats. We observed that SEW2871 (50 ng) reproduced the effects of S1P, reducing the food intake (Figure 2F) and activating the leptin signaling (Figure 2G). Consistent with the role of the hypothalamic S1PR1 activation in the control of food consumption and on STAT3 activation, we observed that acute SEW2871 injection into the third ventricle of rats modulates the expression of neuropeptides involved in the food intake and energy expenditure control. SEW2871 increased POMC mRNA without changing NPY mRNA levels, when compared to vehicle injection (Figure 2H).

Next, we evaluate the impact of intraperitoneal S1P injection (100 ng) on food intake and on energy expenditure. Interestingly, acute intraperitoneal injection of S1P reduced the food consumption and promoted a discrete but significant increase in energy expenditure in C57BL6/J mice (Figure 2I and J). Taken together, these data suggests that hypothalamic S1P/S1PR1 axis activation induces anorexia and increases the energy expenditure in rodents.

Anorexigenic action of S1PR1 requires the Jak2/STAT3 signaling

It has been demonstrated that Jak2/STAT3 signaling activators, including IL-6, promotes a transient STAT3 activation while S1PR1 induces a persistent STAT3 phosphorylation [13]. Based in this information, we suspected that S1P could induce a potent anorexigenic effect through the persistent STAT3 activation. To address this question, we performed a time-course study to examine STAT3 phosphorylation upon acute leptin or S1P ICV injections. The Western blotting analysis revealed that ICV infusion of leptin promoted a transient effect on STAT3 phosphorylation. Leptin increased the tyrosine phosphorylation of STAT3 after 15 and 30 minutes, returning at the basal levels after 60

minutes (Figure 3 A and B). Notably, the ICV infusion of S1P promoted potent and persistent phosphorylation of this molecule, increasing STAT3 phosphorylation after 5 minutes and peaking at 120 minutes later (Figure 3 A and B). We also monitored the food consumption after acute leptin or S1P ICV injection in control rats and we observed that S1P promoted a potent and persistent anorexigenic effect when compared to leptin (Figure 3C). We also performed chronic (3 days) of ICV S1P (50 ng) injections and we observed a consistent anorexigenic effect during 3 consecutive days in control rats, on the other hand, after this injections, these animals rapidly returned to the baseline of food intake, in a similar fashion as observed in the pair-feeding group, suggesting that S1P ICV injection does not evoked a toxic effect (Figure 3D).

To confirm whether S1PR1 anorexigenic action depends on the Jak2 and STAT3 signaling, we used the pretreatment with the inhibitor of STAT3, JSI124, before the S1P injection. To test the efficacy of JSI124 in the hypothalamus, we performed an acute injection using 50 μM into the third ventricle of control animals. Western blot analysis revealed that JSI124 reduced STAT3 tyrosine phosphorylation, when compared to vehicle injection (Figure 3E). Interestingly, the JSI124 pretreatment, thirty minutes before S1P ICV injection, was sufficient to block, at least in part, the anorexigenic action of S1P injection (Figure 3F) and reduced the S1P-induced STAT3 phosphorylation in the hypothalamus (Figure 3G). Acutely, ICV JSI124 alone did not change the food consumption (data not shown). These data indicate that an anorexigenic effect of hypothalamic S1P/S1PR1 axis requires the STAT3 activation.

STAT3 controls the hypothalamic S1PR1 protein levels.

It has been demonstrated that STAT3 directly binds to the *S1pr1* promoter, increasing the S1PR1 mRNA levels in tumor cells [13]. To address whether STAT3 activation modulates S1PR1 protein levels in neurons, we employed the cell culture system using neuronal GT1-7 cells treated with leptin. We carried out a time-course study and observed that leptin (40 μmol/L) increased S1PR1 protein levels in GT1-7 cells in a time-dependent manner (Figure 4A). Next, we used STAT3 siRNA in GT1-7 cells (Figure 4B) to evaluate

the requirement of STAT3 on leptin-induced S1PR1 expression. We observed that leptin failed to increase S1PR1 protein levels in GT1-7 cells transfected with STAT3 siRNA (Figure 4C).

We then evaluated the effects of leptin in the control of S1PR1 protein levels *in vivo*. Similar to GT1-7 cells, acute ICV infusion of leptin increased S1PR1 protein levels in the hypothalamus of control rats, as demonstrated in the time-course study (Figure 4D). In the other hand, ICV JSI-124 pretreatment blocked the effect leptin on hypothalamic S1PR1 protein levels in rats (Figure 4E). In order to confirm the role of leptin in the control of hypothalamic S1PR1 expression *in vivo*, we examine the S1PR1 protein content in the hypothalamus of mice lacking leptin (*ob/ob*). Interestingly, *ob/ob* mice display dramatic reduction on S1PR1 expression (88%) in the hypothalamus, when compared to wild-type mice (Figure 4F). Similar results were found in mice with deficiency in the leptin signaling, *db/db* mice (Figure 4G). These data demonstrate that STAT3 activation plays a reciprocal role in the regulation of hypothalamic S1PR1 protein levels.

Disruption of hypothalamic S1PR1 protein levels in obese rodents.

Based in our results we hypothesized that S1PR1 expression could be affected in the hypothalamus under obesity condition, as a reflex of the leptin resistance. Thus, we then examined the effects of high-fat diet (HFD) on hypothalamic leptin resistance and S1PR1 protein levels. Initially, we observed that high-fat diet reduced the hypothalamic S1PR1 protein levels by about 35%, 50% and 75%, in wistar rats, C57BL6/J and Swiss mice, respectively (Figure 5A-C). Consistent with the S1PR1 protein levels, the real time PCR revealed low S1PR1 mRNA levels in the hypothalamus of wistar rats after HFD treatment (Figure 5D).

We next evaluate the effects of leptin to induce S1PR1 protein levels in the hypothalamus of lean and obese rats. Initially, we observed that ICV infusion of leptin promoted a strong STAT3 tyrosine phosphorylation in control but not in obese animals, as expected (Supplemmentary figure S3A). Thereafter we note that in contrast to lean animal, acute ICV injection of leptin failed to increase S1PR1 mRNA (Supplemmentary figure S4A) and protein levels (Supplemmentary figure S4B) in the hypothalamus of obese rats.

These data demonstrate that central leptin resistance is associated with the downregulation of S1PR1 in the hypothalamus of obese rats.

We then monitored the S1P serum levels in obese animals, using ELISA kit specific to detect S1P in the serum of mouse. We observed that obese mice (C57BL6/J) displayed an augment in the serum levels of S1P when compared to control group (Figure 5E). The high levels of S1P suggest that it could be a compensatory mechanism to subvert the downregulation of S1PR1 and defective STAT3 activity in the hypothalamus of obese mice. Thus, we investigated whether the forced activation of S1PR1 in the hypothalamus of obese animals through S1P ICV injection is capable of overcoming leptin resistance, activate STAT3 and recapitulate the anorexigenic signals in obese rats. We performed an acute injection of S1P (50 ng) into the third ventricle of obese rats to evaluate the energy intake and STAT3 phosphorylation. As observed in control animals, S1P was sufficient to reduce the food intake in obese rats (Figure 5F), similar results were found when we used a specific S1PR1 activator, SEW2871 (Figure 5F). Western blotting analysis demonstrated that acute S1P or SEW2871 ICV injections were able to increase STAT3 tyrosine phosphorylation in the hypothalamus of obese rats (Figure 5G). Furthermore, acute S1P injection increased POMC mRNA levels in the hypothalamus of obese rats (Figure 5H). However, we did not observe difference in the energy expenditure after acute S1P ICV injection in obese rats when compared to vehicle group (Figure 5I) and no difference was found in the physical activity pattern (Figure 5J). Apparently, the anorexigenic and thermogenic effects of S1P were more robust in lean than obese animals.

Thereafter to evaluate the impact of chronic S1P administration in obese rats, we performed a chronic micro infusion of S1P (0.25 μL/hr with 50 ng/day) in the hypothalamus using osmotic mini-pump. We observed that chronic S1P delivery in the hypothalamus promoted reduction on daily-food intake (Figure 5K), body weight (Figure 5L) and epididymal fat pad weight in obese rats (Figure 5M), when compared to vehicle infusion. These data demonstrated that chronic S1P administration into the third ventricle of animals reduced the food consumption and body weight in obese rats.

Cancer-induced anorexia is mediated by S1P/S1PR1 axis.

Several studies have demonstrated that S1P levels are involved in persistent Stat3 activation in cancer cells and in tumor microenvironment and for malignant progression [13-14]. In addition, high levels of circulating S1P levels were found in tumor-bearing animals [14,20]. In line with these evidences, we sought to investigate whether S1P contributes to anorexia in tumor-bearing rodents. We examined S1PR1 protein levels in the hypothalamus of rats injected with Walker-256 tumor cells and in mice injected with Lewis lung carcinoma (LLC) during tumor-induced anorexia. The LLC cells induced severe anorexia and weight loss in mice (Figure 6A-C). Interestingly, four days after onset of anorexia, high levels of S1PR1 protein levels and hyper-phosphorylation of STAT3 were found in hypothalamic tissue of anorectic mice (Figure 6D). These data were accompanied by high S1P serum levels in mice during cancer-induced anorexia (Figure 6E). Furthermore, we detected the presence of high levels of S1P in cerebrospinal fluid (CSF) in tumor-bearing mice (Figure 6F), suggesting that S1P could be involved in the aberrant anorexigenic signals in these animals. We also investigate S1PR1 protein level and STAT3 in different models of cancer-induced anorexia. For instance, Walker-256 tumor cells also induced anorexia and weight loss in rats (Figure 6G and H) and high protein levels of S1PR1 and hyper-phosphorylation of STAT3 were found in the hypothalamus of anorectic rats with Walker-256 tumor (Figure 6I). Similar results were found in the hypothalamus of mice with colon cancer induced by azoxymethane (AOM) and dextran sodium sulfate (DSS) treatment (data not shown).

To determine whether the presence of S1P in CSF of tumor-bearing animals induces anorexia, we collected CSF from control and anorectic wistar rats and injected the CSF (2 μL) into the third ventricle of control rats. We observed that acute ICV injection of CSF from control animals did not change the food consumption (Figure 6J), conversely, ICV injection of CSF from anorectic animals promoted a strong anorexigenic effect in control animals (Figure 6J). Interestingly, the anorexigenic effect of CSF from anorectic rats was blunted in animals that received ICV S1PR1 inhibitor (FTY720) pretreatment 60 minutes before the CSF injection.

In order to confirm the role of hypothalamic S1PR1 on cancer-induced anorexia, we performed ICV injection of FTY720, a functional antagonist of S1PR1, in wistar rats during cancer-induced anorexia. FTY720 is an immunosuppressant compound known to modulate the immune system by acting as a functional antagonist of S1PR1 and inducing its internalization and degradation [21-22]. After the onset of anorexia, we injected FTY720 (50 μM) daily. Our results revealed that FTY720 increased the daily food consumption (Figure 6K) and cumulative energy intake 4 days after the onset of anorexia (Figure 6L), when compared to vehicle treated animals. Moreover, FTY720 ICV treatment reduced hypothalamic STAT3 tyrosine phosphorylation in tumor-bearing animals (Figure 6M). This treatment also attenuated cancer-induced weight loss in tumor-bearing rats (Figure 6N). We observed that ICV FTY720 treatment did not change the tumor growth (data not shown).

Finally, we investigated whether the hypothalamic S1PR1 inhibition increases survival in tumor-bearing animals. After the onset of the anorexia, tumor-bearing animals received a daily ICV injection of vehicle or FTY720 (50 μM). As shown in the Kaplan-Maier graphs, a daily central infusion of FTY720 statistically prolonged the survival in tumor-bearing animals, whereas the median survival of tumor-bearing rats that received vehicle was 6 days and chronic administration of FTY720 increased the median survival to 10 days (Figure 6O). These data suggests that S1P produced during the tumor development triggers hypothalamic anorexigenic signals through the S1PR1/STAT3 axis contributing to cancer-induced anorexia.

DISCUSSION

In the present study we reported that neuronal S1P/S1PR1 axis play an important role in the control of the energy homeostasis in mammals. We identify that hypothalamic cells are enriched with S1PR1 and that the nutritional availability modulates hypothalamic S1PR1 protein levels in rats. Upon S1P stimulus, S1PR1 interacts with and increase Jak2 tyrosine phosphorylation, contributing to the persistent activation of hypothalamic STAT3, reducing the food intake and increasing the energy expenditure. We also demonstrated that STAT3 exerts reciprocal effects, controlling S1PR1 expression in hypothalamic cells *in vivo* and *in vitro*. Our study identified that central leptin resistance is associated with low protein levels of S1PR1 in the hypothalamus, whereas chronic S1PR1 activation in the hypothalamus reduced the food consumption and increased the energy expenditure in obese animals. On the other hand, high S1PR1 protein levels and hyper-phosphorylation of STAT3 were found in the hypothalamus of different models of tumor-induced anorexia. This phenomenon was also accompanied by high levels of circulating S1P.

Sphingosine-1-phosphate receptors (S1PRs) family is composed by five different receptors (1-5) that activate diverse downstream signaling pathways in response to the common sphingosine-1-phosphate (S1P) [15]. S1PR1 is unique in that it couples exclusively to the G_i protein. This receptor is widely expressed, controlling the cellular process in heart, skeletal muscle, colon [14] and other tissues. Although S1P receptors have been identified in the central nervous system [23-24] the presence and function of S1PR1 specifically in the hypothalamus was not reported. Surprisingly, our study revealed that hypothalamic nuclei are highly enriched with S1PR1 protein levels, when compared to peripheral tissues. We identify that S1PR1 is localized in POMC neurons, conversely low expression of S1PR1 was found in NPY neurons. We also observed that the nutritional availability modulates the hypothalamic S1PR1 expression, whereas under fasting condition hypothalamic S1PR1 protein levels was reduced and refeeding was sufficient to increase S1PR1 in the hypothalamus and this phenomenon was accompanied by STAT3 activity, suggesting the participation of this receptor on the control of the energy homeostasis.

The relationship between lysophospholipids and Jak/STAT3 signaling was initially observed in endothelial progenitor cells, once S1P increased Jak2 phosphorylation through

the activation of lipid receptor S1PR3 [25], similar results were found in ventricular cardiomyocytes, in this case, S1P increased STAT3 phosphorylation through S1PR2 [11]. Our study demonstrated that in hypothalamic neurons, ICV injection of S1P promoted a strong Jak2/STAT3 activation, inducing anorexigenic signal in rats. Although we did not evaluate the presence/function of the others subunits of S1PRs in the hypothalamus, our data revealed that specific S1PR1 activator (SEW2871) promoted consistent STAT3 activation and induced anorexigenic and thermogenic response. In contrast, S1P ICV injection did not change IRS1 tyrosine and Akt serine phosphorylation, suggesting that the effects of S1P/S1PR1 on food intake occurred through the leptin but not insulin signaling. Our data is in accordance with Lee and colleagues that showed that S1PR1 interacts with Jak2 leading to persistent STAT3 activation in human tumor cells, whereas pharmacological Jak2 inhibitor abrogated S1PR1-mediated STAT3 tyrosine phosphorylation in MB49 tumor cells [13].

Using *in vivo* and *in vitro* models we observed that leptin increased S1PR1 protein levels in GT1-7 neuronal cells and in the hypothalamus of rats. Importantly, we demonstrated that STAT3 inhibition blunted leptin-induced S1PR1 protein levels in both models. These data could be explained once STAT3 directly binds to and activates the *S1pr1* promoter [13]. In addition, our results revealed that leptin deficiency or leptin receptor deficiency lead a strong reduction in S1PR1 hypothalamic levels, as observed in *ob/ob* and *db/db* mice. Furthermore, high-fat diet treatment induced central leptin resistance and this phenomenon was accompanied by low hypothalamic S1PR1 protein levels, showing the reciprocal requirement among S1PR1 and STAT3 in hypothalamic neurons.

Circulating molecules able to activate Jak2/STAT3 in hypothalamic neurons through the crosstalk mechanisms generally affects food intake and energy expenditure in mammals [4-6,26]. It has been proposed that, alternative activation of Jak2/STAT3 in the hypothalamus through the glycoprotein 130 receptor (gp130R) ligands, for instance, may play as potential therapeutic targets in obesity [27]. Our study provide substantial evidences that a circulating bioactive lipid metabolite, sphingosine-1-phosphate (S1P), could be considerate the alternative molecule capable to activates Jak2/STAT3 signaling pathway and recapitulates the anorexigenic signals in the hypothalamus. We demonstrated that even

with low expression of hypothalamic S1PR1 observed in obese rats, ICV injection of S1P or SEW2871 was sufficient to activate STAT3, and induce anorexigenic effects in obese animals. On the other hand, we detect augment of S1P serum levels in obese mice, when compared to the lean group. These data are in accordance with previous study that demonstrated that plasma levels of S1P were increased in *ob/ob* mice [28]. The augment in S1P levels in obese mice may occur as a compensatory mechanism despite the low hypothalamic S1PR1levels. However, the increased S1P levels observed in obese animals is not enough to activate the S1PR1/STAT3 signaling in the hypothalamus. The downregulation of S1PR1 protein level in obese rodents seems to be associated with the impairment of central S1P anorexigenic and thermogenic action in obese animals, when compared to lean ones. In addition, the molecular mechanisms of leptin resistance, such as, the protein tyrosine phosphatase 1B (PTP1B) [29-31] and IKKβ [32-34], activation may contribute to the impairment of S1PR1/Jak2 association and STAT3 activation in response to S1P; however, this issue deserves further investigations. Furthermore, using the osmotic mini-pump to delivery S1P specifically in the hypothalamus of obese rats resulted in a significant reduction in the food intake and body weight.

Although, high levels of S1P were found in obese and in tumor-bearing mice, probably, the S1P sources are distinct in each situation. The S1P synthesis occurs by phosphorylation of sphingosine, in a reaction catalysed by sphingosine kinases, SPHK1 and SPHK2 [15]. An elegant study demonstrated in model of colitis-associated cancer in mice that S1P is produced by upregulation of sphingosine kinase 1 (SPHK1) in cancer cells [14]. However in the obesity state, the cells types that promote the S1P synthesis remain unknown.

Anorexic cancer patients often report early satiety, which together with a reduced appetite has been postulated to be caused by the production of factors by the tumor that exerts their effects by acting on hypothalamic cells, amplifying the anorexigenic signals [35-36]. The pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1β), and interferon gamma (IFN γ) are the major products secreted by the tumor proposed to play a role in the etiology of cancer-induced anorexia [35]. Studies have reported

that aberrant S1P serum levels were found in animal models of cancer [14,20]. Moreover, serum levels of S1P were inversely correlated with chemotherapy-induced weight gain in women with breast cancer [37]. During the tumor development, high levels of S1P are produced through the upregulation of SPHK1 activity in cancer cells [14]. In addition, it has been proposed that serum S1P elevation represents the communication between cancer cells and host organism [38]. In our study, high serum and CSF S1P levels were found in tumor-bearing rats and it was associated with the upregulation of S1PR1 protein levels and STAT3 phosphorylation in the hypothalamus. In addition, intraperitoneal injection of S1P induced anorexigenic and thermogenic effects. Interestingly, the disruption of S1P/S1PR1 signaling in the hypothalamus reversed the anorexia in tumor-bearing animals and prolonged the survival in these animals. We suggested that in the late-stage of cancer, S1P produced by cancer- or stromal cells, acts in the hypothalamus contributing to cancer-induced anorexia.

Taken together, our study shows that neuronal S1P/S1PR1 axis affects the energy homeostasis in rodents. S1P acts as Jak2/STAT3 activator in hypothalamic neurons through its own receptor. Interestingly, the mediobasal hypothalamus is enriched with S1PR1. We determine that S1PR1 and STAT3 play reciprocal effects in the hypothalamus. We also report that neuronal S1P/S1PR1/STAT3 signaling plays a crucial role in distinct conditions of abnormal feeding behavior, such as obesity or cancer-induced anorexia. Thus, S1P/S1PR1 axis is a newly defined central regulator of appetite and a potential target for the treatment of obesity and cancer-induced anorexia as well.

METHODS

Animals

Wistar rats, Swiss, C57BL/6J, *ob/ob* and *db/db* mice were obtained from the University of Campinas Breeding Center. The investigation was approved by the ethics committee and followed the university guidelines for the use of animals in experimental studies and experiments conform to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication no. 85-23 revised 1996). For detailed information about animal care, diets and tumor inoculations, see the Supplemental Experimental Procedures.

Cell culture

The mouse hypothalamic tumor cell line GT1-7 (Pamela Mellon, San Diego, California, USA [39] were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% of fetal bovine serum with the addition of antibiotics and fungicides in a humid atmosphere with 37 °C, 5% CO_2. GT1-7 cells were transfected with STAT3 siRNA. For detailed information, see the Supplemental Experimental Procedures.

The Lewis lung carcinoma (LLC) cells were obtained from ATCC, Philadelphia, PA, USA and the Walker-256 tumor cell line (originally obtained from the Christ Hospital Line, National Cancer Institute Bank, Cambridge, UK). Cells were cultured in RPMI containing 10% fetal bovine serum and glutamine without addition of antibiotics or fungicides; they were maintained at 37 °C, 5% CO_2.

Intracerebroventricular (ICV) cannulation

After intraperitoneal injection of a mix of ketamin (10 mg) and diazepam (0.07 mg) (0.2 mL/100 g body weight), the rats were stereotaxically instrumented with a chronic 26-gauge stainless steel indwelling guide cannula aseptically placed into the third ventricle. For detailed information, see the Supplemental Experimental Procedures.

Intracerebroventricular (ICV) injections

Rats were deprived of food for 2 h with free access to water and received 2 μL of bolus injections into the third ventricle at 6:00 p.m to evaluate the food consumption or Western blotting analysis. For detailed information of each ICV injection, see the Supplemental Experimental Procedures.

Mini pump

For chronic S1P ICV administration, obese rats were submitted to ICV cannulae, which were implanted under ketamin and diazepam as described above. A catheter tube was connected from the brain infusion cannulae to a mini-osmotic pump (model 2002, Alzet®, DURECT Corporation, Cupertino, CA) infusing 2.08 ng/hr of S1P (50 ng within 24 hours) or vehicle 0.25 μL/hr. A subcutaneous incision on the dorsal surface of the animal was created where the pump was inserted. Thus, the incision was closed as previous described [40-41].

Intraperitoneal S1P injection

Single intraperitoneal S1P (100 ng) or vehicle injection was performed in C57BL6/J lean mice. These injections were performed at 6:00 p.m. and then food consumption and oxygen consumption/carbon dioxide production were measured in LE405 Gas Analyzer (Panlab – Harvard Apparatus, Holliston, MA, USA).

Oxygen Consumption and Locomotor Activity Determination

Oxygen consumption and carbon dioxide production were measured in fed animals through a computer-controlled, open circuit calorimeter system LE405 Gas Analyzer (Panlab – Harvard Apparatus, Holliston, MA, USA). For detailed information see the Supplemental Experimental Procedures.

Immunohistochemistry

Hypothalamic tissue of rats was submitted to single- or double-immunofluorescence staining as previously described protocol [6]. For detailed information, see the Supplemental Experimental Procedures.

Dissection of the hypothalamic regions

Hypothalamic nuclei of rats were quickly dissected in a stainless steel matrix with razor blades and frozen in liquid nitrogen. Later on, each region of the hypothalamus was dissected from 1 mm thick sagittal sections of fresh brain as previously described [6]. For detailed information, see the Supplemental Experimental Procedures.

Statistical analysis

All numeric results are expressed as the means ± SEM of the indicated number of experiments. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by optical densitometry (UN-SCAN-IT gel, 6.1). Statistical analysis was performed using the ANOVA test with the Bonferroni post test. Significance was established at the $p<0.05$ level. The survival curves were estimated using Kaplan-Meier's estimates, and curves were compared using the log-rank test and the level of significance was set at $p<0.001$.

ACKNOWLEDGMENTS

This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - 2011/09656-0 and 2011/13828-1), Fundação de Apoio ao Ensino, Pesquisa e Extensão (FAEPEX –N°519.292) and Conselho Nacional de desenvolvimento científico e tecnológico (CNPq).

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

V.R.R.S. researched data contributed to discussion and reviewed/edited manuscript. T.O.M. researched data. G.D.P. researched data. C.K.K. researched data. L.L. researched data. J.M. researched data. M.C.M. researched data. D.R. researched data. G.Z.R. researched data. C.T.S. researched data. P.O.P. researched data. L.A.V. contributed to discussion. J.B.C.C. contributed to discussion. J.R.P. contributed to discussion and reviewed/edited manuscript. D.E.C. researched data and contributed to discussion and reviewed/edited manuscript. E.R.R. wrote the manuscript, contributed to discussion and reviewed/edited manuscript.

REFERENCES

1 Schwartz, M. W., Woods, S. C., Porte, D., Jr., Seeley, R. J. & Baskin, D. G. Central nervous system control of food intake. *Nature* **404**, 661-671, doi:10.1038/35007534 (2000).

2 Munzberg, H., Huo, L., Nillni, E. A., Hollenberg, A. N. & Bjorbaek, C. Role of signal transducer and activator of transcription 3 in regulation of hypothalamic proopiomelanocortin gene expression by leptin. *Endocrinology* **144**, 2121-2131 (2003).

3 Myers, M. G., Cowley, M. A. & Munzberg, H. Mechanisms of leptin action and leptin resistance. *Annu Rev Physiol* **70**, 537-556, doi:10.1146/annurev.physiol.70.113006.100707 (2008).

4 Janoschek, R. *et al.* gp130 signaling in proopiomelanocortin neurons mediates the acute anorectic response to centrally applied ciliary neurotrophic factor. *Proc Natl Acad Sci U S A* **103**, 10707-10712, doi:0600425103 [pii] 10.1073/pnas.0600425103 (2006).

5 Johnen, H. *et al.* Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1. *Nat Med* **13**, 1333-1340, doi:nm1677 [pii] 10.1038/nm1677 (2007).

6 Ropelle, E. R. *et al.* 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**, doi:10.1371/journal.pbio.1000465 (2010).

7 El-Haschimi, K., Pierroz, D. D., Hileman, S. M., Bjorbaek, C. & Flier, J. S. Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *J Clin Invest* **105**, 1827-1832, doi:10.1172/JCI9842 (2000).

8 Ernst, M. B. *et al.* Enhanced Stat3 activation in POMC neurons provokes negative feedback inhibition of leptin and insulin signaling in obesity. *J Neurosci* **29**, 11582-11593, doi:29/37/11582 [pii] 10.1523/JNEUROSCI.5712-08.2009 (2009).

9 Gao, Q. *et al.* Anorectic estrogen mimics leptin's effect on the rewiring of melanocortin cells and Stat3 signaling in obese animals. *Nat Med* **13**, 89-94, doi:nm1525 [pii] 10.1038/nm1525 (2007).

10 Ghilardi, N. *et al.* Defective STAT signaling by the leptin receptor in diabetic mice. *Proc Natl Acad Sci U S A* **93**, 6231-6235 (1996).

11 Frias, M. A., James, R. W., Gerber-Wicht, C. & Lang, U. Native and reconstituted HDL activate Stat3 in ventricular cardiomyocytes via ERK1/2: role of sphingosine-

1-phosphate. *Cardiovasc Res* **82**, 313-323, doi:cvp024 [pii] 10.1093/cvr/cvp024 (2009).

12 Gurgui, M., Broere, R., Kalff, J. C. & van Echten-Deckert, G. Dual action of sphingosine 1-phosphate in eliciting proinflammatory responses in primary cultured rat intestinal smooth muscle cells. *Cell Signal* **22**, 1727-1733, doi:S0898-6568(10)00178-6 [pii] 10.1016/j.cellsig.2010.06.013 (2010).

13 Lee, H. *et al.* STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors. *Nat Med* **16**, 1421-1428, doi:nm.2250 [pii] 10.1038/nm.2250 (2010).

14 Liang, J. *et al.* Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer. *Cancer Cell* **23**, 107-120, doi:S1535-6108(12)00492-8 [pii] 10.1016/j.ccr.2012.11.013 (2013).

15 Spiegel, S. & Milstien, S. The outs and the ins of sphingosine-1-phosphate in immunity. *Nat Rev Immunol* **11**, 403-415, doi:nri2974 [pii] 10.1038/nri2974 (2011).

16 Spiegel, S. & Milstien, S. Functions of the multifaceted family of sphingosine kinases and some close relatives. *J Biol Chem* **282**, 2125-2129, doi:R600028200 [pii] 10.1074/jbc.R600028200 (2007).

17 Loh, K. C. *et al.* Sphingosine-1-phosphate enhances satellite cell activation in dystrophic muscles through a S1PR2/STAT3 signaling pathway. *PLoS One* **7**, e37218, doi:10.1371/journal.pone.0037218 PONE-D-11-25533 [pii] (2012).

18 Lopez, M. *et al.* Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. *Cell Metab* **7**, 389-399, doi:S1550-4131(08)00077-6 [pii] 10.1016/j.cmet.2008.03.006 (2008).

19 Means, C. K. & Brown, J. H. Sphingosine-1-phosphate receptor signalling in the heart. *Cardiovasc Res* **82**, 193-200, doi:cvp086 [pii] 10.1093/cvr/cvp086 (2009).

20 Nagahashi, M. *et al.* Sphingosine-1-phosphate produced by sphingosine kinase 1 promotes breast cancer progression by stimulating angiogenesis and lymphangiogenesis. *Cancer Res* **72**, 726-735, doi:72/3/726 [pii] 10.1158/0008-5472.CAN-11-2167 (2012).

21 Brinkmann, V. *et al.* Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov* **9**, 883-897, doi:nrd3248 [pii] 10.1038/nrd3248 (2010).

22 Graler, M. H. & Goetzl, E. J. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J* **18**, 551-553, doi:10.1096/fj.03-0910fje 03-0910fje [pii] (2004).

23 Guo, H. *et al.* An activated protein C analog stimulates neuronal production by human neural progenitor cells via a PAR1-PAR3-S1PR1-Akt pathway. *J Neurosci* **33**, 6181-6190, doi:33/14/6181 [pii] 10.1523/JNEUROSCI.4491-12.2013 (2013).

24 Ishii, I., Fukushima, N., Ye, X. & Chun, J. Lysophospholipid receptors: signaling and biology. *Annu Rev Biochem* **73**, 321-354, doi:10.1146/annurev.biochem.73.011303.073731 (2004).

25 Walter, D. H. *et al.* Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor. *Arterioscler Thromb Vasc Biol* **27**, 275-282, doi:01.ATV.0000254669.12675.70 [pii] 10.1161/01.ATV.0000254669.12675.70 (2007).

26 Grossberg, A. J. *et al.* Arcuate nucleus proopiomelanocortin neurons mediate the acute anorectic actions of leukemia inhibitory factor via gp130. *Endocrinology* **151**, 606-616, doi:en.2009-1135 [pii] 10.1210/en.2009-1135 (2010).

27 Febbraio, M. A. gp130 receptor ligands as potential therapeutic targets for obesity. *J Clin Invest* **117**, 841-849, doi:10.1172/JCI30453 (2007).

28 Samad, F., Hester, K. D., Yang, G., Hannun, Y. A. & Bielawski, J. Altered adipose and plasma sphingolipid metabolism in obesity: a potential mechanism for cardiovascular and metabolic risk. *Diabetes* **55**, 2579-2587, doi:55/9/2579 [pii] 10.2337/db06-0330 (2006).

29 Bence, K. K. *et al.* Neuronal PTP1B regulates body weight, adiposity and leptin action. *Nat Med* **12**, 917-924, doi:nm1435 [pii] 10.1038/nm1435 (2006).

30 Chiarreotto-Ropelle, E. C. *et al.* Acute exercise suppresses hypothalamic PTP1B protein level and improves insulin and leptin signaling in obese rats. *Am J Physiol Endocrinol Metab* **305**, E649-659, doi:ajpendo.00272.2013 [pii] 10.1152/ajpendo.00272.2013 (2013).

31 Picardi, P. K. *et al.* Reduction of hypothalamic protein tyrosine phosphatase improves insulin and leptin resistance in diet-induced obese rats. *Endocrinology* **149**, 3870-3880, doi:en.2007-1506 [pii] 10.1210/en.2007-1506 (2008).

32 Milanski, M. *et al.* Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus:

implications for the pathogenesis of obesity. *J Neurosci* **29**, 359-370, doi:29/2/359 [pii] 10.1523/JNEUROSCI.2760-08.2009 (2009).

33 Purkayastha, S., Zhang, G. & Cai, D. Uncoupling the mechanisms of obesity and hypertension by targeting hypothalamic IKK-beta and NF-kappaB. *Nat Med* **17**, 883-887, doi:nm.2372 [pii] 10.1038/nm.2372 (2011).

34 Zhang, X. *et al.* Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. *Cell* **135**, 61-73, doi:S0092-8674(08)01008-8 [pii] 10.1016/j.cell.2008.07.043 (2008).

35 Laviano, A., Meguid, M. M. & Rossi-Fanelli, F. Cancer anorexia: clinical implications, pathogenesis, and therapeutic strategies. *Lancet Oncol* **4**, 686-694, doi:S1470204503012476 [pii] (2003).

36 Tisdale, M. J. Biology of cachexia. *J Natl Cancer Inst* **89**, 1763-1773 (1997).

37 Pchejetski, D. *et al.* Circulating sphingosine-1-phosphate inversely correlates with chemotherapy-induced weight gain during early breast cancer. *Breast Cancer Res Treat* **124**, 543-549, doi:10.1007/s10549-010-0968-y (2010).

38 Ponnusamy, S. *et al.* Communication between host organism and cancer cells is transduced by systemic sphingosine kinase 1/sphingosine 1-phosphate signalling to regulate tumour metastasis. *EMBO Mol Med* **4**, 761-775, doi:10.1002/emmm.201200244 (2012).

39 Mellon, P. L. *et al.* Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron* **5**, 1-10, doi:0896-6273(90)90028-E [pii] (1990).

40 Lopez, M. *et al.* Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance. *Nat Med* **16**, 1001-1008, doi:nm.2207 [pii] 10.1038/nm.2207 (2010).

41 Sangiao-Alvarellos, S. *et al.* Influence of ghrelin and growth hormone deficiency on AMP-activated protein kinase and hypothalamic lipid metabolism. *J Neuroendocrinol* **22**, 543-556, doi:JNE1994 [pii] 10.1111/j.1365-2826.2010.01994.x (2010).

Figure 1

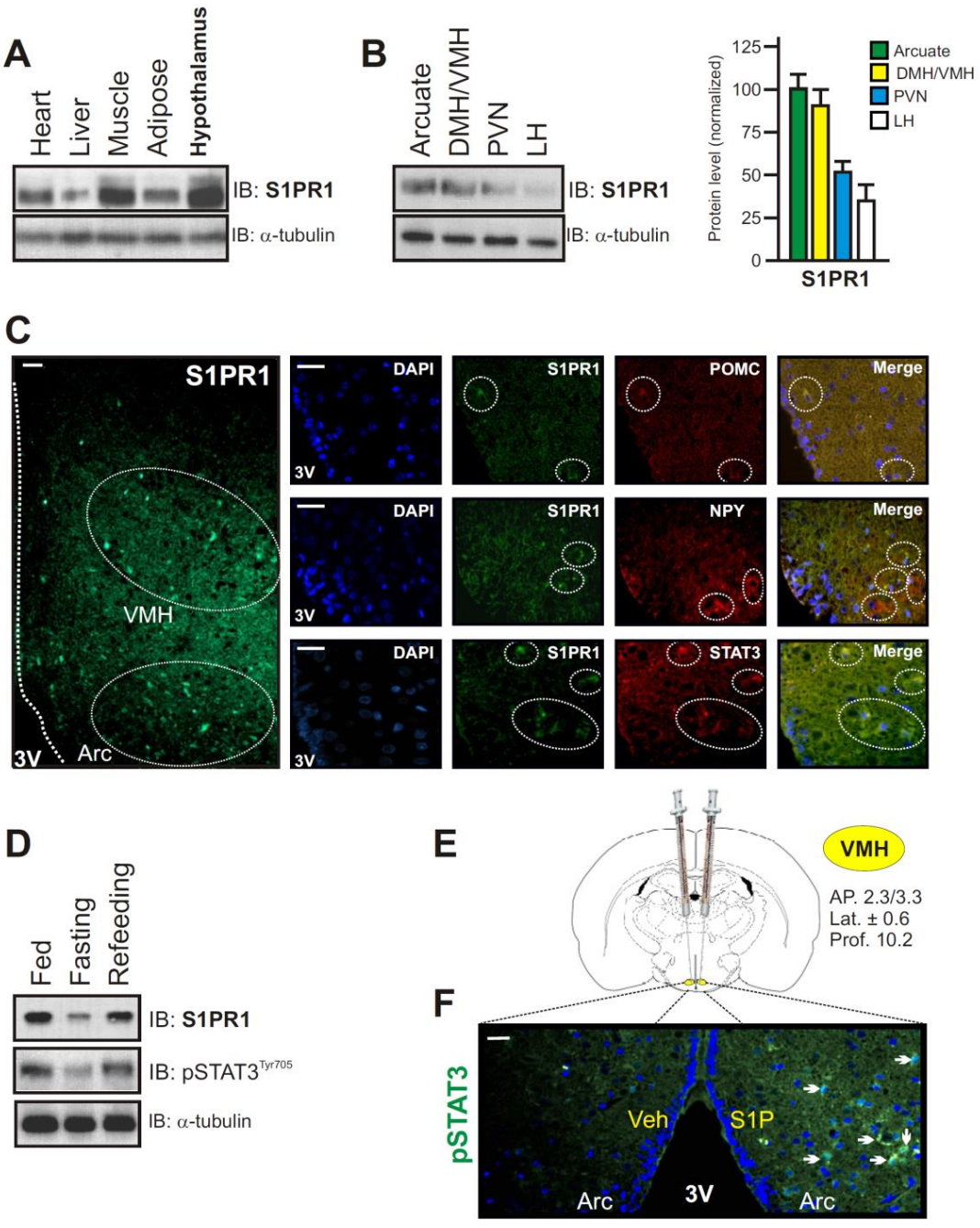

A
Heart
Liver
Muscle
Adipose
Hypothalamus
IB: S1PR1
IB: α-tubulin
B
Arcuate
DMH/VMH
PVN
LH
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
125
100
75
50
25
0
Arcuate
DMH/VMH
PVN
LH
S1PR1
C
S1PR1
VMH
3V
Arc
DAPI
S1PR1
POMC
Merge
NPY
STAT3
D
Fed
Fasting
Refeeding
IB: S1PR1
IB: pSTAT3Tyr705
IB: α-tubulin
E
VMH
AP. 2.3/3.3
Lat. ± 0.6
Prof. 10.2
F
pSTAT3
Veh
S1P
Arc
3V
Arc

Figure 2

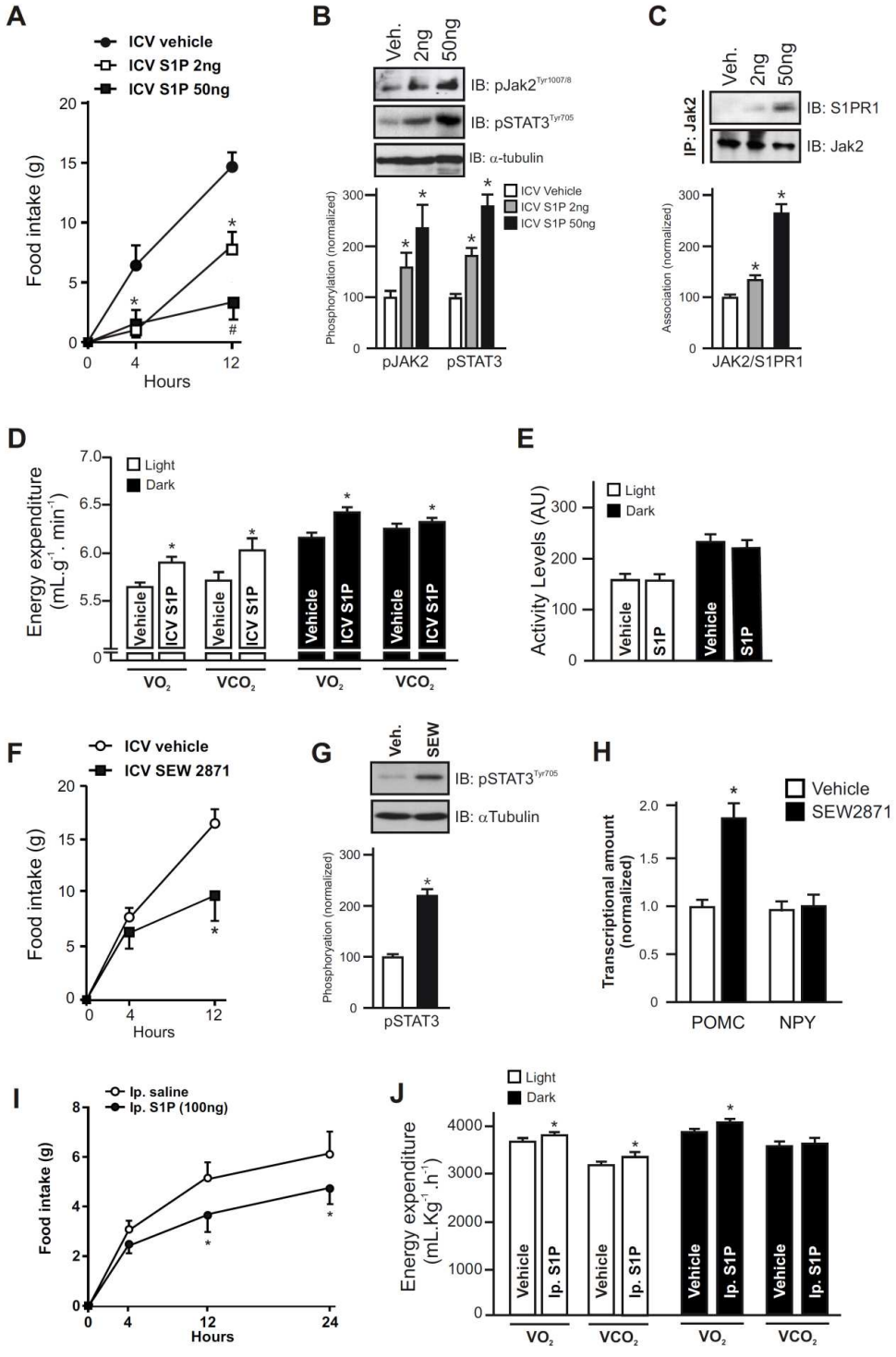

A
ICV vehicle
ICV S1P 2ng
ICV S1P 50ng
Food intake (g)
Hours
B
Veh.
2ng
50ng
IB: pJak2Tyr1007/8
IB: pSTAT3Tyr705
IB: α-tubulin
ICV Vehicle
ICV S1P 2ng
ICV S1P 50ng
Phosphorylation (normalized)
pJAK2
pSTAT3
C
IP: Jak2
IB: S1PR1
IB: Jak2
Association (normalized)
JAK2/S1PR1
D
Light
Dark
Energy expenditure (mL.g-1. min-1)
Vehicle
ICV S1P
VO2
VCO2
E
Activity Levels (AU)
S1P
F
ICV vehicle
ICV SEW 2871
Food intake (g)
Hours
G
Veh.
SEW
IB: pSTAT3Tyr705
IB: αTubulin
Phosphorylation (normalized)
pSTAT3
H
Vehicle
SEW2871
Transcriptional amount (normalized)
POMC
NPY
I
Ip. saline
Ip. S1P (100ng)
Food intake (g)
Hours
J
Light
Dark
Energy expenditure (mL.Kg-1.h-1)
Vehicle
Ip. S1P
VO2
VCO2

Figure 3

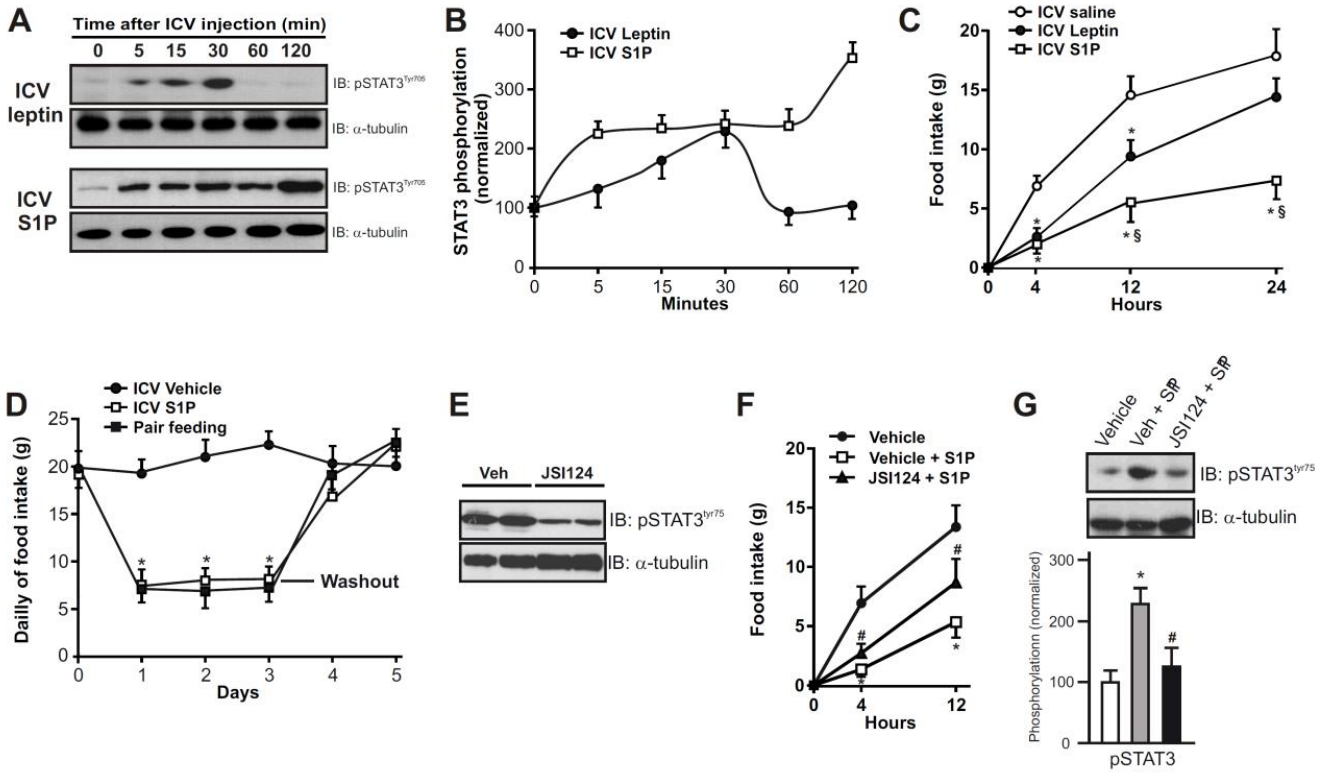

A
Time after ICV injection (min)
0 5 15 30 60 120
ICV leptin
IB: pSTAT3Tyr705
IB: α-tubulin
ICV S1P
IB: pSTAT3Tyr705
IB: α-tubulin
B
ICV Leptin
ICV S1P
STAT3 phosphorylation (normalized)
Minutes
C
ICV saline
ICV Leptin
ICV S1P
Food intake (g)
Hours
D
ICV Vehicle
ICV S1P
Pair feeding
Dailly of food intake (g)
Washout
Days
E
Veh
JSI124
IB: pSTAT3tyr75
IB: α-tubulin
F
Vehicle
Vehicle + S1P
JSI124 + S1P
Food intake (g)
Hours
G
Vehicle
Veh + S1P
JSI124 + S1P
IB: pSTAT3tyr75
IB: α-tubulin
Phosphorylationn (normalized)
pSTAT3

Figure 4

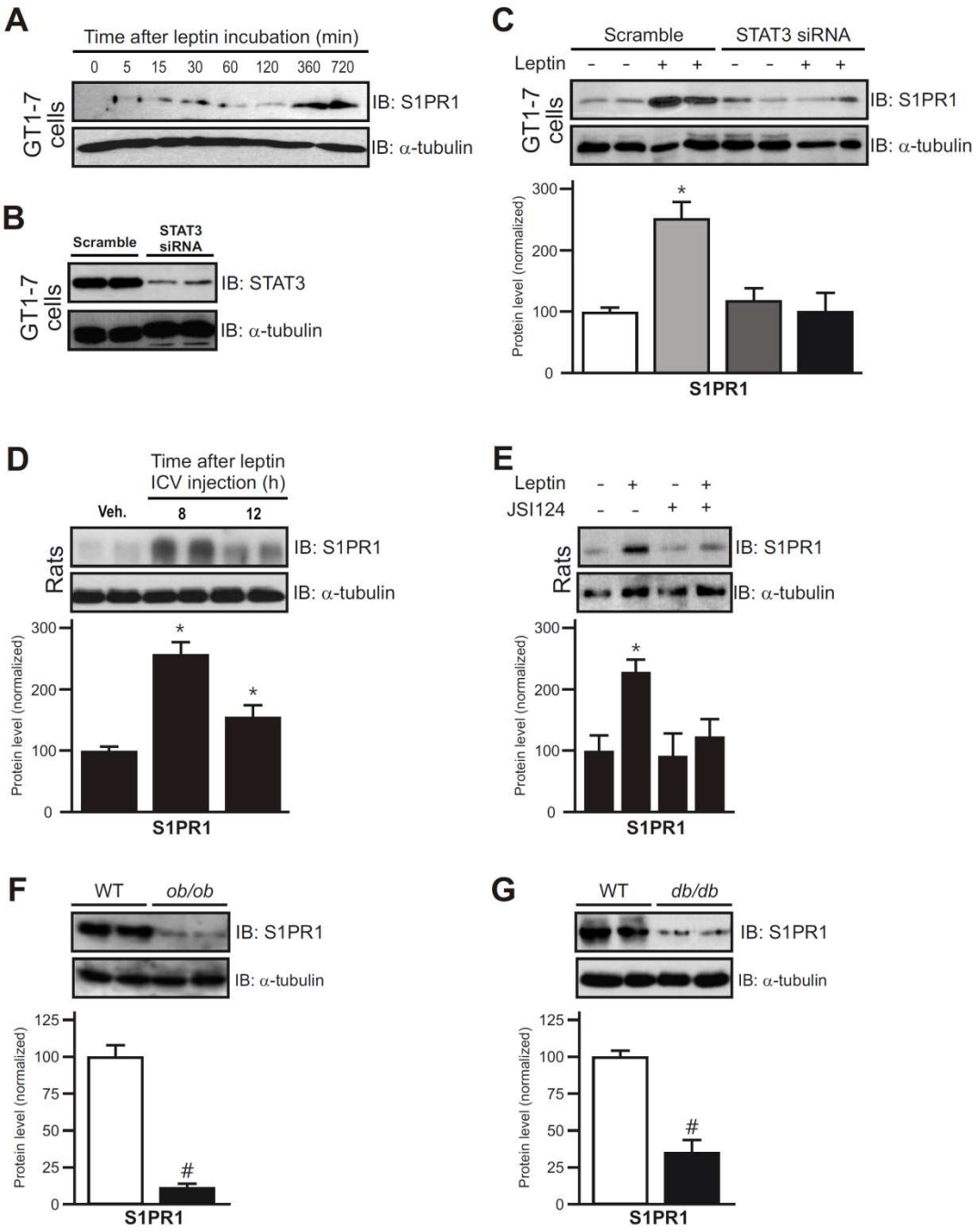

A
Time after leptin incubation (min)
0 5 15 30 60 120 360 720
GT1-7 cells
IB: S1PR1
IB: α-tubulin
B
Scramble
STAT3 siRNA
GT1-7 cells
IB: STAT3
IB: α-tubulin
C
Scramble
STAT3 siRNA
Leptin - - + + - - + +
GT1-7 cells
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
300
200
100
0
*
S1PR1
D
Time after leptin ICV injection (h)
Veh. 8 12
Rats
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
300
200
100
0
*
*
S1PR1
E
Leptin - + - +
JSI124 - - + +
Rats
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
300
200
100
0
*
S1PR1
F
WT ob/ob
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
125
100
75
50
25
0
#
S1PR1
G
WT db/db
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
125
100
75
50
25
0
#
S1PR1

Figure 5

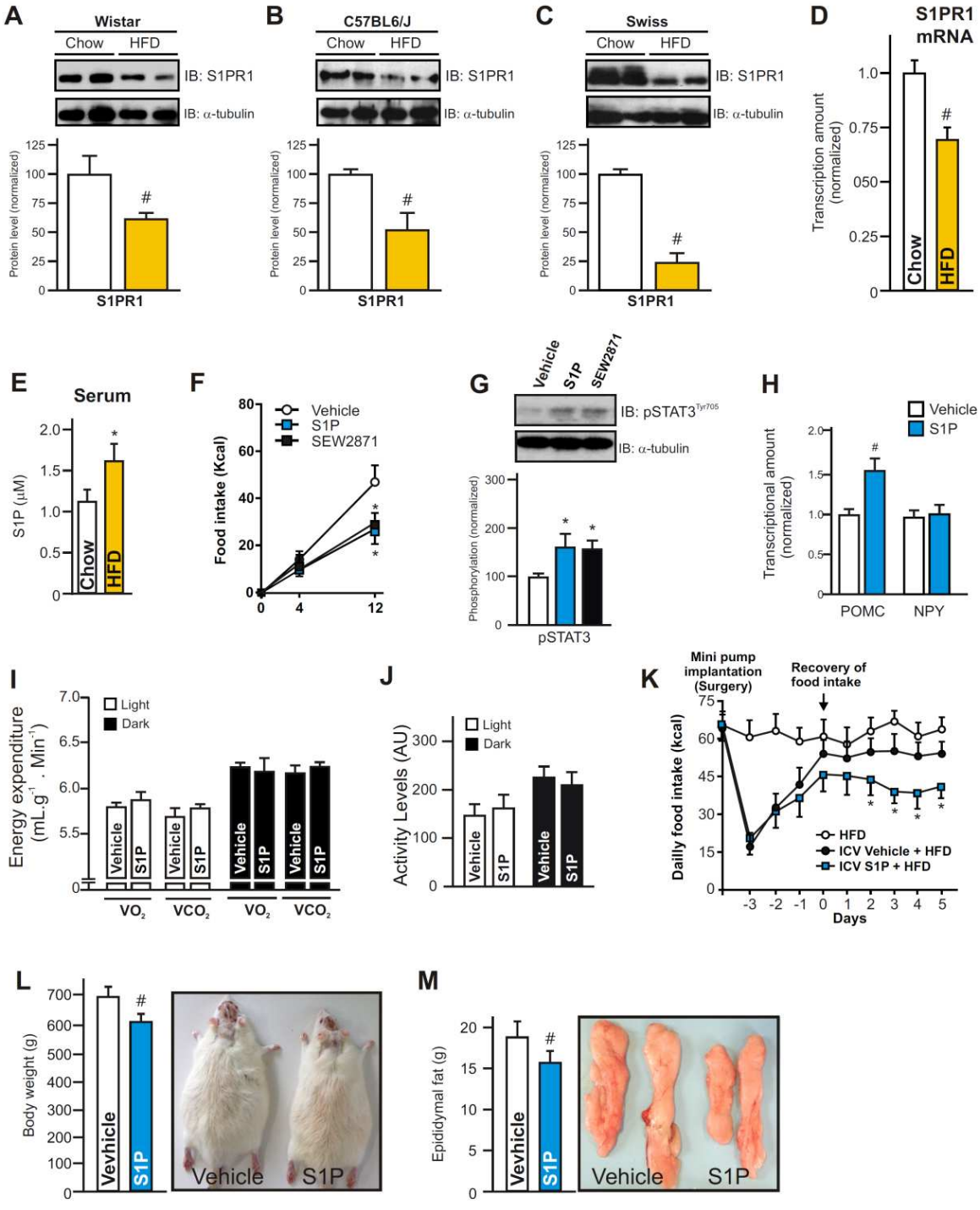

A
Wistar
Chow
HFD
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
S1PR1
B
C57BL6/J
C
Swiss
D
S1PR1 mRNA
Transcription amount (normalized)
Chow
HFD
E
Serum
S1P (μM)
F
Vehicle
S1P
SEW2871
Food intake (Kcal)
G
Vehicle
S1P
SEW2871
IB: pSTAT3Tyr705
Phosphorylation (normalized)
pSTAT3
H
Transcriptional amount (normalized)
POMC
NPY
I
Light
Dark
Energy expenditure (mL.g-1 . Min-1)
VO2
VCO2
J
Activity Levels (AU)
K
Mini pump implantation (Surgery)
Recovery of food intake
Dailly food intake (kcal)
HFD
ICV Vehicle + HFD
ICV S1P + HFD
Days
L
Body weight (g)
Vevhicle
S1P
M
Epididymal fat (g)

Figure 6

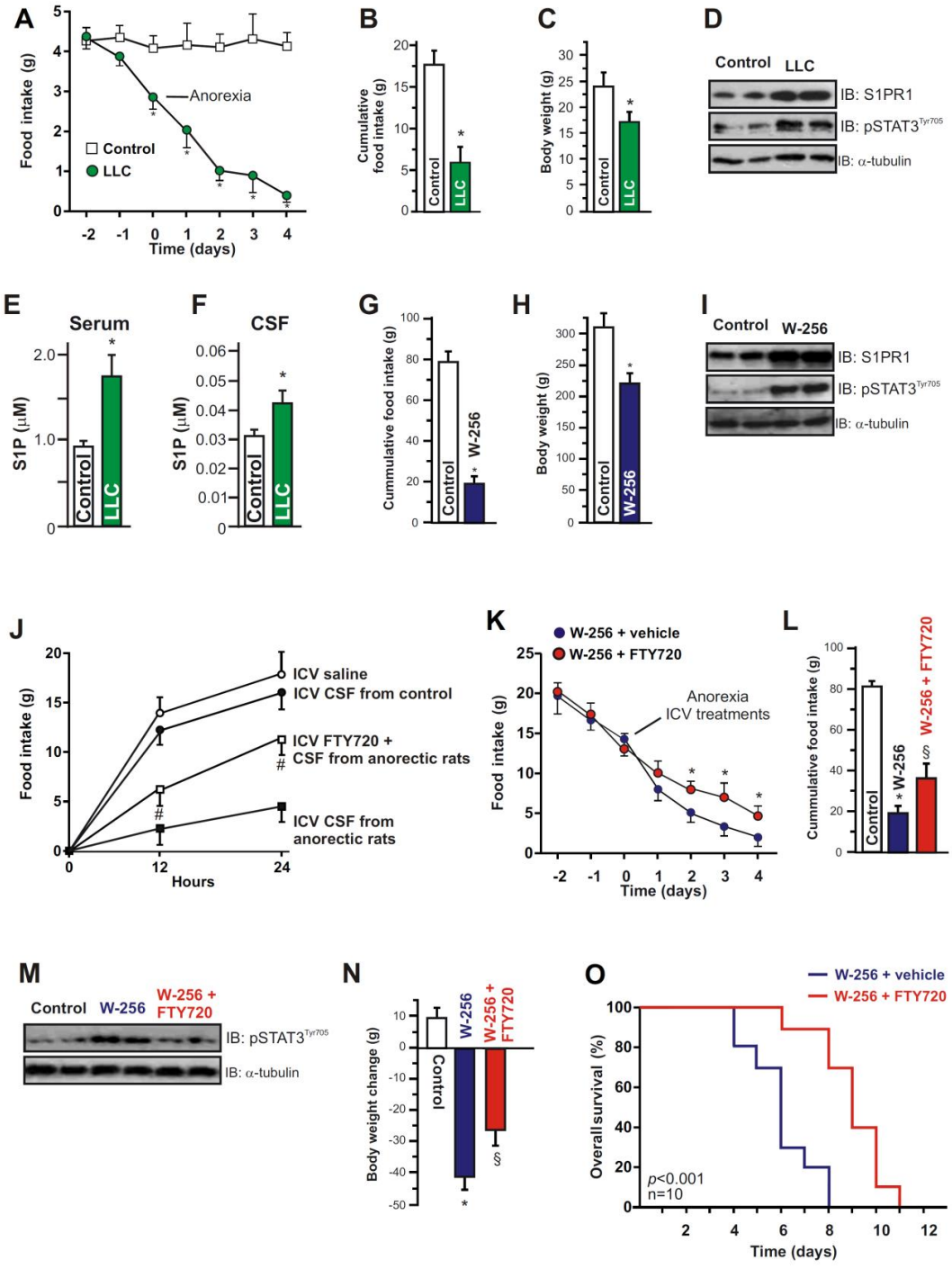

A
Food intake (g)
Anorexia
Control
LLC
Time (days)
B
Cumulative food intake (g)
Control
LLC
C
Body weight (g)
Control
LLC
D
Control
LLC
IB: S1PR1
IB: pSTAT3Tyr705
IB: α-tubulin
E
Serum
S1P (μM)
Control
LLC
F
CSF
S1P (μM)
Control
LLC
G
Cummulative food intake (g)
Control
W-256
H
Body weight (g)
Control
W-256
I
Control
W-256
IB: S1PR1
IB: pSTAT3Tyr705
IB: α-tubulin
J
Food intake (g)
ICV saline
ICV CSF from control
ICV FTY720 +
CSF from anorectic rats
ICV CSF from
anorectic rats
Hours
K
W-256 + vehicle
W-256 + FTY720
Anorexia
ICV treatments
Food intake (g)
Time (days)
L
Cummulative food intake (g)
Control
W-256
W-256 + FTY720
M
Control
W-256
W-256 +
FTY720
IB: pSTAT3Tyr705
IB: α-tubulin
N
Body weight change (g)
Control
W-256
W-256 +
FTY720
O
W-256 + vehicle
W-256 + FTY720
Overall survival (%)
p<0.001
n=10
Time (days)

Figure S1

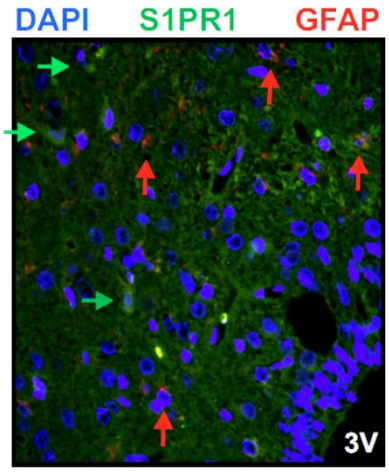


Figure S2

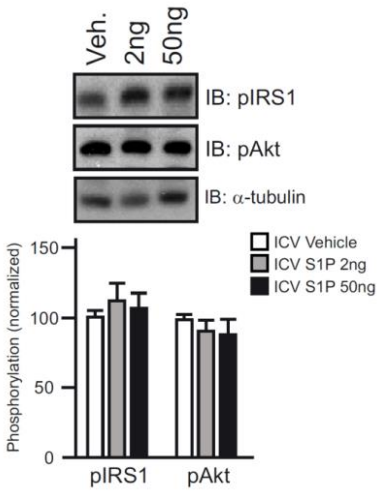

Veh.
2ng
50ng
IB: pIRS1
IB: pAkt
IB: α-tubulin
ICV Vehicle
ICV S1P 2ng
ICV S1P 50ng
Phosphorylation (normalized)
150
100
50
0
pIRS1
pAkt

Figure S3

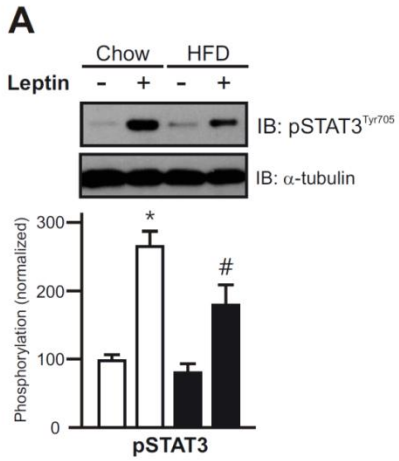
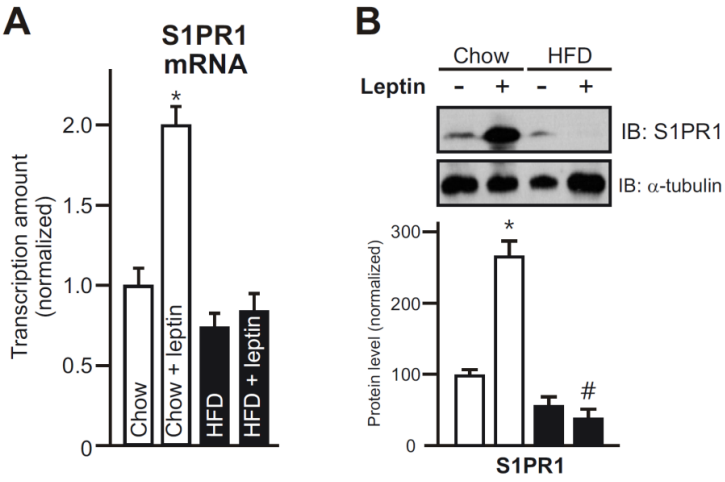

A
Chow
HFD
Leptin
- + - +
IB: pSTAT3Tyr705
IB: α-tubulin
Phosphorylation (normalized)
300
200
100
0
*
#
pSTAT3

Figure S4


A
S1PR1 mRNA
Transcription amount (normalized)
2.0
1.5
1.0
0.5
0
*
Chow
Chow + leptin
HFD
HFD + leptin
B
Chow
HFD
Leptin
- + - +
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
300
200
100
0
*
#
S1PR1

SUPPLEMENTARY INFORMATION

Figure legends.

Figure S1. Double-immunostaining was performed to evaluate the co-localization of S1PR1 (green) GFAP (red) in the arcuate nuclei of control rats, with 200x magnification (scale bar, 20 μm).

Figure S2. Western blots show; S1P-induced (A) IRS1 tyrosine and (B) Akt serine phosphorylation in hypothalamic samples of Wistar rats 30 min after ICV S1P (50 ng) injection (n=6). Data were expressed by using mean ± S.E.M.

Figure S3. (A) Western blots show; leptin-induced STAT3 tyrosine phosphorylation in hypothalamic samples of control and obese Wistar rats 30 min after ICV leptin (10^{-6} M) injection (n=6). Data were expressed by using mean ± S.E.M. * $p<0.05$, vs. chow plus vehicle. # $p<0.05$, vs. chow plus leptin.

Figure S4. S1PR1 (A) mRNA and (B) protein levels in control rats injected with saline (2 μL), leptin (10^{-6} M) or JSI124 plus leptin. Hypothalamic samples were obtained 8 hours after leptin injection (n=4). Data were expressed by using mean ± S.E.M.* $p<0.05$, vs. chow, # $p<0.05$, vs. chow plus leptin.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals and diets

Male 5-wk-old Wistar rats and 10-wk-old Swiss mice were obtained from the University of Campinas Breeding Center. The investigation was approved by the ethics committee and followed the university guidelines for the use of animals in experimental studies and experiments conform to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication no. 85-23 revised 1996). The animals were maintained in 12h: 12h artificial light-dark cycles, with lights on at 06:00 a.m., and were housed in individual cages. The animals were randomly divided into two groups: control, fed on standard rodent chow (3.948 $Kcal.Kg^{-1}$) or high-fat diet (HFD) (5.358 $Kcal.Kg^{-1}$) *ad libitum* for 3 months. The high-fat diet composition was previously described [1].

Male (10-wk-old) *ob/ob* and *db/db* mice and their respective control C57BL/6J background mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were bred under specific pathogen-free conditions at the Central Breeding Center of the University of Campinas and were fed on standard rodent chow (3.948 $Kcal.Kg^{-1}$). The number of animals used in each experiment is specified in the legend figure.

Tumor xenograft models

Male C57BL6/J mice were implanted with 5.0×10^5 LLC cells into the dorsal subcutis of the right flank. Four days after the onset of anorexia, the mice were killed, the hypothalamic tissue was excised and mice were weighed with and without tumor.

Walker-256 tumor cells were obtained from the ascitic fluid of the peritoneal cavity of Wistar rats, 5 days after the intraperitoneal injection of 20×10^6 carcinoma cells. After cell harvesting, the percentage of viable cells was determined by using 1% Trypan blue solution in a Neubauer chamber. Tumor cells (2×10^6 cells in 1 mL saline solution) were

injected in the right flank after the surgical implantation of the intracerebroventricular (ICV) cannula.

Definition of cancer anorexia

Each animal's individual baseline 24-h food intake was defined as the average daily food intake over a period of 3 consecutive days. Subsequent food intake data are expressed as individual percentages and baseline daily food intake. In tumor-bearing animals, cancer anorexia was defined as a single value of less than 70% of baseline occurring after a steady decline of at least 3 days duration, as previously described [2].

Transfection

A total of 5.10^5 cells were seeded in a tissue culture plate in complete growth medium and incubated overnight. Transfection with small interference RNA (siRNA) targeted to STAT3 (si-STAT3 Silencer® Select Pre-designed s744) or scrambled control siRNA (Silencer® Select Pre-designed 4309843) was performed. GT1-7 cells were washed twice with serum- and glucose-free medium (Opti-MEM, Life Technologies) and then incubated with 2 mL of the same medium containing the siRNA (final concentration of 100 nM) previously mixed with 5 µL of Lipofectamine 2000 (Life Technologies). After 7 h, 1 mL of Opti-MEM medium containing 30% FBS was added to the culture containing siRNAs and Lipofectamine. After 17h, cells were cultured in DMEM with 10% of FBS for 12h. After this period, cells were starved overnight and treated with of leptin (40 µmol/L) for 12 hours. Subsequently, the cells were used for Western blot analysis.

Antibodies and chemicals

Anti-EDG-1 (rabbit polyclonal, SC-25489), Anti-Jak2 (rabbit polyclonal, SC-278), anti-STAT3 (rabbit polyclonal, SC-483), anti-phospho-IRS-1 (rabbit polyclonal, SC-17199) anti-NPY (goat polyclonal, SC-14728) and anti-POMC (rabbit polyclonal, SC-20148) antibodies were from Santa Cruz Biotechnology, Inc. Anti-phospho-Akt (rabbit polyclonal, #9271s), anti-α tubulin (rabbit polyclonal, #2144), anti-phospho-STAT3 (rabbit

polyclonal, #9131), anti-phospho-Jak2 (rabbit polyclonal, #3771), was from Cell Signalling Technology (Beverly, MA, USA).

Leptin was from Calbiochem (San Diego, CA, USA). Protein A-Sepharose 6 MB and nitrocellulose paper (Hybond ECL, 0.45 mm) were from Amersham Pharmacia Biotech United Kingdom Ltd. (Buckinghamshire, United Kingdom). Sphingosine-1 Phosphate (S1P) was from Avanti Polar Lipids Inc (Alabama, EUA). SEW2871 was from Cayman Chemical (Michigan,USA), FTY720 was from Cayman Chemical Company (Michigan,USA) and Cucurbitacin JSI 124 was from Sigma Chemical Co. (St.Louis,MO). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Determination of S1P levels.

After six hours of fasting, blood was collected from the cava vein. Plasma was separated by centrifugation (1.100 x g) for 15 min at 4 °C and stored at -80 °C until the assay. Cerebrospinal fluid (CSF) was obtained from rats as previously described [3] with minor modifications. For serum and CSF S1P determination we employed a commercially available Sphingosine- 1 phosphate Assay kit (Echelon Biosciences Inc.).

Intracerebroventricular (ICV) cannulation

After intraperitoneal injection of a mix of ketamin (10 mg) and diazepam (0.07 mg) (0.2 mL/100 g body weight), the rats were stereotaxically instrumented with a chronic 26-gauge stainless steel indwelling guide cannula aseptically placed into the third ventricle at the midline coordinates of 0.5 mm posterior to the bregma and 8.5 mm below the surface of the skull of the rats using the Stoelting stereotaxic apparatus. The ventromedial hypothalamus (VMH) of rats was accessed bilaterally using a 25-gauge needle connected to Hamilton 25 μL syringes.

VMH S1P injection.

The vehicle and S1P injections were directed to stereotaxic coordinates 2.3/3.3 mm posterior to the bregma, ± 0.6 mm lateral to midline and 10.2 mm below the surface of the skull, as previously described [4].

Intracerebroventricular (ICV) injections

Rats were deprived of food for 2 h with free access to water and received 2 μL of bolus injections into the third ventricle, as follow:

Leptin injection. Rats received ICV infusion of vehicle (saline) or leptin (10^{-6} M) at 6:00 p.m. to evaluate the food consumption. Food intake was determined by measuring the difference between the weight of chow given and the weight of chow at the end of a 4- and 12-h period. To evaluate the effects of leptin on Jak/STAT signaling the hypothalamic tissue was removed 30 minutes later. To evaluate the effects of leptin on S1PR1 protein levels, the hypothalamic tissue was removed or 8 and 12 hours after leptin ICV injection.

S1P. For Western blot analysis, ICV S1P (2 and 50 ng) was injected into the third ventricle. To evaluate the effects of S1P on Jak/STAT signaling the hypothalamic tissue was removed 30 minutes later. To evaluate the effects of S1P on food intake in rats, ICV injection of S1P (50 ng) was performed at 6:00 p.m.

SEW2871. For Western blot analysis, ICV SEW2871 (50 ng) was injected into the third ventricle. To evaluate the effects of SEW2871 on STAT3 phosphorylation the hypothalamic tissue was removed 30 minutes later. To evaluate the effects of SEW2871 on food intake in rats, ICV injection of S1P (50ng) was performed at 6:00 p.m.

JSI124. For Western blot analysis, ICV JSI124 (50 μM) was injected into the third ventricle. To evaluate the effects of JSI124 on STAT3 phosphorylation the hypothalamic tissue was removed 60 minutes later. To evaluate the effects of JSI124 on food intake in rats, ICV JSI124 injection was performed 30 minutes before the S1P injection.

FTY720. To evaluate the effects of FTY720 on food intake in tumor bearing rats, daily ICV injections (50 μM) was performed at 6:00 p.m. Thirty minutes after the last injection, the hypothalamic tissue was removed for Western blotting analysis.

Cerebrospinal fluid (CSF) injection. CSF from control or from tumor-bearing wistar rats were obtained through the introduction of a needle into the cisterna magna through the skin and/or dura mater using a stereotactic micromanipulator. Immediately after the liquor sampling, 2 μL were injected into the third ventricle of control rats to evaluate the food intake. One group of rats received ICV injection of FTY720 (50 μM) 120 minutes before the CSF injection. CSF injections were performed at 6:00 p.m.

Oxygen Consumption and Locomotor Activity Determination

Oxygen consumption and carbon dioxide production were measured in fed animals through a computer-controlled, open circuit calorimeter system LE405 Gas Analyzer (Panlab – Harvard Apparatus, Holliston, MA, USA). Animals were singly housed in clear respiratory chambers and room air was passed through chambers at a flow rate of 10 times the respective weight of each animal. The air flow within each chamber was monitored by a sensor Air Supply & Switching (Panlab – Harvard Apparatus). Gas sensors were calibrated prior to the onset of experiments with primary gas standards containing known concentrations of O_2, CO_2 and N_2 (Air Liquid, Sao Paulo, Brazil). The analyses were performed in triplicates of 6 min for each chamber.

Therefore, each animal was evaluated for 24 hours. Outdoor air reference values were sampled after every four measurements. Sample air was sequentially passed through O_2 and CO_2 sensors to determine O_2 and CO_2 content, from which measures of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. The VO_2 and VCO_2 were calculated by Metabolism® 2.2v software and expressed in $mL.g^{-1}.min^{-1}$, based on Withers equation. The spontaneous locomotor activity was evaluated over a 24 h period using a computer-controlled detection system from Panlab – Harvard Apparatus, Holliston, MA, USA.

Immunohistochemistry

Paraformaldehyde-fixed hypothalami were sectioned (5 μm). The sections were obtained from the hypothalami of five rats per group in the same localization (antero-posterior = -1.78 from bregma) and were subjected to regular single- or double-immunofluorescence staining using DAPI, anti-S1PR1, anti-POMC, anti-NPY, anti-STAT3 and anti-GFAP antibodies, according to a previously described protocol [5]. Analysis and photodocumentation of results were performed using a LSM 510 laser confocal microscope (Zeiss, Jena, Germany). The anatomical correlations were made according to the landmarks given in a stereotaxic atlas.

Dissection of the hypothalamic regions

Hypothalamic nuclei of rats were quickly dissected in a stainless steel matrix with razor blades and frozen in liquid nitrogen. Later on, each region of the hypothalamus was dissected from 1 mm thick sagittal sections of fresh brain. PVN, Arc, and VMH plus DMH were dissected from the first sections from the midline of the brain. Coordinates for each hypothalamic region are as follows; PVN: square area with anterior margin (posterior region of anterior commisure), dorsal margin (border with thalamus), ventral margin, and posterior margin (white matter separating PVN/anterior hypothalamus and VMH/DMH); VMH plus DMH: triangular area with anterior margin (white matter separating PVN/anterior hypothalamus and VMH/DMH), posterior margin (border with mammilary body), and ventral margin (border with Arc); Arc: ventral part of the medial hypothalamus with anterior and dorsal margin and posterior margin (border with mammilary body).

mRNA Isolation and Real Time PCR

Hypothalamic total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's recommendations. Total RNA was rendered genomic DNA free by digestion with Rnase-free Dnase (RQ1, Promega, Madison, WI, USA). Rats were deprived of food for 9 h for real time PCR analysis. Real time PCR and mRNA isolation were performed using a commercial kit, as follows: POMC:

Rn00595020_m1, NPY: Rn00561681_m1, GAPD, #4352338E, S1PR1: Rn.PT.56a.12493708 for rat and RPS-29 (NCBI: NM012876), sense: 59-AGGCAAGATGGGTCACCAGC-39, antisense: 59-AGTCGAATCATCCATTCAGGTCfG- 39.

Western blotting analysis and immunoprecipitation

The animals were anesthetized, and the hypothalamus, liver, heart, gastrocnemius muscle and adipose tissue were quickly removed, minced coarsely, and homogenized immediately in a freshly prepared ice-cold buffer (1% Triton X-100, 100 mmol/L Tris pH 7.4, 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L phenyl methylsulphonyl fluoride, and 0.1 mg aprotinin) suitable for preserving the phosphorylation states of enzymes. Western blotting was performed as previously described [1].

Jak2 was immunoprecipitated from rat hypothalami. Antibodies used for immunoblotting were anti-S1PR1 and anti-Jak2. Blots were exposed to preflashed Kodak XAR film. Band intensities were quantified by optical densitometry (UN-SCAN-IT gel, 6.1) of the developed autoradiographs.

SUPPLEMENTAL REFERENCES

1 Ropelle, E. R. *et al.* Reversal of diet-induced insulin resistance with a single bout of exercise in the rat: the role of PTP1B and IRS-1 serine phosphorylation. *J Physiol* **577**, 997-1007, doi:jphysiol.2006.120006 [pii] 10.1113/jphysiol.2006.120006 (2006).

2 Ropelle, E. R. *et al.* A central role for neuronal adenosine 5'-monophosphate-activated protein kinase in cancer-induced anorexia. *Endocrinology* **148**, 5220-5229, doi:en.2007-0381 [pii] 10.1210/en.2007-0381 (2007).

3 Lebedev, S. V., Blinov, D. V. & Petrov, S. V. Spatial characteristics of cisterna magna in rats and novel technique for puncture with a stereotactic manipulator. *Bull Exp Biol Med* **137**, 635-638, doi:doi [pii] (2004).

4 Lopez, M. *et al.* Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. *Cell Metab* **7**, 389-399, doi:S1550-4131(08)00077-6 [pii] 10.1016/j.cmet.2008.03.006 (2008).

5 Ropelle, E. R. *et al.* 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**, doi:10.1371/journal.pbio.1000465 (2010).

FIGURE LEGENDS

Figure 1. Characterization of S1PR1 in the hypothalamus of rats. Western blots show; (A) S1PR1 protein levels in different tissues of control rats (n=6). (B) S1PR1 protein levels in the arcuate (Arc), dorsomedial/vetromedial (DMH/VMH), paraventricular (PVN) nucleus and in the lateral hypothalamus (LH) of control rats (n=6). Bars represent the mean ± S.E.M. (C) Single and double-immunostaining was performed to evaluate the co-localization of S1PR1 (green) with POMC, NPY and STAT3 (red) in the arcuate nuclei of rats, with 50x magnification (left panel) or 200x magnification (scale bar, 20 μm). (D) Western blot shows the S1PR1 protein levels and STAT3 tyrosine phosphorylation in hypothalamic samples of rats at 12-h of fasting and after 6-h of refeeding (n=5). (E) A schematic representation showing the localization of S1P injection. S1P was injected into the VMH of rats (coordinates 2.3/3.3 mm posterior to the bregma, ± 0.6 mm lateral to midline and 10.2 mm below the surface of the skull). (F) Single-immunostaining was performed to evaluate STAT3 tyrosine phosphorylation (green) in the hypothalamus of rats 30 minutes after vehicle (left) or S1P 50 ng (right) injections in the same rat, with 100x magnification (scale bar, 20 μm).

Figure 2. Neuronal S1PR1 activation induces anorexigenic and thermogenic response. (A) Determination of food consumption after intracerebroventricular injection of S1P (2 or 50 ng) in control rats (n=8). (B) Western blots show; S1P-induced $Jak2^{tyr1007/1008}$ and $STAT3^{tyr705}$ phosphorylation in hypothalamic samples of Wistar rats 30 min after S1P injections (n=6). (C) Immunoprecipitation assay was performed to evaluate S1PR1/Jak2 association 30 min after S1P injections (n=6). (D) Oxygen consumption/carbon dioxide production after single injection of S1P into the third ventricle of control rats. (E) Locomotor activity. (F) Determination of food consumption after intracerebroventricular injection of SEW2871 (50ng) in control rats (n=6). (G) Western blot shows SEW-induced $STAT3^{tyr705}$ phosphorylation in hypothalamic samples of Wistar rats 30 min after SEW injection (n=4). (H) POMC and NPY mRNA were examined using real time PCR assay 9 hours after intracerebroventricular SEW2871 (50 ng) injection (n=8). Effects of acute intraperitoneal S1P injection (100 ng) on: (I) food intake and (J) VO_2 consumption and

VCO_2 production in C57BL6/J mice. Data were expressed by using mean ± S.E.M.* $p<0.05$, vs. vehicle and # $p<0.05$, vs. S1P 2 ng.

Figure 3. S1PR1 requires STAT3 activation to induce anorexia. (A) Western blots show leptin-induced STAT3 phosphorylation (upper panel) and S1P-induced STAT3 phosphorylation (lower panel) in control rats after ICV stimulation (n=4 in each point). (B) Representation of STAT3 phosphorylation pattern after leptin or S1P intracerebroventricular injections. (C) Determination of food consumption after intracerebroventricular injection of saline (2 μL), leptin (10^{-6} M) or S1P (50 ng) in control rats (n=10). (D) Daily food intake evaluation during 3 consecutive injections of S1P and after washout period. In parallel, pair feeding group was monitored during S1P treatment and washout period (n=5). (E) Western blot shows the effects of JSI124 (50 μM) on STAT3 phosphorylation in the hypothalamus of control rats (n=4). (F) Food intake evaluation was performed in rats injected with vehicle (2μL), S1P (50 ng) and JSI124 (50 μM) plus S1P. JSI124 injection was performed 30 minutes before S1P. (G) Western blot shows the effects of JSI124 (50 μM) on S1P-induced STAT3 phosphorylation in the hypothalamus of control rats (n=4). Data were expressed by using mean ± S.E.M.* $p<0.05$, vs. vehicle, § $p<0.05$, vs. leptin group. # $p<0.05$, vs. vehicle plus S1P.

Figure 4. STAT3 controls S1PR1 protein levels in neurons. Western blots show; (A) S1PR1 protein levels in GT1-7 cells after leptin (40 μmol/L) incubation it was performed 3 independent experiments. (B) Effect of STAT3 siRNA transfection on STAT3 protein levels in GT1-7 cells. (C) Effect of leptin (40 μmol/L) incubation on S1PR1 protein levels in GT1-7 cells transfected with STAT3 siRNA. (D) Effect of leptin on S1PR1 protein levels in the hypothalamus of control rats (n=4). Hypothalamic samples were obtained 8 and 12-h after acute leptin (10^{-6} M) injection. (E) S1PR1 protein levels in control rats injected with saline (2 μL), leptin (10^{-6} M) or JSI124 plus leptin. Hypothalamic samples were obtained 8 hours after leptin injection (n=4). (F) Evaluation of S1PR1 protein levels on the hypothalamus of *ob/ob* mice and (G) *db/db* mice and theirs wild-type littermates (C57BL6/J) (n=4). Data were expressed by using mean ± S.E.M.* $p<0.05$, vs. vehicle, # $p<0.05$, vs. wild-type.

Figure 5. Defective S1PR1 expression in the hypothalamus of obese rodents. Western blots show S1PR1 protein levels in the hypothalamus of; (A) Wistar rats (n=8), (B) C57BL6/J mice (n=4) and (C) Swiss mice (n=4) after high-fat diet treatment. (D) Hypothalamic S1PR1 mRNA levels in control and obese wistar rats (n=10). (E) Determination of S1P serum levels in control and obese C57BL6/J mice (n=6-8). (F) Determination of food consumption after intracerebroventricular injection of vehicle (DMSO), S1P (50 ng) and SEW2871 (50 ng) in obese rats (n=8). (G) Western blot shows S1P- and SEW2871-induced STAT3 ^{tyr705} phosphorylation in hypothalamic samples of obese wistar rats (n=6). (H) POMC and NPY mRNA were examined using real time PCR assay 9 hours after intracerebroventricular S1P (50 ng) injection (n=8). (I) Oxygen consumption/carbon dioxide production after single injection of S1P into the third ventricle of obese rats (n=5). (J) Locomotor activity (n=5). (K) Determination of food consumption after chronic intracerebroventricular infusion of S1P 2.08 ng/hr or vehicle 0.25 μL/hr through the osmotic mini-pump during 9 days. (L) total body weight and (M) epididymal fat pad weight after chronic delivery of S1P in the hypothalamus of obese rats (n=6). Data were expressed by using mean ± S.E.M. # $p<0.05$, vs. chow.* $p<0.05$, vs. obese plus vehicle.

Figure 6. Hyper-activation of S1PR1/STAT3 signaling in the hypothalamus during cancer-induced anorexia. (A) Daily of food intake determination in C57BL6/J mice injected with Lewis Lung Carcinoma (n=6) (B) Cumulative food intake (4 days) after the onset of anorexia in C57BL6/J mice injected with Lewis Lung Carcinoma (n=6). (C) Total body weight 4 days after onset of anorexia in mice with LLC cancer, excluding the tumor weight (n=6). (D) Western Blotting analysis was performed to evaluate hypothalamic S1PR1 protein level (upper panel) and STAT3 tyrosine phosphorylation (middle panel) in tumor-bearing mice 4 days after the onset of anorexia. (E) S1P serum levels in anorectic mice. These samples were obtained 4 days after the onset of anorexia. (F) Cerebrospinal fluid (CSF) levels in control and in anorectic mice. (G) Cumulative food intake (4 days) after the onset of anorexia in wistar rats (n=8). (H) Total body weight 4 days after onset of anorexia in wistar rats, excluding the tumor weight (n=8). (I) Western Blotting analysis was performed to evaluate hypothalamic S1PR1 protein level (upper panel) and STAT3 tyrosine phosphorylation (middle panel) in tumor-bearing rats 4 days after the onset of anorexia. (J)

CSF from control and anorectic rats were injected into the third ventricle of control wistar rats (2μL) and then the food consumption was monitored. One group received ICV injection of FTY720 (50 μM) 120 minutes before the injection of CSF from anorectic rats (n=5). (K) Effects of daily ICV FTY720 (50 μM) injection in tumor-bearing rats on: (K) daily food consumption (n=5), (L) cumulative food intake during 4 days after the onset of anorexia (n=5), (M) STAT3 tyrosine phosphorylation in the hypothalamic tissue (n=4), (N) body weight change (n=5) and (O) survival (n=10). Data were expressed by using mean ± S.E.M. *p<0.05, vs. respective control group. #p<0.05, vs. group injected with CSF from anorectic rats. § p<0.05, vs. W-256 group. The survival curves were estimated using Kaplan-Meier's estimates, and curves were compared using the log-rank test, p<0.001 (n=10).

ARTIGO 2

Exercise activates hypothalamic Jak2/STAT3 through the S1P/S1PR1 axis in obese rats.

Vagner R. R. Silva[1], Carlos K. Katashima[2], Luciene Lenhare[2], Thayana O. Micheletti[2], Gustavo D. Pimentel[2], Eloize C. Chiarreotto Ropelle[1], José Rodrigo Pauli[1], Dennys E. Cintra[1], Eduardo R. Ropelle[1-2].

1. School of Applied Science, University of Campinas (UNICAMP), Limeira, São Paulo, Brazil.
2. Department of Internal Medicine, Faculty of Medical Sciences, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil.

Running head: Hypothalamic S1PR1 in the control of energy intake

Key words: Obesity, hypothalamus, exercise, S1P, S1PR1.

Please address correspondence to:

Eduardo Rochete Ropelle
School of Applied Sciences.
University of Campinas (UNICAMP),
Limeira, São Paulo, Brazil.
Phone: + 55 - 19 37016706
Email: eduardoropelle@gmail.com

ABSTRACT

The Jak2/STAT3 signaling plays a critical role in the control of food consumption and energy expenditure in the hypothalamus. Here, we demonstrated that acute exercise induces Jak/STAT activation in the hypothalamus through the bioactive lipid molecule, sphingosine-1-phosphate (S1P). Acute exercise was able to increase Jak2 and STA3 tyrosine phosphorylation, the energy expenditure and reduced hyperphagia in rats fed on high-fat diet. In parallel we observed that exercise increased S1P levels in serum, cerebrospinal fluid (CSF) and in hypothalamic tissue of obese rats and hypothalamic sphingosine-1-phosphate receptor 1 (S1PR1) protein levels. Interestingly, intracerebroventricular (ICV) administration of cerebrospinal fluid (CSF) of exercised rats reduced the food intake in obese rats at rest. However, the disruption of hypothalamic-specific S1PR1 through the ICV administration of FTY720, blocked at least in part, the anorexigenic effects of exercise in obese rats. Taken together, our results report that physical exercise activates Jak2/STAT3signaling in hypothalamic tissue through S1P/S1PR1 axis. Overall, these results provide new insights into the mechanism by which exercise controls the anorexigenic and thermogenic signals in the central nervous system.

INTRODUCTION

The sedentary lifestyle are among the of risk most important factors that lead to an unprecedented increase in the prevalence of obesity. The prevalence of obesity is associated with various types of diseases, such as type 2 diabetes, cardiovascular disease, respiratory and cancer (1-4). The hypothalamus plays a critical role in the control of energy homeostasis, controlling feeding and energy expenditure. Hypothalamus integrates hormonal (5), and nutritional signals to control the energy balance (6-12). In this context, Jak/STAT signaling plays a fine tune in the control of anorexigenic and thermogenic signal upon hormonal stimulus (13). Several studies showed that the impairment on hypothalamic Jak/STAT signaling is associated to hyperphagia and obesity (14-16) .

On the other hand, studies indicated that activity physical is pivotal target therapeutics for treatment of obesity and type 2 diabetes (17-18). Recent studies have demonstrated that the beneficial metabolic effects of exercise are associated with the energy expenditure induction and anorexigenic effects, as observed in animal models (19-21) and in humans (22-25). It has been demonstrated that that physical exercise modulates the food consumption in obese rodents acting as a leptin mimetic in hypothalamus, inducing STAT3 activation (21, 26-30) or improving the leptin action (21, 30-32). However the mechanism by which exercise induces the leptin signaling remains unclear. In the present study we hypothesized that the bioactive lipid, sphingosine-1-phosphate (S1P) could activate Jak2/STAT3 in the hypothalamus and contributes to the anorexigenic and thermogenic exercise action.

S1P is a naturally occurring bioactive lysophospholipid that controls diverse physiological functions in a variety of cell types, binding to its cell surface receptors sphingosine 1 phosphate receptors (S1PRs) (33). Accumulating evidences demonstrated that S1P/S1PRs signaling induces Jak2/STAT3 activation in different cell types (34-36). In particular, the type 1 of these receptors, S1PR1 (also called EDG1), induces the persistent STAT3 phosphorylation through its direct physical association with Jak2, as demonstrated in tumor cells (35). However, the function of S1P/S1PR1 axis in the hypothalamus is not examined. Thus, in the present study, we examine the circulating S1P levels and

hypothalamic expression of S1PR1 in exercised animals. In parallel, we evaluate whether S1P/S1PR1 axis induces Jak2/STAT3 activation and anorexigenic signals in the hypothalamic tissue of obese rats in response of acute exercise protocol.

MATERIALS AND METHODS

Animals and diets

Male 5-wk-old Wistar rats were obtained from the University of Campinas Breeding Center. The investigation was approved by the ethics committee and followed the university guidelines for the use of animals in experimental studies and experiments conform to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication no. 85-23 revised 1996). The animals were maintained in 12h:12h artificial light-dark cycles, with lights on at 06:00 a.m., and were housed in individual cages. The animals were randomly divided into two groups: control, fed on standard rodent chow (3.948 kcal.Kg^{-1}) or high-fat diet (HFD) (5.358 kcal.Kg^{-1}) *ad libitum* for 3 months. The high-fat diet composition was previously described (37). The number of animals used in each experiment is specified in the legend figure.

Antibodies and chemicals

Anti-EDG1 (rabbit polyclonal, SC-25489), antibody was purchased from Santa Cruz Biotechnology, Inc. anti-α tubulin (rabbit polyclonal, #2144), anti-phospho-STAT3 (rabbit polyclonal, #9131), anti-phospho-Jak2 (rabbit polyclonal, #3771), were from Cell Signalling Technology (Beverly, MA, USA).

FTY720 was from Cayman Chemical Company (Michigan,USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Intracerebroventricular cannulation

After intraperitoneal injection of a mix of ketamin (10 mg) and diazepam (0.07 mg) (0.2 ml/100 g body weight), the rats were stereotaxically instrumented with a chronic 26-gauge stainless steel indwelling guide cannula a septically placed into the third ventricle at the midline coordinates of 0.5 mm posterior to the bregma and 8.5 mm below the surface of the skull of the rats using the Stoelting stereotaxic apparatus. After a 5-d recovery period, cannula placement was confirmed by a positive drinking response after administration of angiotensin II (40ng per 2 μL), and animals that did not drink 5 ml of water within 15 min after angiotensin injection were not included in the experiments.

Intracerebroventricular (ICV) injections

Rats were deprived of food for 2 h with free access to water and received 2 μl of bolus injection into the third ventricle, as follows:

S1P. For Western blot analysis, ICV S1P (50 ng) was injected into the third ventricle. To evaluate the effects of S1P on Jak/STAT signaling the hypothalamic tissue was removed 30 minutes later. To evaluate the effects of S1P on food intake in rats, ICV injection of S1P (50 ng) was performed at 6:00 p.m.

FTY720. Animals received ICV infusion of vehicle or FTY720 (50μM) 120 minutes before the swimming protocol.

Cerebrospinal fluid (CSF) injection. CSF was obtained from Wistar rats at rest or immediately after acute exercise through the introduction of a needle into the cisterna magna through the skin and/or duramater using a stereotactic micromanipulator as previously described (38) with minor modifications. Immediately after the liquor sampling, 2 (μL) of CSF were injected into the third ventricle of obese rats to evaluate the food intake during 12 hours period. CSF injections were performed at 6:00 p.m.

Acute exercise protocol

Animals were acclimated to swimming for 2 d (10 min per day).Water temperature was maintained at 32 °C. Rats performed two 3-h exercise bouts, separated by one 45-min rest period. The rats swam in groups of three in plastic barrels of 45 cm in diameter that were filled to a depth of 50 cm. This protocol was conducted between 11:00 a.m. and 6:00 p.m. After the exercise protocol rats were submitted to food consumption evaluation or sacrificed for removal of hypothalamic tissue.

Food intake determination

After acute exercise or CSF ICV infusions the food intake was determined by measuring the difference between the weight of chow given and the weight of chow at the end of a12-h period. The food consumption data were presented in Kcal.

Oxygen consumption and locomotor activity determination

Oxygen consumption/carbon dioxide production were measured in fed animals through a computer-controlled, open circuit calorimeter system LE405 Gas Analyzer (Panlab – Harvard Apparatus, Holliston, MA, USA). Animals were singly housed in clear respiratory chambers and room air was passed through chambers at a flow rate of 10 times the respective weight of each animal. The air flow within each chamber was monitored by a sensor Air Supply & Switching (Panlab – Harvard Apparatus). Gas sensors were calibrated prior to the onset of experiments with primary gas standards containing known concentrations of O2, CO2 and N2 (Air Liquid, Sao Paulo, Brazil). The analyses were performed in triplicates of 6 min for each chamber.

Therefore, each animal was evaluated for 24hr. Outdoor air reference values were sampled after every four measurements. Sample air was sequentially passed through O2 and CO2 sensors to determine O2 and CO2 content, from which measures of oxygen consumption (VO2) and carbon dioxide production (VCO2) were estimated. The VO2 and VCO2 were calculated by Metabolism® 2.2v software and expressed in $mL.g^{-1}.min^{-1}$, based on Withers equation.

Determination of insulin, leptin, lactate and S1P levels.

Immediately after acute exercise protocol, blood was collected from the cava vein. Plasma was separated by centrifugation (1,100 x g) for 15 min at 4 °C and stored at -80 °C until the assay. Leptin and insulin concentrations were determined using a commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit (Crystal Chem Inc., Chicago, IL). For serum, CSF and hypothalamic S1P determination we employed a commercially available Sphingosine 1 phosphate Assay kit (Echelon Biosciences Inc.). Blood lactate was measured using Accutrend Plus equipment (Roche); sample blood was obtained from the tails every 15 min during the exercise protocol.

mRNA Isolation and Real Time PCR

Hypothalamic total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's recommendations. Total RNA was rendered genomic DNA free by digestion with Rnase-free Dnase (RQ1, Promega, Madison, WI, USA). Three hours after the exercise protocol, hypothalamic samples were obtained for real time PCR analysis. Real time PCR and mRNA isolation were performed using a commercial kit, as follows: POMC: Rn00595020_m1, NPY: Rn00561681_m1, GAPD, #4352338E, S1PR1: Rn.PT.56a.12493708 for rat and RPS-29 (NCBI: NM012876), sense: 59-AGGCAAGATGGGTCACCAGC-39, antisense: 59-AGTCGAATCATCCATTCAGGTCfG- 39.

Western blotting analysis

The animals were anesthetized, and the hypothalamus, were quickly removed, minced coarsely, and homogenized immediately in a freshly prepared ice-cold buffer (1% Triton X-100, 100 mmol/l Tris pH 7.4, 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium vanadate, 2 mmol/l phenyl methylsulphonyl fluoride, and 0.1 mg aprotinin) suitable for preserving the phosphorylation states of enzymes. Western blotting was performed as previously described (37). Blots were

exposed to preflashed Kodak XAR film. Band intensities were quantified by optical densitometry (UN-SCAN-IT gel 6.1) of the developed autoradiographs.

Statistical analysis

All numeric results are expressed as the means ± SEM of the indicated number of experiments. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by optical densitometry (Scion Image). Statistical analysis was performed using the ANOVA test with the Bonferroni post test. Significance was established at the $p < 0.05$ level.

RESULTS

Effects of acute exercise on food consumption and energy expenditure.

Initially, we sought to evaluate the effect of exercise on food consumption in obese rats. Rats were fed on HFD for 3 mouths and then submitted to acute swimming exercise protocol. Immediately after the exercise the food intake was monitored during 12 hours. We observed that exercise reduced food intake about 25%, when compared to obese animals at rest (Figure 1A). Hypothalamic samples obtained immediately after exercise revealed that exercise increased Jak2 and STAT3 tyrosine phosphorylation (Figure 1B and C). These data were accompanied by high levels of POMC and low level of NPY mRNA in exercised group (Figure 1D). Furthermore, exercise increased the energy expenditure in the dark phase, as demonstrated by the O_2 and CO_2 analysis (Figure 1E).

This exercise protocol did not change the total body weight and epididymal fat pad weight (Figure 1F and G). Thereafter, we measured the insulin and leptin serum levels. Exercise reduced insulin but did not change leptin levels (Figure 1H). These data demonstrated that exercise induces anorexigenic and thermogenic response in obese rats. These phenomena were accompanied by hypothalamic Jak2/STAT3 activation, independently of the serum leptin variation.

Exercise increases the S1P levels.

As previously described, S1P is capable to induces its biological effects through the STAT3 signaling (34-36). We next evaluate the role of S1P on Jak2/STAT3 signaling in the hypothalamus of obese rats after acute exercise. First, we evaluated the effect of exercise of S1P levels in obese rats. Using the ELISA assay we monitored S1P levels in serum, cerebrospinal fluid (CSF) and in the hypothalamic tissue of obese rats. Exercise increased S1P levels in serum of obese rats when compared to obese group at rest (Figure 2A). Interestingly, we detected the presence of high levels of S1P in the CSF (Figure 2B) and in the hypothalamic tissue (Figure 2C) of exercised animals.

To address the effect of S1P on hypothalamic Jak2/STAT3 activation and on food intake, we performed an acute S1P ICV injection (50ng) into the third ventricle of obese

rats. ICV S1P injection increased Jak2 (Figure 2D) and STAT3 (Figure 2E) tyrosine phosphorylation in the hypothalamus of obese rats, when compared to vehicle injection. S1P injection increased POMC mRNA levels in the hypothalamus, but we did not observed the modulation of NPY mRNA levels after S1P ICV injection (Figure 2F). In addition, we monitored the food intake after S1P ICV injection. We observed that S1P promoted a strong anorexigenic effect, reducing the food intake in obese rats (Figure 2G). These data demonstrated that exercise increased S1P levels, including in the CSF and in hypothalamic tissue and that central action of S1P induced Jak2 and STAT3 activation and reduced the food consumption in obese rats.

Exercise increased S1PR1protein levels in the hypothalamus

The presence and the function of S1PR1 in hypothalamic neurons are unknown. Thus, we examined the S1PR1 expression in some hypothalamic nuclei. The combination of dissection of hypothalamic nuclei and Western blotting assay revealed high protein levels of S1PR1 in the arcuate (Arc), dorsomedial (DMH) and ventromedial (VMH) nucleus of hypothalamus in control wistar rats (Figure 3A). Thereafter, we compared the hypothalamic expression of S1PR1 among lean and obese rats. Surprising, S1PR mRNA and protein levels were reduced in hypothalamic tissue of obese animals (Figure 3B and C).

Next, we checked the impact of physical activity on hypothalamic S1PR1 protein levels in control and obese animals. In the time-course study, we observed that 4 hours after acute exercise, S1PR1 protein levels were normalized in obese rats, while, exercise did not change hypothalamic S1PR1 expression in control animals (Figure 3D).

CSF from exercised animals reduces food intake in obese rats.

We next evaluated S1P levels in CSF from control and obese rats at rest or after acute exercise. At rest, S1P levels were slightly elevated in CSF from obese rats when compared to control group, however, no statistical was observed (Figure 4A). After exercise no difference of S1P in CSF was observed among lean and obese groups (Figure 4A). To determine whether lean and obese animals were swimming in the same intensity,

we evaluated lactate production every 15 min during the exercise protocol. We did not find any difference in the lactate production between lean and obese rats (Figure 4B).

Thereafter we removed the CSF from exercised animals and injected into the third ventricle of obese rats at rest to evaluate the food intake. This strategy demonstrated that acute ICV injection of CSF from animals at rest did not change the food intake in obese rats, conversely, ICV injection of CSF from exercised animals reduced the food consumption in obese rats (Figure 4C), no difference was observed in the total body weight 12 hours after ICV CSF injection (Figure 4D). This data suggests that the presence of S1P in CSF in exercised obese animals is related to reduction of food intake in obese.

Pharmacological S1PR1 inhibition blunts the effects of exercise in hypothalamus.

Although we used ICV injection of exogenous S1P to evaluate the effect of this sphingolipid on food intake in obese rats, the dose of S1P used (50 ng) is relatively high and this approach does not reflect the same physiological conditions observed after exercise. Thus, we hypothesized that if exercise requires hypothalamic S1P/S1PR1 axis to reduce food intake, inhibiting the hypothalamic S1PR1 expression, under physiological conditions, should diminish the appetite suppressive action mediated by S1P in exercised obese rats. To address this hypothesis, we developed an experimental strategy aimed at antagonizing the central action of S1PR1 in the presence of a systemic elevation in S1P concentration after physical exercise. For this, we performed an acute ICV S1PR1 inhibitor, FTY720, 120 minutes before the exercise protocol. FTY720 is an immunosuppressant compound known to modulate the immune system by acting as a functional antagonist of S1PR1 and inducing its internalization and degradation (39-40). First, we observed that ICV FTY720 injection reduced the hypothalamic S1PR1 protein levels in rats at rest and in exercised animals (Figure 5A). In addition, FTY720 prevented exercise-induced Jak2 (Figure 5B) and STAT3 (Figure 5C) tyrosine phosphorylation in the hypothalamus. We observed that FTY720 ICV injection did not change the lactate and S1P production during exercise (data not shown). Interestingly, the reduction of S1PR1 protein levels induced by FTY720, blocked the anorexigenic effects mediated by exercise (Figure 5D), without

change the total body weight (Figure 5E). Taken together, these data demonstrated that exercise requires the hypothalamic S1PR1 expression to induce the anorexigenic response.

DISCUSSION

In the present study we investigated the role of S1P/S1PR1 axis in the hypothalamus of obese rats after acute exercise. Our data revealed that swimming exercise increased the circulating S1P levels in obese rats. We observed that this sphingolipid is capable to activate Jak2/STAT3 and induce anorexigenic signals in the hypothalamus through its receptor S1PR1. We found that the S1PR1 protein is highly enriched in the mediobasal hypothalamus of rats, and that HFD diminished hypothalamic S1PR1 protein levels. However, physical exercise normalized hypothalamic S1PR1 protein levels. Finally, we demonstrated that exercise requires S1PR1 protein levels in the hypothalamus to reduce the food intake in obese animals.

The effect of exercise in the control of food intake is a complex phenomenon and depends on many circumstances, amongst them, intensity, volume, type of exercise and mainly the subjects enrolled in the exercise program. Recent studies have demonstrated that exercise has an appetite suppressive action in overweight and obese subjects (22-23, 25, 41), including in adolescents and post-menopausal women (42). However, the mechanism by which exercise modulates the energy intake in unclear. Accumulating studies have demonstrated that exercise induces the secretion of muscle-derived biomolecules, including interleukins (43), apelin (44) , protein acidic and rich in cysteine (SPARC) (45) and Irisin (46) promoting the beneficial effects of physical exercise. Using an animal models, we previously demonstrated that single bout of moderate exercise improves insulin and leptin action in the hypothalamus of lean (31) and obese rats (8, 21, 30) reducing the energy intake in an Interleukin-6 (IL-6) dependent-manner, suggesting that exercise mediates the peripheral and central integration through the specialized molecules. In the present study we described that a class of sphingolipid, S1P, could acts in the central nervous system as a signaling molecule in response of exercise.

The S1P synthesis occurs by phosphorylation of sphingosine, in a reaction catalysed by sphingosine kinases, SPHK1 and SPHK2. S1P mediates its biological effects through the paracrine or autocrine mechanism (33). Under pathological condition, Liang and colleagues showed that aberrant S1P levels is produced by upregulation of sphingosine kinase 1 (SPHK1) in cancer cells, contributing to the inflammation and the cancer colon

progression (36). In addition, alteration of S1P synthesis was reported under obesity condition, whereas high levels of serum S1P were found in obesity animal models (47-48) and in obese subjects (48). In the present study we found high levels of S1P in the plasma, liquor and in hypothalamic tissue of exercise rats, however, the sphingosine kinases activity in the metabolic tissues in the obesity state or in response of exercise is unknown, thus, the source of S1P synthesis both situations deserves further investigations.

Our results demonstrates that systemic S1P elevation was associate with anorexigenic and thermogenic effects mediated by physical exercise, on the other hand, high levels of S1P were found in hyperphagic mice, *ob/ob* (47-48). This apparent paradox could be explained by the strong downregulation of S1PR1 expression observed in the hypothalamus of obese rats, when compared to lean group. Probably, the low S1PR1 protein levels in the hypothalamus of obese animal is related to the impairment of hypothalamic STAT3 signaling (49-50) , once STAT3 is a direct transcriptional activator of *S1pr1* promoter, as demonstrated in NIH-3T3 cells (35). Interestingly, STAT3 induces S1PR1 expression, as well as S1P/S1PR1 axis, is important for persistent STAT3 phosphorylation, playing a positive feedback circuitry (35) . Thus, the upregulation of S1P levels in the hypothalamus of exercise animals seems sufficient to activate S1PR1/Jak2/STAT3 cascade and normalize the S1PR1 protein levels in hypothalamic tissue of obese animals. This mechanism could help to reorganize the set point of nutritional balance and therefore aid in counteracting the energy imbalance induced by overnutrition in hypothalamic neurons.

Sphingosine-1-phosphate receptors (S1PRs) family is composed by five different receptors (1-5) that activate diverse downstream signaling pathways in response to S1P (33). However, the expression and functions of these receptors in hypothalamic neurons is not documented. In preliminary experiment we observed higher S1PR1 protein levels in the hypothalamus, when compared to several peripheral tissues, such as, gastrocnemius muscle, adipose tissue, liver and heart (data not shown). After this observation, we performed the micro dissection of hypothalamic nuclei to determine the specific S1PR1 localization. This technique revealed that S1PR1 is expressed predominantly in the arcuate and ventromedial/dorsomedial nuclei in the hypothalamus. Interestingly, these nuclei are considered the main hypothalamic region that controls the energy homeostasis, through the

balance of anorexigenic and orexigenic neuropeptides production (12). Our result demonstrated that acute S1P ICV administration increased POMC mRNA without change NPY mRNA levels, this result suggests that S1PR1 induces anorexigenic effects through the POMC elevation, once STAT3 controls POMC expression (13) and that, probably, S1PR1 is expressed in POMC neurons.

AUTHOR CONTRIBUTIONS

V.R.R.S. researched data contributed to discussion and reviewed/edited manuscript. C.K.K. researched data. L.L. researched data. T.O.M. researched data. G.D.P. researched data. E.C.C.R. researched data. J.R.P. contributed to discussion and reviewed/edited manuscript. D.E.C. researched data and contributed to discussion and reviewed/edited manuscript. E.R.R. wrote the manuscript, contributed to discussion and reviewed/edited manuscript.

ACKNOWLEDGMENTS

This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP- 2011/13828-1), Fundação de Apoio ao Ensino, Pesquisa e Extensão (FAEPEX –N°519.292) and Conselho Nacional de desenvolvimento científico e tecnológico (CNPq).

REFERENCES

1. Farooqui AA, Farooqui T, Panza F,Frisardi V, Metabolic syndrome as a risk factor for neurological disorders. Cell Mol Life Sci 2012;69:(5):741-62
2. Ramachandrappa S,Farooqi IS, Genetic approaches to understanding human obesity. J Clin Invest 2011;121:(6):2080-6
3. Kahn SE, Hull RL,Utzschneider KM, Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006;444:(7121):840-6
4. Kopelman PG, Obesity as a medical problem. Nature 2000;404:(6778):635-43
5. Zhang Y, Zhou J, Corll C, Porter JR, Martin RJ,Roane DS, Evidence for hypothalamic K+(ATP) channels in the modulation of glucose homeostasis. Eur J Pharmacol 2004;492:(1):71-9
6. Lopez M, Nogueiras R, Tena-Sempere M,Dieguez C, Orexins (hypocretins) actions on the GHRH/somatostatin-GH axis. Acta Physiol (Oxf) 2010;198:(3):325-34
7. Lam TK, Gutierrez-Juarez R, Pocai A, Bhanot S, Tso P, Schwartz GJ,Rossetti L, Brain glucose metabolism controls the hepatic secretion of triglyceride-rich lipoproteins. Nat Med 2007;13:(2):171-80
8. Ropelle ER, Pauli JR, Fernandes MF, Rocco SA, Marin RM, Morari J, Souza KK, Dias MM, Gomes-Marcondes MC, Gontijo JA, Franchini KG, Velloso LA, Saad MJ,Carvalheira JB, A central role for neuronal AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) in high-protein diet-induced weight loss. Diabetes 2008;57:(3):594-605
9. Dagon Y, Hur E, Zheng B, Wellenstein K, Cantley LC,Kahn BB, p70S6 kinase phosphorylates AMPK on serine 491 to mediate leptin's effect on food intake. Cell Metab 2012;16:(1):104-12
10. Kahn BB, Alquier T, Carling D,Hardie DG, AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 2005;1:(1):15-25
11. Coll AP, Farooqi IS,O'Rahilly S, The hormonal control of food intake. Cell 2007;129:(2):251-62
12. Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ,Baskin DG, Central nervous system control of food intake. Nature 2000;404:(6778):661-71
13. Munzberg H, Huo L, Nillni EA, Hollenberg AN,Bjorbaek C, Role of signal transducer and activator of transcription 3 in regulation of hypothalamic proopiomelanocortin gene expression by leptin. Endocrinology 2003;144:(5):2121-31
14. Thaler JP, Yi CX, Schur EA, Guyenet SJ, Hwang BH, Dietrich MO, Zhao X, Sarruf DA, Izgur V, Maravilla KR, Nguyen HT, Fischer JD, Matsen ME, Wisse BE, Morton GJ, Horvath TL, Baskin DG, Tschop MH,Schwartz MW, Obesity is associated with hypothalamic injury in rodents and humans. J Clin Invest 2012;122:(1):153-62
15. Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE, Tsukumo DM, Anhe G, Amaral ME, Takahashi HK, Curi R, Oliveira HC, Carvalheira JB, Bordin S, Saad MJ,Velloso LA, Saturated fatty acids produce an inflammatory response

predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. J Neurosci 2009;29:(2):359-70
16. Munzberg H, Flier JS,Bjorbaek C, Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. Endocrinology 2004;145:(11):4880-9
17. Egan B,Zierath JR, Exercise metabolism and the molecular regulation of skeletal muscle adaptation. Cell Metab 2013;17:(2):162-84
18. Roberts CK, Little JP,Thyfault JP, Modification of insulin sensitivity and glycemic control by activity and exercise. Med Sci Sports Exerc 2013;45:(10):1868-77
19. Bi S, Scott KA, Hyun J, Ladenheim EE,Moran TH, Running wheel activity prevents hyperphagia and obesity in Otsuka long-evans Tokushima Fatty rats: role of hypothalamic signaling. Endocrinology 2005;146:(4):1676-85
20. Mainardi M, Scabia G, Vottari T, Santini F, Pinchera A, Maffei L, Pizzorusso T,Maffei M, A sensitive period for environmental regulation of eating behavior and leptin sensitivity. Proc Natl Acad Sci U S A 2010;107:(38):16673-8
21. Ropelle ER, Flores MB, Cintra DE, Rocha GZ, Pauli JR, Morari J, de Souza CT, Moraes JC, Prada PO, Guadagnini D, Marin RM, Oliveira AG, Augusto TM, Carvalho HF, Velloso LA, Saad MJ,Carvalheira JB, IL-6 and IL-10 anti-inflammatory activity links exercise to hypothalamic insulin and leptin sensitivity through IKKbeta and ER stress inhibition. PLoS Biol 2010;8:(8):
22. Hagobian TA, Yamashiro M, Hinkel-Lipsker J, Streder K, Evero N,Hackney T, Effects of acute exercise on appetite hormones and ad libitum energy intake in men and women. Appl Physiol Nutr Metab 2013;38:(1):66-72
23. Schubert MM, Desbrow B, Sabapathy S,Leveritt M, Acute exercise and subsequent energy intake. A meta-analysis. Appetite 2013;63:(92-104
24. Thivel D, Isacco L, Taillardat M, Rousset S, Boirie Y, Morio B,Duche P, Gender effect on exercise-induced energy intake modification among obese adolescents. Appetite 2011;56:(3):658-61
25. Thivel D, Isacco L, Montaurier C, Boirie Y, Duche P,Morio B, The 24-h energy intake of obese adolescents is spontaneously reduced after intensive exercise: a randomized controlled trial in calorimetric chambers. PLoS One 2012;7:(1):e29840
26. Zhao JB, Zhang Y, Li GZ, Su XF,Hang CH, Activation of JAK2/STAT pathway in cerebral cortex after experimental traumatic brain injury of rats. Neurosci Lett 2011;498:(2):147-52
27. Shapiro A, Cheng KY, Gao Y, Seo DO, Anton S, Carter CS, Zhang Y, Tumer N,Scarpace PJ, The act of voluntary wheel running reverses dietary hyperphagia and increases leptin signaling in ventral tegmental area of aged obese rats. Gerontology 2011;57:(4):335-42
28. Patterson CM, Bouret SG, Dunn-Meynell AA,Levin BE, Three weeks of postweaning exercise in DIO rats produces prolonged increases in central leptin sensitivity and signaling. Am J Physiol Regul Integr Comp Physiol 2009;296:(3):R537-48
29. Park S, Jang JS, Jun DW,Hong SM, Exercise enhances insulin and leptin signaling in the cerebral cortex and hypothalamus during dexamethasone-induced stress in diabetic rats. Neuroendocrinology 2005;82:(5-6):282-93
30. Chiarreotto-Ropelle EC, Pauli LS, Katashima CK, Pimentel GD, Picardi PK, Silva VR, de Souza CT, Prada PO, Cintra DE, Carvalheira JB, Ropelle ER,Pauli JR,

Acute exercise suppresses hypothalamic PTP1B protein level and improves insulin and leptin signaling in obese rats. Am J Physiol Endocrinol Metab 2013;305:(5):E649-59
31. Flores MB, Fernandes MF, Ropelle ER, Faria MC, Ueno M, Velloso LA, Saad MJ,Carvalheira JB, Exercise improves insulin and leptin sensitivity in hypothalamus of Wistar rats. Diabetes 2006;55:(9):2554-61
32. Krawczewski Carhuatanta KA, Demuro G, Tschop MH, Pfluger PT, Benoit SC,Obici S, Voluntary exercise improves high-fat diet-induced leptin resistance independent of adiposity. Endocrinology 2011;152:(7):2655-64
33. Spiegel S,Milstien S, The outs and the ins of sphingosine-1-phosphate in immunity. Nat Rev Immunol 2011;11:(6):403-15
34. Frias MA, James RW, Gerber-Wicht C,Lang U, Native and reconstituted HDL activate Stat3 in ventricular cardiomyocytes via ERK1/2: role of sphingosine-1-phosphate. Cardiovasc Res 2009;82:(2):313-23
35. Lee H, Deng J, Kujawski M, Yang C, Liu Y, Herrmann A, Kortylewski M, Horne D, Somlo G, Forman S, Jove R,Yu H, STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors. Nat Med 2010;16:(12):1421-8
36. Liang J, Nagahashi M, Kim EY, Harikumar KB, Yamada A, Huang WC, Hait NC, Allegood JC, Price MM, Avni D, Takabe K, Kordula T, Milstien S,Spiegel S, Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer. Cancer Cell 2013;23:(1):107-20
37. Ropelle ER, Pauli JR, Prada PO, de Souza CT, Picardi PK, Faria MC, Cintra DE, Fernandes MF, Flores MB, Velloso LA, Saad MJ,Carvalheira JB, Reversal of diet-induced insulin resistance with a single bout of exercise in the rat: the role of PTP1B and IRS-1 serine phosphorylation. J Physiol 2006;577:(Pt 3):997-1007
38. Lebedev SV, Blinov DV,Petrov SV, Spatial characteristics of cisterna magna in rats and novel technique for puncture with a stereotactic manipulator. Bull Exp Biol Med 2004;137:(6):635-8
39. Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S,Burtin P, Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat Rev Drug Discov 2010;9:(11):883-97
40. Graler MH,Goetzl EJ, The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. FASEB J 2004;18:(3):551-3
41. Holmstrup ME, Fairchild TJ, Keslacy S, Weinstock RS,Kanaley JA, Satiety, but not total PYY, Is increased with continuous and intermittent exercise. Obesity (Silver Spring) 2013;
42. Borer KT, Wuorinen E, Ku K,Burant C, Appetite responds to changes in meal content, whereas ghrelin, leptin, and insulin track changes in energy availability. J Clin Endocrinol Metab 2009;94:(7):2290-8
43. Pedersen BK,Febbraio MA, Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat Rev Endocrinol 2012;8:(8):457-65
44. Besse-Patin A, Montastier E, Vinel C, Castan-Laurell I, Louche K, Dray C, Daviaud D, Mir L, Marques MA, Thalamas C, Valet P, Langin D, Moro C,Viguerie N, Effect of endurance training on skeletal muscle myokine expression in obese men: identification of apelin as a novel myokine. Int J Obes (Lond) 2013;

45. Aoi W, Naito Y, Takagi T, Tanimura Y, Takanami Y, Kawai Y, Sakuma K, Hang LP, Mizushima K, Hirai Y, Koyama R, Wada S, Higashi A, Kokura S, Ichikawa H,Yoshikawa T, A novel myokine, secreted protein acidic and rich in cysteine (SPARC), suppresses colon tumorigenesis via regular exercise. Gut 2013;62:(6):882-9
46. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, Rasbach KA, Bostrom EA, Choi JH, Long JZ, Kajimura S, Zingaretti MC, Vind BF, Tu H, Cinti S, Hojlund K, Gygi SP,Spiegelman BM, A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 2012;481:(7382):463-8
47. Samad F, Hester KD, Yang G, Hannun YA,Bielawski J, Altered adipose and plasma sphingolipid metabolism in obesity: a potential mechanism for cardiovascular and metabolic risk. Diabetes 2006;55:(9):2579-87
48. Kowalski GM, Carey AL, Selathurai A, Kingwell BA,Bruce CR, Plasma sphingosine-1-phosphate is elevated in obesity. PLoS One 2013;8:(9):e72449
49. El-Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C,Flier JS, Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. J Clin Invest 2000;105:(12):1827-32
50. Bence KK, Delibegovic M, Xue B, Gorgun CZ, Hotamisligil GS, Neel BG,Kahn BB, Neuronal PTP1B regulates body weight, adiposity and leptin action. Nat Med 2006;12:(8):917-24

Figure 1

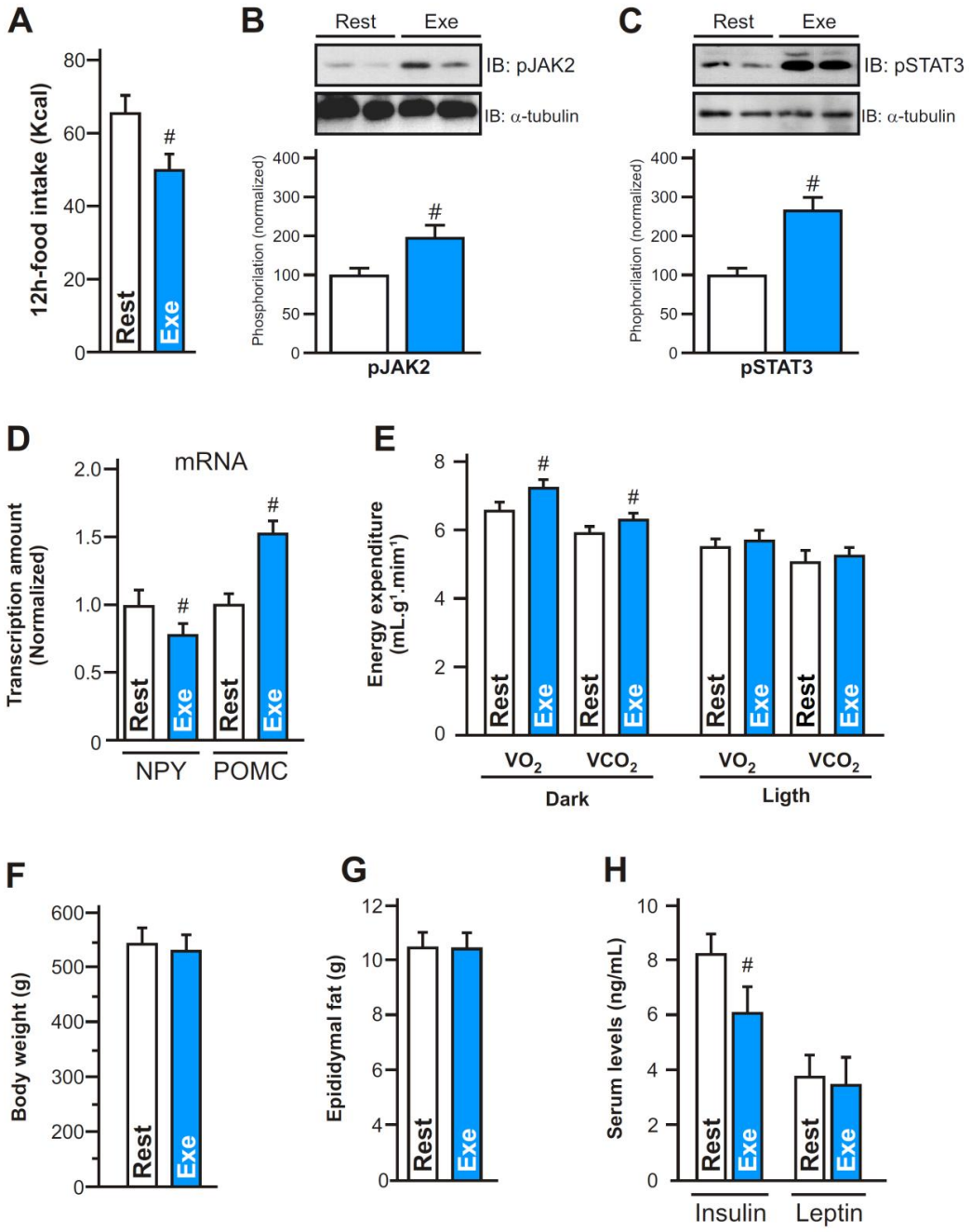

A
12h-food intake (Kcal)
80
60
40
20
0
Rest
Exe
#
B
Rest
Exe
IB: pJAK2
IB: α-tubulin
Phosphorilation (normalized)
400
300
200
100
50
0
#
pJAK2
C
Rest
Exe
IB: pSTAT3
IB: α-tubulin
Phophorilation (normalized)
400
300
200
100
50
0
#
pSTAT3
D
mRNA
Transcription amount (Normalized)
2.0
1.5
1.0
0.5
0
Rest
Exe
Rest
Exe
#
#
NPY
POMC
E
Energy expenditure (mL.g-1.mim-1)
8
6
4
2
0
#
#
Rest
Exe
Rest
Exe
Rest
Exe
Rest
Exe
VO2
VCO2
VO2
VCO2
Dark
Ligth
F
Body weight (g)
600
500
400
300
200
0
Rest
Exe
G
Epididymal fat (g)
12
10
8
6
4
0
Rest
Exe
H
Serum levels (ng/mL)
10
8
6
4
2
0
#
Rest
Exe
Rest
Exe
Insulin
Leptin

Figure 2

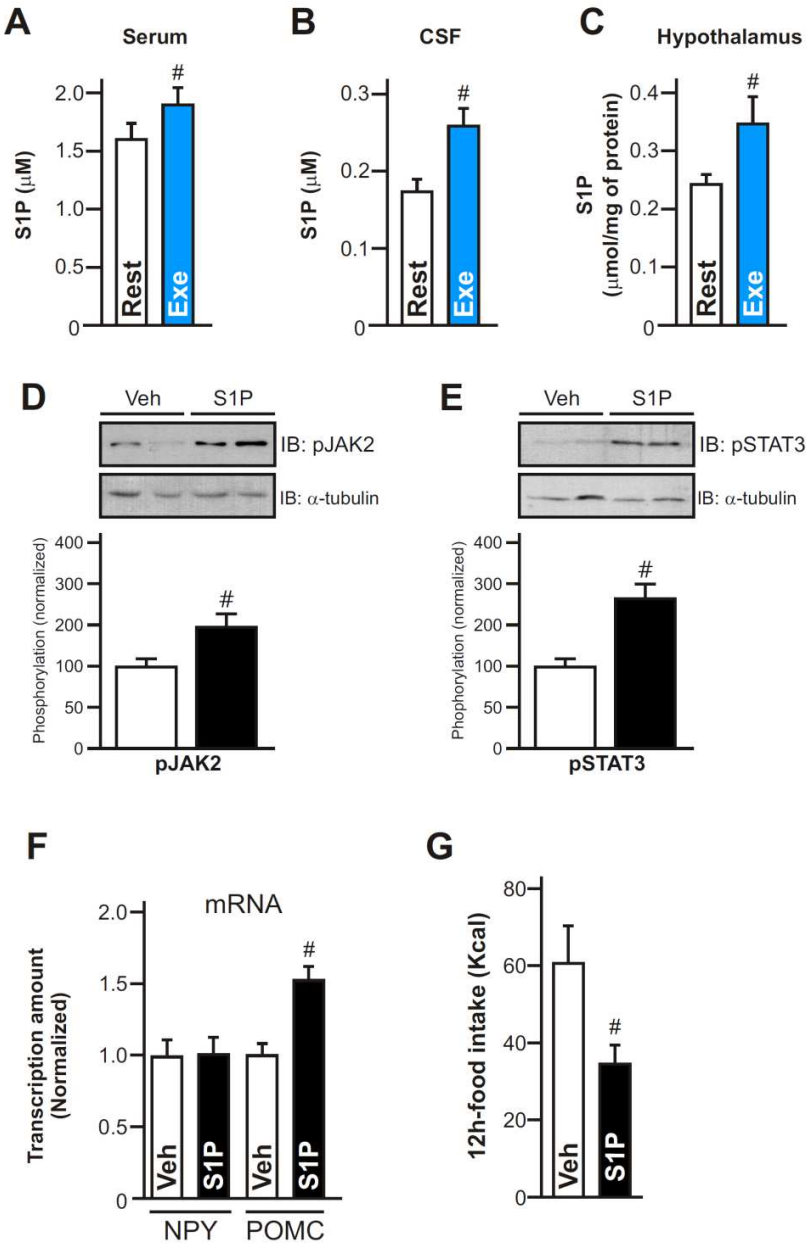

A
Serum
S1P (μM)
2.0
1.5
1.0
0.5
0
Rest
Exe
#
B
CSF
S1P (μM)
0.3
0.2
0.1
0
Rest
Exe
#
C
Hypothalamus
S1P (μmol/mg of protein)
0.4
0.3
0.2
0.1
0
Rest
Exe
#
D
Veh
S1P
IB: pJAK2
IB: α-tubulin
Phosphorylation (normalized)
400
300
200
100
50
0
#
pJAK2
E
Veh
S1P
IB: pSTAT3
IB: α-tubulin
Phophorylation (normalized)
400
300
200
100
50
0
#
pSTAT3
F
mRNA
Transcription amount (Normalized)
2.0
1.5
1.0
0.5
0
Veh
S1P
Veh
S1P
#
NPY
POMC
G
12h-food intake (Kcal)
80
60
40
20
0
Veh
S1P
#

Figure 3

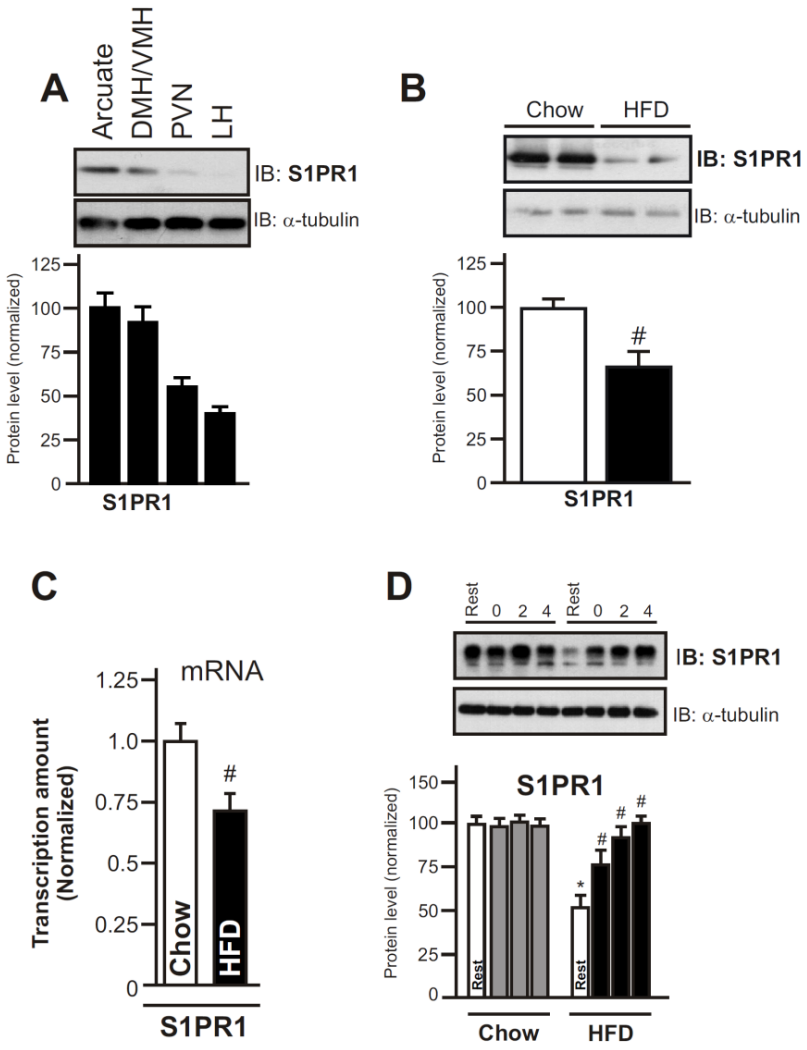

A
Arcuate
DMH/VMH
PVN
LH
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
125
100
75
50
25
0
S1PR1
B
Chow
HFD
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
125
100
75
50
25
0
#
S1PR1
C
mRNA
Transcription amount (Normalized)
1.25
1.0
0.75
0.5
0.25
0
Chow
HFD
#
S1PR1
D
Rest
0
2
4
Rest
0
2
4
IB: S1PR1
IB: α-tubulin
S1PR1
Protein level (normalized)
150
100
75
50
25
0
Rest
Rest
*
#
#
#
Chow
HFD

Figure 4

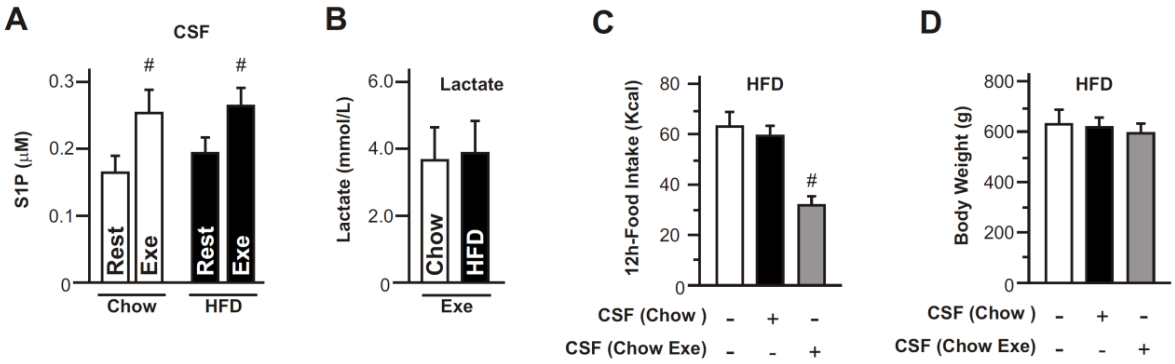

A
CSF
S1P (μM)
0.3
0.2
0.1
0
#
#
Rest
Exe
Rest
Exe
Chow
HFD
B
Lactate
Lactate (mmol/L)
6.0
4.0
2.0
0
Chow
HFD
Exe
C
HFD
12h-Food Intake (Kcal)
80
60
40
20
0
#
CSF (Chow) - + -
CSF (Chow Exe) - - +
D
HFD
Body Weight (g)
800
600
400
200
0
CSF (Chow) - + -
CSF (Chow Exe) - - +

Figure 5

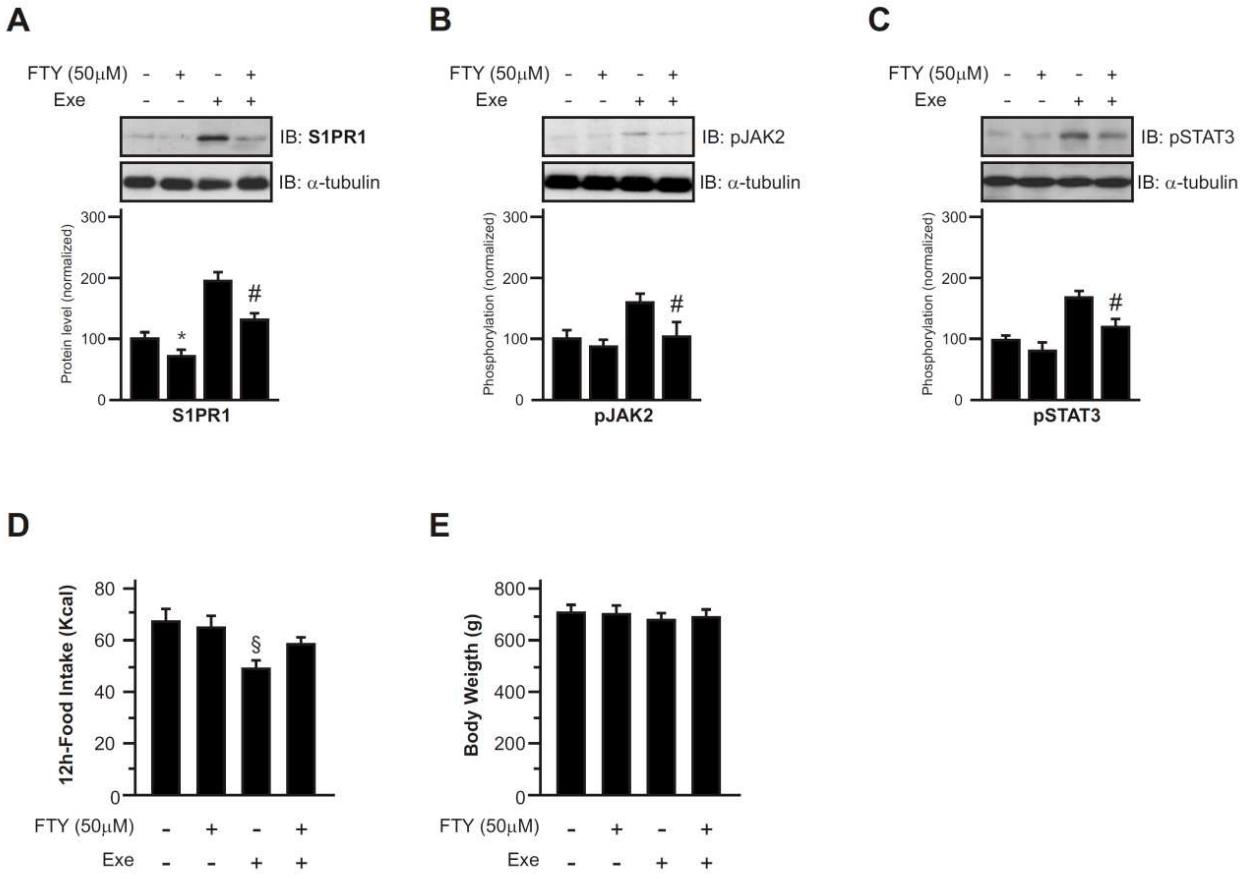

A
FTY (50μM) - + - +
Exe - - + +
IB: S1PR1
IB: α-tubulin
Protein level (normalized)
300
200
100
0
*
#
S1PR1
B
FTY (50μM) - + - +
Exe - - + +
IB: pJAK2
IB: α-tubulin
Phosphorylation (normalized)
300
200
100
0
#
pJAK2
C
FTY (50μM) - + - +
Exe - - + +
IB: pSTAT3
IB: α-tubulin
Phosphorylation (normalized)
300
200
100
0
#
pSTAT3
D
12h-Food Intake (Kcal)
80
60
40
20
0
§
FTY (50μM) - + - +
Exe - - + +
E
Body Weigth (g)
800
600
400
200
0
FTY (50μM) - + - +
Exe - - + +

FIGURE LEGENDS

Figure 1. Acute exercise reduces food consumption and energy expenditure in obese rats. (A) 12h of food intake (Kcal) (n=8-10 per group). Western blots show; (B and C) JAK2 tyrosine phosphorylation and STAT3 tyrosine phosphorylation (n=6 per group). Rats were fasted during 9h and the hypothalamic levels (D) NPY and POMC mRNA were examined using real time PCR assay. The animals remain in fasted during 9h after the exercise for analysis hypothalamic levels. (n= 6 per group). (E) VO_2 consumption and VCO_2 production in period dark and light. (F) Body weight and (G) Epididymal fat pad weight. (H) Serum level of Insulin and leptin (n= 8-10 per group). Data were expressed by using mean ± S.E.M. # $p<0.05$, vs. Rest.

Figure 2. Increasing level of S1P in obese rats exercised. S1P level, (A) serum (B) cerebrospinal fluid (CSF) and (C) hypothalamus tissue in obese rats exercised and in rest. (D)Western blots show; S1P-induced $Jak2^{tyr1007/1008}$ and (E) $STAT3^{tyr705}$ phosphorylation in hypothalamic samples of Wistar rats 30 min after S1P injections (50ng) (n=4).(F) NPY and POMC mRNA were examined using real time PCR assay 9 hours after intracerebroventricular S1P (50 ng) injection (n=6-8 per group). (G) 12h food intake (Kcal) (n= 6). Data were expressed by using mean ± S.E.M. # $p<0.05$, vs. Rest. # $p<0.05$, vs. vehicle.

Figure 3. Exercise increased S1PR1 level in the hypothalamus of obese rats. (A) S1PR1 protein levels in the arcuate (Arc), dorsomedial/vetromedial (DMH/VMH), paraventricular (PVN) nucleus and in the lateral hypothalamus (LH) of control rats (n=6). Bars represent the mean ± S.E.M. (B) Western blots show S1PR1 protein levels in the hypothalamus in control and obese wistar (n=5).(C) Hypothalamic S1PR1 mRNA levels in control and obese wistar rats (n=6-8). (D) Western blot show S1PR1 protein level in hypothalamus lean rats exercised and obese rats exercised and respective control. Data were expressed by using mean ± S.E.M. # $p<0.05$, vs. respective controls. # $p<0.05$, vs *.

Figure 4. CSF reduces food intake in obese rats. (A) S1P level. Cerebrospinal fluid (CSF) levels in control and in obese rats exercised and your respective controls. (B) Lactate (mmol/L) (C) Determination of 12h food intake (Kcal) after intracerebroventricular injection of vehicle (saline) or CSF(2µl) of control rats exercised in obese rats (n=4-6 per group) and (D) Body weight. Data were expressed by using mean ± S.E.M. # $p<0.05$, vs. others groups.

Figure 5. Pharmacological S1PR1 inhibitor blocks the effects of exercise in hypothalamus of obese rats. (A) Western blots show; S1PR1 protein level and (B) $Jak2^{tyr1007/1008}$ and (C) $STAT3^{tyr705}$ phosphorylation in hypothalamic (D) 12h food intake (Kcal) and (E) Body weight. The animals received injection of vehicle (DMSO) or FTY720 (50µl) 120 minutes before of exercise protocol. Data were expressed by using mean ± S.E.M. # $p<0.05$, vs. FTY720*. # $p<0.05$, vs. others groups. § $p<0.05$, vs. others groups.

DISCUSSÃO (ARTIGO 1)

A ingestão alimentar e o gasto energético são regulados por neurônios específicos localizados no hipotálamo. O processo inflamatório subclínico observado em modelos experimentais de obesidade está diretamente associado a distintos mecanismos de resistência à leptina e da sua via de sinalização anorexigênica Jak/STAT no hipotálamo, culminando no aumento da ingestão alimentar e no ganho do peso corporal (Carvalheira et al., 2003; De Souza et al., 2005; El-Haschimi et al., 2000; Milanski et al., 2009; Zhang et al., 2008). Paralelamente a isso, a anorexia através da indução de tumor, está também associada à ativação da via anorexigênica de maneira crônica, levando a um estado de caquexia. Esse estado anoréxico se deve parcialmente pela ativação de citoquinas inflamatórias, capazes de ativar a via anorexigênica Jak/STAT demasiadamente (Laviano et al., 2003). Tomados em conjuntos, entender os mecanismos e os meios de controle da sinalização da via Jak/STAT no controle da ingestão alimentar e do gasto energético vem ganhando destaque no âmbito cientifico nos últimos anos, seja como alvo terapêutico da obesidade ou como alvo no tratamento da anorexia induzida pelo câncer (Febbraio, 2007; Laviano et al., 2003; Lee et al., 2010; Liang et al., 2013; Ropelle et al., 2010).

Recentemente alguns estudos têm relatado a proteína S1PR1 como um importante modulador na via de sinalização Jak2/STAT3 em células tumorais através do eixo S1P/S1PR1 (Lee et al., 2010; Liang et al., 2013; Liu et al., 2012). No entanto, até o presente momento pouco se sabe a respeito do papel do eixo S1P/S1PR1 hipotalâmico no controle anorexigênico. Em nosso estudo, observamos que o eixo S1P/S1PR1 parece estar relacionado com a anorexia, por outro lado, a falha do eixo S1P/S1PR1 hipotalâmico em mamíferos parece ter uma estreita relação no controle do peso corporal e da ingestão alimentar.

Neste estudo mostramos que o eixo neuronal S1P/S1PR1/STAT3 desempenha um importante papel no controle da homeostase energética de mamíferos. No primeiro estudo avaliamos a expressão do receptor S1PR1 em diferentes tecidos (músculo, coração, tecido adiposo, rins e hipotálamo) e observamos que o hipotálamo expressa o receptor S1PR1 de maneira abundante, quando comparado aos demais tecidos e que sua expressão é

dependente do estado nutricional. A partir disso, avaliamos o tratamento intracerebroventricular (ICV) com S1P, o principal ligante do receptor S1PR1, e observamos que, após o estímulo com S1P, o S1PR1 foi capaz de interagir com a proteína Jak2 levando a ativação persistente da proteína STAT3 no hipotálamo de roedores aumentando o gasto energético e reduzindo a ingestão alimentar.

Mostramos também que a proteína STAT3 exerce efeitos recíprocos, controlando a expressão do receptor S1PR1 em células hipotalâmicas *in vivo* e *in vitro.* Nosso estudo identificou que a resistência central a leptina está associada com baixos níveis do receptor S1PR1 no hipotálamo de animais obesos, por outro lado, a ativação crônica do S1PR1 no hipotálamo reduziu a ingestão alimentar e aumentou o gasto energético em roedores obesos. De maneira interessante encontramos elevados níveis da proteína S1PR1 e da proteína STAT3 no hipotálamo de diferentes modelos de anorexia induzidos por tumor, e alto nível sistêmico de S1P.

A esfingosina 1 fosfato (S1P), é composta por uma família de cinco receptores (1-5) acoplados á proteína G, capazes de ativar diferentes vias de sinalizações celulares (Lepine et al., 2011). O receptor S1PR1 vem ganhando destaque por estar envolvido em diversas funções fisiológicas, como inflamação, migração celular e carcinogênese (Camerer et al., 2009; Konig et al., 2010; Lee et al., 2010; Liang et al., 2013; Schulte et al., 2001; Yamaguchi et al., 2003; Yoshida et al., 2010). Alguns estudos encontraram receptores de S1P no sistema nervoso central, no entanto a presença e função do receptor S1PR1 especificamente no hipotálamo não foi documentado até o presente momento (Baudhuin et al., 2004; Guo et al., 2013). De maneira interessante, nosso estudo mostrou que os núcleos hipotalâmicos são altamente enriquecidos da proteína S1PR1 comparados aos demais tecidos periféricos. Encontramos alto nível de S1PR1 em neurônios POMC e baixos níveis em neurônios NPY. Observamos que o receptor S1PR1 é modulado pelo estado nutricional no hipotálamo, dados similares à proteína STAT3, sugerindo a participação do receptor S1PR1 no controle da homeostase energética em conjunto com a STAT3.

Outros receptores da família S1PRs possuem capacidade de ativar a via de sinalização Jak2/STAT3. Por exemplo, em células progenitoras endoteliais foi demonstrado que a via

de sinalização Jak2/STAT3 é ativada de maneira robusta através do receptor S1PR3 (Walter et al., 2007), por outro lado, em cardiomiocitos, a ativação da via Jak2/STAT3 ocorre através do receptor S1PR2 (Frias et al., 2009). Nosso estudo revelou que a injeção intracerebroventricular (ICV) de S1P foi suficiente para ativar a via Jak2/STAT3 no hipotálamo, induzindo a sinalização anorexigênica em ratos. Apesar de não avaliar a presença e funções das outras subunidades de receptores S1PRs no hipotálamo, nossos dados mostraram que o ativador específico do receptor S1PR1, a saber, o agonista SEW2871, promoveu ativação da proteína STAT3 no hipotálamo, reduzindo a ingestão alimentar. Em contrapartida, a injeção ICV de S1P não alterou a fosforilação da via de insulina IRS1 e AKT, sugerindo que os efeitos da ingestão alimentar sejam mediados pela via da leptina e não pela via da insulina. Esses dados corroboram com Lee e colaboradores, mostrado em células tumorais (Lee et al., 2010) .O tratamento com leptina *in vivo* e *in vitro* aumentou os níveis proteicos do S1PR1 em células neuronais GT1-7 e no hipotálamo de ratos. De maneira interessante observamos que animais deficientes de leptina (*ob/ob*) ou deficientes do receptor de leptina (*db/db*), possuem baixos níveis proteicos do receptor S1PR1 no hipotálamo, esses dados também foram encontrados em vários modelos de obesidade com resistência central á leptina (Wistar, Swiss, C57BL/6J). Esses dados revelam que a expressão do receptor S1PR1 é recíproca a ativação da proteína STAT3 no hipotálamo, conforme descrito em células tumorais (Lee et al., 2010).

Alguns estudos mostraram mecanismos alternativos de ativação e de controle da via Jak2/STAT3 em neurônios hipotalâmicos no controle da ingestão alimentar e do gasto energético em mamíferos (Grossberg et al., 2010a; Janoschek et al., 2006; Johnen et al., 2007; Ropelle et al., 2010). O receptor glicoproteína 130 (gp130R) tem sido proposto como uma alternativa para ativação da via Jak2/STAT3 no hipotálamo, podendo ser um alvo terapêutico da obesidade (Febbraio, 2007). Mediante a isso, o lipídio bioativo esfingosina 1 fosfato (S1P), pode ser considerado uma molécula alternativa capaz de ativar a via de sinalização Jak2/STAT3 e restaurar os sinais anorexigênicos no hipotálamo. Demonstramos que o receptor S1PR1 mesmo estando em baixa expressão no hipotálamo de roedores obesos, a injeção (ICV) de S1P ou de SEW2871 no hipotálamo foi suficiente para ativar a STAT3 e neurônios POMC, responsáveis pelo controle da fome e da termogênese.

Detectamos aumento nos níveis circulantes de S1P em ratos obesos, quando comparados ao grupo controle magro. Esses dados são similares aos resultados encontrados por Samad e colaboradores que mostraram aumento nos níveis plasmáticos de S1P em camundongos *ob/ob (Samad et al., 2006).* O aumento nos níveis de S1P em ratos obesos pode ter ocorrido como um mecanismo de compensação, contudo, esse fenômeno não foi avaliado em nosso estudo. No entanto, observamos que o aumento dos níveis de S1P observados em animais obesos não é suficiente para ativar a via de sinalização S1PR1/STAT3 hipotalâmica. A baixa regulação dos níveis do S1PR1 em roedores obesos parece estar associada ao comprometimento na ação central anorexigênica e termogênica do S1P em roedores obesos, quando comparado aos roedores magros.

Mecanismos moleculares de resistência a leptina no hipotálamo, como por exemplo, proteína tirosina fosfatase 1B (PTP1B) (Bence et al., 2006; Chiarreotto-Ropelle et al., 2013) IKKβ (Milanski et al., 2009; Purkayastha et al., 2011; Zhang et al., 2008), poderiam ao menos em parte explicar a diminuição da associação Jak2/S1PR1, em resposta a ação do S1P em animais obesos, no entanto mais investigações devem ser feitas sobre esta questão. Contudo, quando utilizamos a bomba de infusão osmótica com o tratamento de S1P cronicamente no hipotálamo de ratos obesos, resultou em redução significativa na ingestão alimentar e no peso corporal.

Embora tenhamos encontrado elevados níveis de S1P em roedores obesos e em roedores com tumores, provavelmente as fontes de S1P são distintas em cada situação. A síntese de S1P ocorre pela fosforilação de esfingosinas, catalisado pelas quinases SphK1 e SphK2 (Lepine et al., 2011). Esses dados são confirmados em um estudo conduzido por Liang e colaboradores, os quais mostraram em modelos de câncer de colón o aumento na produção de S1P advindo da regulação da esfingosina quinase 1 (SphK1) em células cancerígenas (Liang et al., 2013). No entanto, a síntese que promove a produção do S1P no estado de obesidade permanece desconhecida.

Pacientes com câncer desencadeiam caquexia, caracterizada pela anorexia levando a redução do apetite e da massa muscular. Esses efeitos anorexigênicos parecem ser recebidos pela produção de fatores tumorais agindo sobre células hipotalâmicas, ampliando

os sinais anorexigênicos (Bruera, 1997; Laviano et al., 2003; Tisdale, 1997). As citoquinas pró-inflamatórias, tais como fator de necrose tumoral α (TNF-α), interleucina-1 (IL-1β) e interferon gama (IFN γ), são descritos como moduladores propostos para desempenhar um papel na etiologia da anorexia induzidos pelo câncer (Laviano et al., 2003). Estudos encontraram níveis séricos de S1P aumentados em modelos de animais com câncer (Liang et al., 2013; Nagahashi et al., 2012). Durante o desenvolvimento do tumor, os elevados níveis de S1P são produzidos através da regulação positiva da atividade da quinase SphK1 em células cancerigenas (Liang et al., 2013). Em nosso estudo mostramos que a alta produção de S1P em roedores com tumor foi associado à regulação positiva dos níveis de S1PR1 e na fosforilação da proteína STAT3 no hipotálamo, a injeção intraperitonial de S1P foi suficiente para induzir anorexia e aumentar o gasto energético de roedores. De maneira muito interessante o bloqueio da via S1P/S1PR1 com inibidor farmacológico antagonista FTY720 foi suficiente para reverter o quadro anorexigênico em roedores com tumor, prolongando a sobrevida destes animais. Esses dados sugerem que a produção do S1P através do câncer age no hipotálamo contribuindo para induzir anorexia.

Tomados em conjunto, nosso estudo mostrou que o eixo neuronal S1P/S1PR1 afeta a atividade anorexigênica em mamiferos. O fosfolipidio bioativo S1P é capaz de atuar como ativador da via de sinalização Jak2/STAT3 em neurônios hipotalâmicos através do receptor S1PR1. Mostramos que o S1PR1 e a proteína STAT3 têm efeitos reciprocos no hipotálamo, proporcionando uma retroalimentação positiva. A sinalização neuronal S1PR1/STAT3 desempenha um papel fundamental no controle da obesidade e da anorexia induzida pelo câncer. Desta maneira, o eixo S1P/S1PR1 é um regulador central do apetite e um potencial alvo no tratamento da obesidade e da anorexia induzida pelo câncer.

DISCUSSÃO (ARTIGO 2)

Neste estudo, investigamos o papel do eixo S1P/S1PR1 no hipotálamo de ratos obesos após o exercício físico agudo. No primeiro momento, demonstramos que o exercicio físico de natação aumentou os níveis circulantes de S1P em ratos obesos. Observamos que o esfingolipídio bioativo esfingosina 1 fosfato (S1P) foi capaz de ativar a via de sinalização anorexigênica Jak2/STAT3 por meio do receptor S1PR1 no hipotálamo ratos obesos. De maneira interessante, observamos que a proteína S1PR1 está em grande quantidade no hipotálamo mediobasal (VMH) de ratos magros, por outro lado, quando tratamos os ratos com dieta rica em gordura (HFD), os níveis proteicos de S1PR1 foram diminuidos, dados similares à via Jak/STAT3 hipotalâmica. No entanto, uma única sessão aguda de natação foi suficiente para normalizar os níveis proteicos de S1PR1 hipotalâmico. Finalmente, demonstramos que o exercício fisico requer a ativação do receptor S1PR1 no hipotálamo para reduzir a ingestão alimentar e aumentar o gasto energético de ratos obesos.

O efeito que o exercício físico exerce sobre o controle da ingestão alimentar ainda é pouco explorado, sendo um fenômeno complexo e que depende de muitas circunstâncias, entre elas, a intensidade, o volume, e o tipo de exercício, e principalmente, as caracteristicas fenotípicas do indivíduo engajado no programa de exercício físico. Vários estudos têm demonstrado que o exercício físico tem ação no controle do apetite em individuos com sobrepeso e obeso (Hagobian et al., 2013; Holmstrup et al., 2013; Schubert et al., 2013; Thivel et al., 2012), inclusive em adolescentes e mulheres na pós-menopausa (Borer et al., 2009), porém os mecanismos pelos quais o exercício físico modula a ingestão alimentar não estão claros. Estudos demostraram que o exercício físico induz a secreção de biomoléculas musculares, como interleucinas (Pedersen and Febbraio, 2012), a apelina (Besse-Patin et al., 2013), a secreted protein acidic and rich in cysteine (SPARC) (Aoi et al., 2013) e irisina (Bostrom et al., 2012), levando a promoção de efeitos benéficos do exercício físico. Um estudo conduzido por Flores e colaboradores mostrou que o exercício físico moderado melhorou a ação da insulina e leptina no hipotálamo de roedores (Flores et al., 2006), e essa melhora também foi encontrada em roedores obesos (Chiarreotto-Ropelle et al., 2013; Ropelle et al., 2010; Ropelle et al., 2007), reduzindo a ingestão alimentar

mediado pelo processo anti-inflamatório mediado pela inteleucina-6 (IL-6). Esses dados sugerem que o exercício físico medeia interação central e periférica através de moléculas especializadas. Em nosso estudo, descrevemos que o esfingolipídio S1P poderia atuar no sistema nervoso central como uma molécula sinalizadora em resposta ao exercício físico.

A síntese de S1P ocorre por fosforilação, sendo catalisada por meio das esfingosinas quinases SphK1 e SphK2. O S1P medeia efeitos biológicos através de mecanismos parácrinos ou autócrinos (Lepine et al., 2011). Em condições patológicas, os níveis de S1P são catalisados pela esfingosina quinase 1 (SphK1) contribuindo para inflamação e para o crescimento do câncer (Liang et al., 2013) . Paralelamente a isso, foram encontrados elevados níveis sistêmicos de S1P em modelos de obesidade (Samad et al., 2006) e em indivíduos obesos (Kowalski et al., 2013). Em nosso estudo encontramos elevados níveis plasmáticos de S1P, no líquor (CSF) e em tecidos hipotalâmicos de ratos obesos exercitados, no entanto a atividade da esfingosina-quinases nos tecidos metabólicos no estado de obesidade ou em resposta ao exercício físico permanece desconhecida, e requer mais investigacões.

Os níveis elevados de S1P sistêmico estão associados com o efeito anorexigênico e termogênico produzido pelo exercício físico, paradoxalmente, foram encontrados elevados níveis de S1P em modelos de obesidade (Kowalski et al., 2013; Samad et al., 2006). Provavelmente o aumento dos níveis circulantes de S1P em obesos seja decorrente de um mecanismo compensatório em resposta à redução da expressão do S1PR1. É possível que os baixos níveis de S1PR1 em roedores obesos possam estar também relacionados com a diminuição na sinalização da proteína STAT3 hipotalâmica (Bence et al., 2006; El-Haschimi et al., 2000; Lee et al., 2010). Demonstramos que a regulação positiva dos níveis de S1P no hipotálamo de ratos obesos exercitados parece ser suficiente para ativar a via de sinalização S1PR1/Jak2/STAT3 e normalizar os níveis de S1PR1 hipotalâmico em ratos obesos. Esse equilíbrio de regulação poderia ajudar a reajustar o estado nutricional e o equilíbrio energético induzido pelo excesso de nutrientes no hipotálamo e contribuir para a redução da ingestão alimentar e do peso corporal. Esses efeitos podem de certa forma contribuir para o controle do peso corporal ou manutenção do fenótipo magro.

CONCLUSÃO

Em nossos estudos, mostramos que o eixo S1P/S1PR1 foi capaz de controlar a atividade anorexigênica em roedores magros e obesos, atuando como ativador da via Jak2/STAT3 em neurônios hipotalâmicos através da proteína S1PR1. Adicionalmente, a STAT3 é ativada pelo eixo S1P/S1PR1 de maneira persistente, e de maneira recíproca, a STAT3 aumenta a expressão do receptor S1PR1 no hipotálamo. Encontramos baixos níveis de S1PR1 em roedores obesos. Por outro lado, o tratamento ICV com o esfingolipídio bioativo (S1P) foi capaz de reativar a via de sinalização anorexigênica Jak2/STAT3 no hipotálamo de ratos obesos, reduzindo a ingestão alimentar e aumentando o gasto energético. Encontramos em modelos de câncer induzidos por tumor, o aumento dos níveis sistêmicos de S1P foi estreitamente relacionado com o desenvolvimento da anorexia através do eixo S1PR1/STAT3. O bloqueio com o inibidor farmacológico antagonista do receptor S1PR1, o FTY720, reduziu a ativação anorexigênica e aumentou a sobrevida dos animais com tumor. Finalmente, observamos que o exercício físico agudo foi capaz de aumentar os níveis sistêmicos de S1P em ratos obesos sendo suficiente para ativar o eixo S1PR1/Jak2/STAT3, normalizando os níveis de S1PR1 reestabelecendo a via anorexigênica, no hipotálamo de ratos obesos. Tomados em conjuntos, nossos resultados apontam que o eixo S1P/S1PR1 é um potente regulador da via anorexigênica Jak2/STAT3 no hipotálamo, tanto em condições patológicas como na obesidade e na anorexia induzida por tumor, como em condições fisiológicas como durante o jejum ou o exercício físico.

REFERÊNCIAS

Abrantes, M.M., Lamounier, J.A., and Colosimo, E.A. (2002). Recommendations for the use of body mass index for the classification of overweight and obese children and adolescents. Food and nutrition bulletin *23*, 262-266.

Ahima, R.S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., and Flier, J.S. (1996). Role of leptin in the neuroendocrine response to fasting. Nature *382*, 250-252.

Aoi, W., Naito, Y., Takagi, T., Tanimura, Y., Takanami, Y., Kawai, Y., Sakuma, K., Hang, L.P., Mizushima, K., Hirai, Y*., et al.* (2013). A novel myokine, secreted protein acidic and rich in cysteine (SPARC), suppresses colon tumorigenesis via regular exercise. Gut *62*, 882-889.

Bacha, F., Saad, R., Gungor, N., Janosky, J., and Arslanian, S.A. (2003). Obesity, regional fat distribution, and syndrome X in obese black versus white adolescents: race differential in diabetogenic and atherogenic risk factors. The Journal of clinical endocrinology and metabolism *88*, 2534-2540.

Bado, A., Levasseur, S., Attoub, S., Kermorgant, S., Laigneau, J.P., Bortoluzzi, M.N., Moizo, L., Lehy, T., Guerre-Millo, M., Le Marchand-Brustel, Y*., et al.* (1998). The stomach is a source of leptin. Nature *394*, 790-793.

Baranowski, M., Charmas, M., Dlugolecka, B., and Gorski, J. (2011). Exercise increases plasma levels of sphingoid base-1 phosphates in humans. Acta physiologica *203*, 373-380.

Baskin, M.L., Ard, J., Franklin, F., and Allison, D.B. (2005). Prevalence of obesity in the United States. Obesity reviews : an official journal of the International Association for the Study of Obesity *6*, 5-7.

Baudhuin, L.M., Jiang, Y., Zaslavsky, A., Ishii, I., Chun, J., and Xu, Y. (2004). S1P3-mediated Akt activation and cross-talk with platelet-derived growth factor receptor

(PDGFR). FASEB journal : official publication of the Federation of American Societies for Experimental Biology *18*, 341-343.

Bence, K.K., Delibegovic, M., Xue, B., Gorgun, C.Z., Hotamisligil, G.S., Neel, B.G., and Kahn, B.B. (2006). Neuronal PTP1B regulates body weight, adiposity and leptin action. Nature medicine *12*, 917-924.

Besse-Patin, A., Montastier, E., Vinel, C., Castan-Laurell, I., Louche, K., Dray, C., Daviaud, D., Mir, L., Marques, M.A., Thalamas, C.*, et al.* (2013). Effect of endurance training on skeletal muscle myokine expression in obese men: identification of apelin as a novel myokine. International journal of obesity.

Bi, S., Scott, K.A., Hyun, J., Ladenheim, E.E., and Moran, T.H. (2005). Running wheel activity prevents hyperphagia and obesity in Otsuka long-evans Tokushima Fatty rats: role of hypothalamic signaling. Endocrinology *146*, 1676-1685.

Bjorbaek, C., Elmquist, J.K., Frantz, J.D., Shoelson, S.E., and Flier, J.S. (1998). Identification of SOCS-3 as a potential mediator of central leptin resistance. Molecular cell *1*, 619-625.

Borer, K.T., Wuorinen, E., Ku, K., and Burant, C. (2009). Appetite responds to changes in meal content, whereas ghrelin, leptin, and insulin track changes in energy availability. The Journal of clinical endocrinology and metabolism *94*, 2290-2298.

Bostrom, P., Wu, J., Jedrychowski, M.P., Korde, A., Ye, L., Lo, J.C., Rasbach, K.A., Bostrom, E.A., Choi, J.H., Long, J.Z.*, et al.* (2012). A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature *481*, 463-468.

Bruera, E. (1997). ABC of palliative care. Anorexia, cachexia, and nutrition. Bmj *315*, 1219-1222.

Camerer, E., Regard, J.B., Cornelissen, I., Srinivasan, Y., Duong, D.N., Palmer, D., Pham, T.H., Wong, J.S., Pappu, R., and Coughlin, S.R. (2009). Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. The Journal of clinical investigation *119*, 1871-1879.

Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R., and Burn, P. (1995). Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science *269*, 546-549.

Carroll, S., and Dudfield, M. (2004). What is the relationship between exercise and metabolic abnormalities? A review of the metabolic syndrome. Sports medicine *34*, 371-418.

Carvalheira, J.B., Ribeiro, E.B., Folli, F., Velloso, L.A., and Saad, M.J. (2003). Interaction between leptin and insulin signaling pathways differentially affects JAK-STAT and PI 3-kinase-mediated signaling in rat liver. Biological chemistry *384*, 151-159.

Carvalheira, J.B., and Saad, M.J. (2006). [Insulin resistance/hyperinsulinemia associated diseases not included in the metabolic syndrome]. Arquivos brasileiros de endocrinologia e metabologia *50*, 360-367.

Chi, X.X., and Nicol, G.D. (2010). The sphingosine 1-phosphate receptor, S1PR(1), plays a prominent but not exclusive role in enhancing the excitability of sensory neurons. Journal of neurophysiology *104*, 2741-2748.

Chiarreotto-Ropelle, E.C., Pauli, L.S., Katashima, C.K., Pimentel, G.D., Picardi, P.K., Silva, V.R., de Souza, C.T., Prada, P.O., Cintra, D.E., Carvalheira, J.B.*, et al.* (2013). Acute exercise suppresses hypothalamic PTP1B protein level and improves insulin and leptin signaling in obese rats. American journal of physiology Endocrinology and metabolism *305*, E649-659.

Considine, R.V., Considine, E.L., Williams, C.J., Hyde, T.M., and Caro, J.F. (1996). The hypothalamic leptin receptor in humans: identification of incidental sequence polymorphisms and absence of the db/db mouse and fa/fa rat mutations. Diabetes *45*, 992-994.

De Souza, C.T., Araujo, E.P., Bordin, S., Ashimine, R., Zollner, R.L., Boschero, A.C., Saad, M.J., and Velloso, L.A. (2005). Consumption of a fat-rich diet activates a

proinflammatory response and induces insulin resistance in the hypothalamus. Endocrinology *146*, 4192-4199.

De Vos, P., Saladin, R., Auwerx, J., and Staels, B. (1995). Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. The Journal of biological chemistry *270*, 15958-15961.

El-Haschimi, K., Pierroz, D.D., Hileman, S.M., Bjorbaek, C., and Flier, J.S. (2000). Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. The Journal of clinical investigation *105*, 1827-1832.

Elmquist, J.K., and Flier, J.S. (2004). Neuroscience. The fat-brain axis enters a new dimension. Science *304*, 63-64.

Fan, W., Boston, B.A., Kesterson, R.A., Hruby, V.J., and Cone, R.D. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature *385*, 165-168.

Febbraio, M.A. (2007). gp130 receptor ligands as potential therapeutic targets for obesity. The Journal of clinical investigation *117*, 841-849.

Flores, M.B., Fernandes, M.F., Ropelle, E.R., Faria, M.C., Ueno, M., Velloso, L.A., Saad, M.J., and Carvalheira, J.B. (2006). Exercise improves insulin and leptin sensitivity in hypothalamus of Wistar rats. Diabetes *55*, 2554-2561.

Frederich, R.C., Hamann, A., Anderson, S., Lollmann, B., Lowell, B.B., and Flier, J.S. (1995). Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nature medicine *1*, 1311-1314.

Frias, M.A., James, R.W., Gerber-Wicht, C., and Lang, U. (2009). Native and reconstituted HDL activate Stat3 in ventricular cardiomyocytes via ERK1/2: role of sphingosine-1-phosphate. Cardiovascular research *82*, 313-323.

Gelin, J., Moldawer, L.L., Lonnroth, C., Sherry, B., Chizzonite, R., and Lundholm, K. (1991). Role of endogenous tumor necrosis factor alpha and interleukin 1 for experimental tumor growth and the development of cancer cachexia. Cancer research *51*, 415-421.

Grossberg, A.J., Scarlett, J.M., and Marks, D.L. (2010a). Hypothalamic mechanisms in cachexia. Physiology & behavior *100*, 478-489.

Grossberg, A.J., Scarlett, J.M., Zhu, X., Bowe, D.D., Batra, A.K., Braun, T.P., and Marks, D.L. (2010b). Arcuate nucleus proopiomelanocortin neurons mediate the acute anorectic actions of leukemia inhibitory factor via gp130. Endocrinology *151*, 606-616.

Guo, H., Zhao, Z., Yang, Q., Wang, M., Bell, R.D., Wang, S., Chow, N., Davis, T.P., Griffin, J.H., Goldman, S.A., *et al.* (2013). An activated protein C analog stimulates neuronal production by human neural progenitor cells via a PAR1-PAR3-S1PR1-Akt pathway. The Journal of neuroscience : the official journal of the Society for Neuroscience *33*, 6181-6190.

Hagobian, T.A., Yamashiro, M., Hinkel-Lipsker, J., Streder, K., Evero, N., and Hackney, T. (2013). Effects of acute exercise on appetite hormones and ad libitum energy intake in men and women. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme *38*, 66-72.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., and Friedman, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. Science *269*, 543-546.

Hannun, Y.A., and Obeid, L.M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. Nature reviews Molecular cell biology *9*, 139-150.

Haslett, P.A. (1998). Anticytokine approaches to the treatment of anorexia and cachexia. Seminars in oncology *25*, 53-57.

Havel, P.J. (1998). Leptin production and action: relevance to energy balance in humans. The American journal of clinical nutrition *67*, 355-356.

Heldin, C.H. (1995). Dimerization of cell surface receptors in signal transduction. Cell *80*, 213-223.

Hervey, G.R. (1959). The effects of lesions in the hypothalamus in parabiotic rats. The Journal of physiology *145*, 336-352.

Hla, T., and Maciag, T. (1990). An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. The Journal of biological chemistry *265*, 9308-9313.

Holmstrup, M.E., Fairchild, T.J., Keslacy, S., Weinstock, R.S., and Kanaley, J.A. (2013). Satiety, but not total PYY, Is increased with continuous and intermittent exercise. Obesity *21*, 2014-2020.

Hopkins, S.J., and Rothwell, N.J. (1995). Cytokines and the nervous system. I: Expression and recognition. Trends in neurosciences *18*, 83-88.

Im, D.S., Ungar, A.R., and Lynch, K.R. (2000). Characterization of a zebrafish (Danio rerio) sphingosine 1-phosphate receptor expressed in the embryonic brain. Biochemical and biophysical research communications *279*, 139-143.

Janoschek, R., Plum, L., Koch, L., Munzberg, H., Diano, S., Shanabrough, M., Muller, W., Horvath, T.L., and Bruning, J.C. (2006). gp130 signaling in proopiomelanocortin neurons mediates the acute anorectic response to centrally applied ciliary neurotrophic factor. Proceedings of the National Academy of Sciences of the United States of America *103*, 10707-10712.

Johnen, H., Lin, S., Kuffner, T., Brown, D.A., Tsai, V.W., Bauskin, A.R., Wu, L., Pankhurst, G., Jiang, L., Junankar, S.*, et al.* (2007). Tumor-induced anorexia and weight loss are mediated by the TGF-beta superfamily cytokine MIC-1. Nature medicine *13*, 1333-1340.

Konig, K., Diehl, L., Rommerscheidt-Fuss, U., Golletz, C., Quast, T., Kahl, P., Kolanus, W., Knolle, P., Buettner, R., and Heukamp, L.C. (2010). Four-and-a-half LIM domain

protein 2 is a novel regulator of sphingosine 1-phosphate receptor 1 in CCL19-induced dendritic cell migration. Journal of immunology *185*, 1466-1475.

Kowalski, G.M., Carey, A.L., Selathurai, A., Kingwell, B.A., and Bruce, C.R. (2013). Plasma sphingosine-1-phosphate is elevated in obesity. PloS one *8*, e72449.

Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N.*, et al.* (1998). Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature *393*, 72-76.

Larkin, M. (1998). Thwarting the dwindling progression of cachexia. Lancet *351*, 1336.

Laviano, A., Meguid, M.M., and Rossi-Fanelli, F. (2003). Improving food intake in anorectic cancer patients. Current opinion in clinical nutrition and metabolic care *6*, 421-426.

Lee, H., Deng, J., Kujawski, M., Yang, C., Liu, Y., Herrmann, A., Kortylewski, M., Horne, D., Somlo, G., Forman, S.*, et al.* (2010). STAT3-induced S1PR1 expression is crucial for persistent STAT3 activation in tumors. Nature medicine *16*, 1421-1428.

Lepine, S., Allegood, J.C., Park, M., Dent, P., Milstien, S., and Spiegel, S. (2011). Sphingosine-1-phosphate phosphohydrolase-1 regulates ER stress-induced autophagy. Cell death and differentiation *18*, 350-361.

Liang, J., Nagahashi, M., Kim, E.Y., Harikumar, K.B., Yamada, A., Huang, W.C., Hait, N.C., Allegood, J.C., Price, M.M., Avni, D.*, et al.* (2013). Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer. Cancer cell *23*, 107-120.

Licinio, J., Mantzoros, C., Negrao, A.B., Cizza, G., Wong, M.L., Bongiorno, P.B., Chrousos, G.P., Karp, B., Allen, C., Flier, J.S.*, et al.* (1997). Human leptin levels are pulsatile and inversely related to pituitary-adrenal function. Nature medicine *3*, 575-579.

Licinio, J., and Wong, M.L. (1997). Pathways and mechanisms for cytokine signaling of the central nervous system. The Journal of clinical investigation *100*, 2941-2947.

Liu, C.H., and Hla, T. (1997). The mouse gene for the inducible G-protein-coupled receptor edg-1. Genomics *43*, 15-24.

Liu, Y., Deng, J., Wang, L., Lee, H., Armstrong, B., Scuto, A., Kowolik, C., Weiss, L.M., Forman, S., and Yu, H. (2012). S1PR1 is an effective target to block STAT3 signaling in activated B cell-like diffuse large B-cell lymphoma. Blood *120*, 1458-1465.

Maceyka, M., Harikumar, K.B., Milstien, S., and Spiegel, S. (2012). Sphingosine-1-phosphate signaling and its role in disease. Trends in cell biology *22*, 50-60.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R.E., Lee, G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S.*, et al.* (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nature medicine *1*, 1155-1161.

Mantovani, G., Maccio, A., Lai, P., Massa, E., Ghiani, M., and Santona, M.C. (1998). Cytokine activity in cancer-related anorexia/cachexia: role of megestrol acetate and medroxyprogesterone acetate. Seminars in oncology *25*, 45-52.

Martinez de Morentin, P.B., Gonzalez, C.R., and Lopez, M. (2010). AMP-activated protein kinase: 'a cup of tea' against cholesterol-induced neurotoxicity. The Journal of pathology *222*, 329-334.

Masuzaki, H., Ogawa, Y., Sagawa, N., Hosoda, K., Matsumoto, T., Mise, H., Nishimura, H., Yoshimasa, Y., Tanaka, I., Mori, T.*, et al.* (1997). Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. Nature medicine *3*, 1029-1033.

Matthys, P., and Billiau, A. (1997). Cytokines and cachexia. Nutrition *13*, 763-770.

Mercer, J.G., Hoggard, N., Williams, L.M., Lawrence, C.B., Hannah, L.T., and Trayhurn, P. (1996). Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. FEBS letters *387*, 113-116.

Metcalf, D., and Gearing, D.P. (1989). Fatal syndrome in mice engrafted with cells producing high levels of the leukemia inhibitory factor. Proceedings of the National Academy of Sciences of the United States of America *86*, 5948-5952.

Milanski, M., Degasperi, G., Coope, A., Morari, J., Denis, R., Cintra, D.E., Tsukumo, D.M., Anhe, G., Amaral, M.E., Takahashi, H.K., *et al.* (2009). Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 359-370.

Moldawer, L.L., Rogy, M.A., and Lowry, S.F. (1992). The role of cytokines in cancer cachexia. JPEN Journal of parenteral and enteral nutrition *16*, 43S-49S.

Mori, M., Yamaguchi, K., Honda, S., Nagasaki, K., Ueda, M., Abe, O., and Abe, K. (1991). Cancer cachexia syndrome developed in nude mice bearing melanoma cells producing leukemia-inhibitory factor. Cancer research *51*, 6656-6659.

Munzberg, H., Bjornholm, M., Bates, S.H., and Myers, M.G., Jr. (2005). Leptin receptor action and mechanisms of leptin resistance. Cellular and molecular life sciences : CMLS *62*, 642-652.

Nagahashi, M., Ramachandran, S., Kim, E.Y., Allegood, J.C., Rashid, O.M., Yamada, A., Zhao, R., Milstien, S., Zhou, H., Spiegel, S., *et al.* (2012). Sphingosine-1-phosphate produced by sphingosine kinase 1 promotes breast cancer progression by stimulating angiogenesis and lymphangiogenesis. Cancer research *72*, 726-735.

Nishimura, H., Akiyama, T., Irei, I., Hamazaki, S., and Sadahira, Y. (2010). Cellular localization of sphingosine-1-phosphate receptor 1 expression in the human central nervous system. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society *58*, 847-856.

Ogden, C.L., Carroll, M.D., Curtin, L.R., McDowell, M.A., Tabak, C.J., and Flegal, K.M. (2006). Prevalence of overweight and obesity in the United States, 1999-2004. JAMA : the journal of the American Medical Association *295*, 1549-1555.

Pedersen, B.K., and Febbraio, M.A. (2012). Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nature reviews Endocrinology *8*, 457-465.

Pedersen, B.K., Steensberg, A., and Schjerling, P. (2001a). Exercise and interleukin-6. Current opinion in hematology *8*, 137-141.

Pedersen, B.K., Steensberg, A., and Schjerling, P. (2001b). Muscle-derived interleukin-6: possible biological effects. The Journal of physiology *536*, 329-337.

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. Science *269*, 540-543.

Picardi, P.K., Calegari, V.C., Prada, P.O., Moraes, J.C., Araujo, E., Marcondes, M.C., Ueno, M., Carvalheira, J.B., Velloso, L.A., and Saad, M.J. (2008). Reduction of hypothalamic protein tyrosine phosphatase improves insulin and leptin resistance in diet-induced obese rats. Endocrinology *149*, 3870-3880.

Plata-Salaman, C.R. (1996). Leptin (OB protein), neuropeptide Y, and interleukin-1 interactions as interface mechanisms for the regulation of feeding in health and disease. Nutrition *12*, 718-719.

Purkayastha, S., Zhang, G., and Cai, D. (2011). Uncoupling the mechanisms of obesity and hypertension by targeting hypothalamic IKK-beta and NF-kappaB. Nature medicine *17*, 883-887.

Rex, K., Jeffries, S., Brown, M.L., Carlson, T., Coxon, A., Fajardo, F., Frank, B., Gustin, D., Kamb, A., Kassner, P.D.*, et al.* (2013). Sphingosine kinase activity is not required for tumor cell viability. PloS one *8*, e68328.

Rivera, J., Proia, R.L., and Olivera, A. (2008). The alliance of sphingosine-1-phosphate and its receptors in immunity. Nature reviews Immunology *8*, 753-763.

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 biology *8*.

Ropelle, E.R., Pauli, J.R., Zecchin, K.G., Ueno, M., de Souza, C.T., Morari, J., Faria, M.C., Velloso, L.A., Saad, M.J., and Carvalheira, J.B. (2007). A central role for neuronal adenosine 5'-monophosphate-activated protein kinase in cancer-induced anorexia. Endocrinology *148*, 5220-5229.

Rothwell, N.J., and Hopkins, S.J. (1995). Cytokines and the nervous system II: Actions and mechanisms of action. Trends in neurosciences *18*, 130-136.

Samad, F., Hester, K.D., Yang, G., Hannun, Y.A., and Bielawski, J. (2006). Altered adipose and plasma sphingolipid metabolism in obesity: a potential mechanism for cardiovascular and metabolic risk. Diabetes *55*, 2579-2587.

Schubert, M.M., Desbrow, B., Sabapathy, S., and Leveritt, M. (2013). Acute exercise and subsequent energy intake. A meta-analysis. Appetite *63*, 92-104.

Schulte, K.M., Beyer, A., Kohrer, K., Oberhauser, S., and Roher, H.D. (2001). Lysophosphatidic acid, a novel lipid growth factor for human thyroid cells: over-expression of the high-affinity receptor edg4 in differentiated thyroid cancer. International journal of cancer Journal international du cancer *92*, 249-256.

Schwartz, M.W., Woods, S.C., Porte, D., Jr., Seeley, R.J., and Baskin, D.G. (2000). Central nervous system control of food intake. Nature *404*, 661-671.

Shutter, J.R., Graham, M., Kinsey, A.C., Scully, S., Luthy, R., and Stark, K.L. (1997). Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in obese and diabetic mutant mice. Genes & development *11*, 593-602.

Spiegelman, B.M., and Flier, J.S. (2001). Obesity and the regulation of energy balance. Cell *104*, 531-543.

Stephens, T.W., Basinski, M., Bristow, P.K., Bue-Valleskey, J.M., Burgett, S.G., Craft, L., Hale, J., Hoffmann, J., Hsiung, H.M., Kriauciunas, A., *et al.* (1995). The role of neuropeptide Y in the antiobesity action of the obese gene product. Nature *377*, 530-532.

Sternberg, E.M. (1997). Neural-immune interactions in health and disease. The Journal of clinical investigation *100*, 2641-2647.

Tartaglia, L.A. (1997). The leptin receptor. The Journal of biological chemistry *272*, 6093-6096.

Thivel, D., Isacco, L., Montaurier, C., Boirie, Y., Duche, P., and Morio, B. (2012). The 24-h energy intake of obese adolescents is spontaneously reduced after intensive exercise: a randomized controlled trial in calorimetric chambers. PloS one *7*, e29840.

Tisdale, M.J. (1997). Biology of cachexia. Journal of the National Cancer Institute *89*, 1763-1773.

Walter, D.H., Rochwalsky, U., Reinhold, J., Seeger, F., Aicher, A., Urbich, C., Spyridopoulos, I., Chun, J., Brinkmann, V., Keul, P., *et al.* (2007). Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor. Arteriosclerosis, thrombosis, and vascular biology *27*, 275-282.

Welberg, L. (2010). Metabolism: An appetite for exercise. Nature reviews Neuroscience *11*, 662.

Woods, S.C., Chavez, M., Park, C.R., Riedy, C., Kaiyala, K., Richardson, R.D., Figlewicz, D.P., Schwartz, M.W., Porte, D., Jr., and Seeley, R.J. (1996). The evaluation of insulin as a metabolic signal influencing behavior via the brain. Neuroscience and biobehavioral reviews *20*, 139-144.

Yamaguchi, H., Kitayama, J., Takuwa, N., Arikawa, K., Inoki, I., Takehara, K., Nagawa, H., and Takuwa, Y. (2003). Sphingosine-1-phosphate receptor subtype-specific positive and negative regulation of Rac and haematogenous metastasis of melanoma cells. The Biochemical journal *374*, 715-722.

Yang, X.P., Schaper, F., Teubner, A., Lammert, F., Heinrich, P.C., Matern, S., and Siewert, E. (2005). Interleukin-6 plays a crucial role in the hepatic expression of SOCS3 during acute inflammatory processes in vivo. Journal of hepatology *43*, 704-710.

Yoshida, Y., Nakada, M., Harada, T., Tanaka, S., Furuta, T., Hayashi, Y., Kita, D., Uchiyama, N., Hayashi, Y., and Hamada, J. (2010). The expression level of sphingosine-1-phosphate receptor type 1 is related to MIB-1 labeling index and predicts survival of glioblastoma patients. Journal of neuro-oncology *98*, 41-47.

Zhang, L., Urtz, N., Gaertner, F., Legate, K.R., Petzold, T., Lorenz, M., Mazharian, A., Watson, S.P., and Massberg, S. (2013). Sphingosine kinase 2 (Sphk2) regulates platelet biogenesis by providing intracellular sphingosine 1-phosphate (S1P). Blood *122*, 791-802.

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.

Zhao, Y., Kalari, S.K., Usatyuk, P.V., Gorshkova, I., He, D., Watkins, T., Brindley, D.N., Sun, C., Bittman, R., Garcia, J.G.*, et al.* (2007). Intracellular generation of sphingosine 1-phosphate in human lung endothelial cells: role of lipid phosphate phosphatase-1 and sphingosine kinase 1. The Journal of biological chemistry *282*, 14165-14177.

Zhang, Y., Proenca, R., Maffei, M., Boronem, Leopold L., friedman JM: Positional cloning of mouse obese gene and its human homologue. Nature 1994:372:425-432