JULIANA DE ALMEIDA FARIA

COMUNICAÇÃO INTER-ORGÃO ATIVADA PELA MELATONINA PROMOVE O CONTROLE DA GLICONEOGÊNESE

Campinas
2012

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS MÉDICAS

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Dissertação de Mestrado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas - UNICAMP para obtenção de título de Mestra em Farmacologia.

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA POR JULIANA DE ALMEIDA FARIA E ORIENTADA PELO PROF. DR. GABRIEL FORATO ANHE

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Assinatura do Orientador

Campinas
2012

FICHA CATALOGRÁFICA ELABORADA POR
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F225c

Faria, Juliana de Almeida.
Comunicação inter-orgão ativada pela melatonina promove o controle da gliconeogênese / Juliana de Almeida Faria. -- Campinas, SP : [s.n.], 2012.

Orientador : Gabriel Forato Anhê.
Dissertação (Mestrado) - Universidade Estadual de Campinas, Faculdade de Ciências Médicas.

1. Melatonina. 2. Gluconeogênese. 3. Receptores de melatonina. 4. Fígado. I. Anhê, Gabriel Forato, 1980-. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. IV. Título.

Informações para Biblioteca Digital

Título em inglês: Melatonin-induced activation of hypothalamic AKT activates an inter-organ communication leading to suppression of hepatic gluconeogenesis.
Palavras-chave em inglês:
Melatonin
Gluconeogenesis
Melatonin receptors
Liver
Área de concentração: Farmacologia
Titulação: Mestra em Farmacologia
Banca examinadora:
Gabriel Forato Anhê [Orientador]
Sisi Marcondes Paschoal
José Cipolla Neto
Data da defesa: 17-08-2012
Programa de Pós-Graduação: Farmacologia

Banca Examinadora de Dissertação de Mestrado

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Curso de Pós-Graduação em Farmacologia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 17/08/2012

AGRADECIMENTOS

Agradeço a Deus, pela dádiva da vida.

Agradeço ao Professor Gabriel, pela oportunidade e confiança. Agradeço por sua competência, sua inquietação, sua incessante busca pelo conhecimento. Serei sempre grata por seus ensinamentos.

Agradeço ao professor Lício por me acolher em seu laboratório e por colaborar no desenvolvimento desse estudo.

Agradeço ao secretário da pós-graduação, Bruno, pelo empenho e eficiência no desenvolvimento de suas funções, os quais nos servem de exemplo para o desenvolvimento de nossas atribuições.

Agradeço aos colegas dos laboratórios Labffamel e Labsincel pela convivência.

Agradeço aos amigos Andrezza, Letícia e Thiago que, além de participarem de meu cotidiano acadêmico, participam e compartilham de minha vida. Obrigada por, muitas vezes, abdicarem de suas noites e finais de semana para participarem de experimentos. Obrigada pelos almoços de última hora, pelos cafés da tarde, pelos bares e festas, pelas piadas, pelas risadas, pelos conselhos, enfim, por estarem ao meu lado nessa caminhada.

Agradeço aos meus pais, meus avôs e avós, que se dedicaram a ser exemplo de honestidade e justiça. Agradeço por me amarem incondicionalmente, mesmo em um mundo em que este sentimento parece ser cada vez mais ausente. Seus ensinamentos são luz para a minha vida.

Agradeço ao Lucas pelo amor, companheirismo, pelo otimismo e paciência.

Agradeço às agências de financiamento CapesCNPq e FAPESP.

RESUMO

O aumento da produção hepática de glicose (PHG) é o principal componente que contribui para os elevados valores da glicemia de jejum em indivíduos obesos com Diabetes Mellitus tipo 2 (DM2). Deste modo, a compreensão dos eventos relacionados ao prejuízo no controle da PHG é extremamente importante para o controle glicêmico no DM2. A insulina, através de uma ação direta no fígado e também de uma ação extra-hepática, é o principal hormônio responsável pela supressão da PHG. A capacidade extra-hepática da insulina em reduzir a PHG decorre da capacidade deste hormônio em ativar a AKT no hipotálamo. Este evento segue da ativação do ramo hepático do nervo vago e da ativação do fator de transcrição STAT3 no fígado. Ratos obesos apresentam um prejuízo desta comunicação inter-orgão ativada pela insulina e, conseqüentemente, um aumento da PHG. O mecanismo pelo qual esta comunicação hipotálamo/fígado está prejudicada ainda não está completamente esclarecido. Desta maneira, o presente projeto pretende avaliar se a exposição direta do sistema nervoso central à melatonina auxilia na supressão da PHG, por mecanismo sinérgico à ação hipotalâmica da insulina.

ABSTRACT

The increase in hepatic glucose production (HGP) is the main component that contributes to high values of fasting glucose levels in obese individuals with diabetes mellitus type 2 (DM2). Thus, understanding the events related to impaired control of HGP is extremely important for glycemic control in DM2. Insulin, via a direct action on the liver and also an action extrahepatic, is the main hormone responsible for the suppression of the HGP. The ability extrahepatic insulin reduce PHG stems from the ability of this hormone to activate AKT in the hypothalamus. This event follows the activation of the hepatic branch of the vagus nerve and the activation of the transcription factor STAT3 in the liver. Obese rats have a loss of inter-organ communication activated by insulin and, consequently, an increase in HGP. The mechanism by which this communication hypothalamus / liver is impaired is not yet completely understood. Thus, this project aims to assess whether direct exposure of the central nervous system to melatonin helps in suppressing the HGP, by the synergic mechanism for hypothalamic action of insulin.

LISTA DE ABREVIATURAS

AKT - insulin-stimulated RAC-α serine/threonine-protein kinase

ARC - Arcuate nucleus

CNS - Central nervous system

ChIP - Chromatin immunoprecipitation

GABA - γ-amino butyric acid

GPCR - G protein-coupled receptor

G6Pase - Glucose-6-phosphatase (protein)

G6pc - Glucose 6 phosphatase (gene)

HGP - hepatic glucose production

IpBr - Ipratropium bromide

LH - Lateral hypothalamus

MBH - Mediobasal hypothalamus

MT1 - Melatonin receptor 1

MT2 - Melatonin receptor 2

PVN - Paraventricular nucleus

Pck1 - Phosphoenolpyruvate carboxykinase 1 (gene)

PI3K - Phosphatidylinositol 3-kinase

SCN - Suprachiasmatic nucleus

STAT3 - Signal transducer and activator of transcription 3

SUMÁRIO

INTRODUÇÃO

Ritmicidade circadiana, melatonina e o metabolismo energético

Os ritmos biológicos circadianos, ubíquos nos seres vivos, constituem uma estratégia adaptativa que garante a relação temporal entre os seres vivos e seu meio ambiente, em geral sincronizada pelo claro-escuro do dia e da noite. Para isso, o sistema de temporização circadiana organiza todos os processos fisiológicos e comportamentais de forma a fazer com que os sistemas funcionais no organismo funcionem com intensidades e qualidades diferentes de acordo com a hora do dia, garantindo, assim, essa relação temporal vital entre os seres vivos e seu ambiente e, por extensão, a sobrevivência individual e da espécie. Neste cenário, é principio fundamental que todos os seres vivos, desde procariotos até os mamíferos, mantenham uma relação adequada entre a aquisição, armazenamento e o consumo energético.

No caso dos animais, é absolutamente necessário que o período de procura de alimento seja otimizado, de forma a estar alocado ao período circadiano de atividade da espécie e perfeitamente sincronizado com o momento do dia em que a probabilidade de aquisição energética seja a maior possível. Nos mamíferos, de acordo com a história filogenética da espécie e sua colocação na cadeia alimentar, o processo de aquisição energética tem condicionantes mais complexos, e a sua expressão rítmica circadiana não está estritamente alocada ao dia ou à noite em todas as espécies, mas sim ao surto diário de atividade típico da espécie. Assim, considerando as adaptações particulares de cada espécie, as funções metabólicas se modificam circadianamente de acordo com o período de atividade/aquisição energética e o período de repouso/jejum.

Como a aquisição energética é condição absoluta para a sobrevida, é compreensível que fatores sinalizadores intrínsecos do estado metabólico do organismo (como glicemia, insulinemia, níveis dos estoques energéticos, sinalizadores da presença de nutrientes no trato gastrointestinal, etc.) devam

também, paralelamente aos sincronizadores circadianos clássicos, regular e sincronizar a própria expressão rítmica circadiana da atividade e do repouso e, em particular, dos processos de aquisição, armazenamento, consumo e preservação de energia.

Sabe-se que processos rítmicos associados ao balanço energético e suas alterações metabólicas resultantes variam sazonalmente de acordo com a disponibilidade de alimentos, ciclos reprodutivos sazonais, migratórios, etc. (Scott e Grant, 2006). Uma das estratégias mais importantes, desenvolvidas filogeneticamente pelos vertebrados para garantir a sincronização dos processos rítmicos fisiológicos e comportamentais com o meio ambiente, foi a de acoplar, independentemente das características de atividade da espécie, o processo secretório de melatonina pineal ao período da noite, num processo de secreção que acompanha a duração deste, e que, portanto, que pode variar sazonalmente. Dessa forma, a produção de melatonina adquire, para os vertebrados, a característica funcional de representar e sinalizar para o meio interno, pela sua maior ou menor concentração plasmática, se é noite ou dia no meio exterior, além de sinalizar, pela duração do episódio secretório, a duração da noite externa e, portanto, a estação do ano (Reiter, 1993).

Em mamíferos, a glândula pineal não tem característica de órgão fotoceptor, sendo que o controle dos ciclos ambientais de iluminação sobre a produção de melatonina se dá de forma indireta, por projeções retinianas para estruturas diencefálicas e destas para os neurônios pré-ganglionares que, através da inervação simpática periférica, inervam a glândula pineal (Cipolla-Neto *et al.*, 1995 e 1999, Bartol *et al.*, 1997, Ribeiro-Barbosa *et al.*, 1999). Dessa forma, a melatonina pode regular e sincronizar, circadiana e sazonalmente, processos fisiológicos vitais como atividade e repouso, sono e vigília, processos reprodutivos, secreções hormonais e a aquisição, armazenamento, consumo e preservação de energia e, conseqüentemente, todos os processos envolvidos na regulação do metabolismo energético.

Em trabalho publicado em 1994 (Lima *et al*., 1994), foram relatados os primeiros resultados de experimentos com adipócitos isolados de tecido adiposo branco e incubados na presença de melatonina. Este trabalho mostrou que a incubação de adipócitos isolados de animais expostos, ou a alimentação ad libitum ou a um regime não circadiano de refeições, à melatonina aumentou a captação de glicose estimulada pela insulina. Em continuidade, outros estudos do mesmo grupo demonstraram que a remoção da glândula pineal (pinealectomia) gera um quadro de intolerância a glicose e resistência à insulina. Esta intolerância a glicose encontrada em animais pinealectomizados foi atribuída primeiramente a uma menor capacidade secretora de insulina pelas ilhotas pancreáticas tanto no início quanto no meio da fase clara do ciclo claro/escuro. No entanto, também foi detectado que a capacidade dos adipócitos captarem glicose após o estímulo da insulina estava diminuída em animais pinealectomizados, provavelmente como decorrência de uma menor concentração do transportador de glicose GLUT4 no tecido adiposo (Lima *et al*, 1998).

Após estes achados, foi demonstrado que os animais pinealectomizados apresentavam de fato, resistência a insulina frente a um teste de tolerância a insulina (ITT) e que tanto a resistência a insulina quanto a diminuição da expressão do GLUT4 no tecido adiposo destes animais era recuperada após reposição de melatonina (Zanquetta *et al*., 2003). É importante ressaltar que, ao contrário de outros parâmetros metabólicos, a captação de glicose por adipócitos em animais pinealectomizados está reduzida em diferentes momentos da fase do ciclo claro/escuro (Alonso-Vale *et al*., 2004). Mais interessante, foi a constatação de que a incubação intermitente in vitro de adipócitos com melatonina (12h de presença e 12h de ausência de melatonina in vitro) é um potencializador da insulina mais eficiente do que a exposição continua à melatonina (24h) (Alonso-Vale *et al*., 2006). Após estes achados, foi detectado que a melatonina aumenta também a captação de glicose em células musculares in vitro em paralelo a um aumento da sinalização da insulina (Ha *et al*., 2006) e que a administração de

melatonina in vivo para animais obesos diminui a resistência a insulina (Sartori *et al*., 2009).

Como dito anteriormente, a intolerância à glicose decorrente da pinealectomia decorre tanto da resistência a insulina quanto do prejuízo da capacidade secretora do pâncreas endócrino. Picinato e colaboradores publicaram em 2002 que, apesar de aumentada em termos absolutos, a responsividade à glicose da ilhota pancreática isolada de ratos pinealectomizados está aumentada durante o período claro do ciclo claro/escuro (Picinato *et al*., 2002). Esta maior secreção basal de insulina em animais pinealectomizados pode ser uma mera decorrência dos constantes níveis aumentados de glicemia de jejum.

De maneira geral, todos estes achado sugerem que a ritmicidade de melatonina favorece a sensibilidade a insulina de maneira sincronizada com a capacidade secretora do pâncreas endócrino, de modo a evitar uma intolerância a glicose.

Mecanismos pelos quais a insulina diminui a gliconeogênese

A capacidade do organismo humano poupar reservas energéticas em situações de abundante oferta de alimentos com a finalidade de gerar estoques que possam prover o funcionamento de órgãos vitais durante períodos de escassez nutricional é uma das mais complexas e fascinantes facetas metabólicas de nossa espécie. A insulina, hormônio produzido e secretado pelas células beta pancreáticas, é um dos principais agentes hormonais desta plasticidade metabólica.

A secreção de insulina é estimulada principalmente pelo aumento nas concentrações circulantes de glicose geralmente acima de 8.3 mM, estando a sua liberação extremamente reduzida em períodos de jejum (Meglasson & Matschinsky, 1986). Em uma situação pós-absortiva, entretanto, a insulina promove a captação de glicose por tecidos periféricos, principalmente aqueles que expressam GLUT4, o transportador de glicose cuja translocação para membrana celular é sensível à insulina (i.e.: tecido adiposo e musculatura esquelética e

cardíaca) (Kahn, 1996). Além desta capacidade de depurar a glicose do sangue para tecidos capazes de estocar macromoléculas altamente energéticas tais como glicogênio e triglicerídeos, a insulina também promove de maneira eficiente a redução no débito hepático de glicose.

De maneira geral, a produção hepática de glicose (PHG) é determinada pela taxa de glicogenólise e gliconeogênese. As evidências acumuladas nos últimos 60 anos demonstram que a PHG pode ser inibida diretamente pela insulina e estimulada por hormônios conhecidos como contra-reguladores, cujas concentrações, ao contrário da insulina, estão aumentadas durante o jejum (i.e.: catecolaminas e glicocorticóides). A ação destes hormônios faz do fígado um órgão singular em sua capacidade de controlar o metabolismo de carboidratos, na medida em que apresenta uma capacidade tanto de captar quanto de liberar glicose para a circulação, de acordo com a demanda nutricional do organismo (Moore *et al*., 1998).

A insulina exerce uma potente ação hepática que resulta na supressão da PHG. O mecanismo proposto para esta ação envolve a inibição do fator de transcrição FoxO1 (*Forkhead box-Other 1*), proteína que quando ativa, estimula a expressão das enzimas Fosfoenolpiruvato-Carboxicinase (PEPCK) e glicose-6-fosfatase (G-6-Pase) (Zhang *et al*., 2006). O mecanismo pelo qual a insulina inibe a atividade transcricional do FoxO1 envolve sua fosforilação em resíduos de serina e treonina pela AKT (Brunet *et al*., 1999).

AKT é uma proteína com atividade seril-treonil cinase que é ativada após a ligação da insulina à porção extracelular de seu receptor. Após ser recrutada para a membrana citoplasmática, esta proteína é completamente ativada pela auto-fosforilação em resíduos seril específicos. Camundongos que apresentam deleção funcional para a AKT2 são intolerantes à glicose e resistentes à ação da insulina. Estes camundongos também apresentam uma maior produção hepática de glicose e uma menor captação de glicose no músculo esquelético (Cho *et al*., 2001).

De maneira contrária às ações da insulina, a expressão da PEPCK e da G-6-Pase é estimulada pelo glucagon, pelas catecolaminas e pelos glicocorticóides. Os mecanismos pelos quais estes hormônios controlam a expressão destas enzimas ainda não são consenso na literatura, mas certamente envolvem a ativação conjunta de diversos fatores de transcrição tais como PGC1alfa, GR (*glucocorticoid receptor*), HNF-3 (*hepatocyte nuclear factor*) e HNF-4 (Barthel & Schmoll, 2003).

As comunicações inter-órgãos e a regulação extra-hepática da PHG pela insulina

Apesar de serem relativamente bem esclarecidos os mecanismos intracelulares pelos quais a insulina e os hormônios contra reguladores modulam de maneira direta a gliconeogênese, pouco ainda se sabe sobre os eventos moleculares que compreendem a regulação indireta da PHG mediada pela ação insulina em territórios extra-hepáticos. Neste sentido, trabalhos recentes demonstraram experimentalmente que determinadas regiões hipotalâmicas são importantes alvos cuja ação da insulina repercute negativamente sobre a gliconeogênese hepática (Obici *et al*., 2002; Pocai *et al*., 2005).

Desta maneira, foi demonstrado que a injeção intracerebroventricular (ICV) de pequenas quantidades de insulina é capaz de reduzir a glicemia em um modelo experimental cujas concentrações circulantes de insulina se mantiveram constates. Mais importante, a diminuição da glicemia decorrente da ação hipotalâmica da insulina resulta da supressão da liberação de glicose pelo fígado, e não do aumento da captação de glicose pelo tecido muscular (Obici *et al*., 2002). Além disto, estes mesmos experimentos demonstraram que a transmissão da via de sinalização da insulina a partir do seu receptor transmenbrânico (IR) até a ativação da PI3K/Akt no hipotálamo é um evento molecular necessário para o desencadeamento deste evento biológico (Obici *et al*., 2002). Mais especificamente, pode-se dizer que os efeitos da injeção ICV da insulina sobre a gliconeogênese são reproduzidos por injeções deste mesmo hormônio no

hipotálamo médio-basal e no núcleo hipotalâmico ventromedial (Pocai *et al*., 2005; Iguchi *et al*., 1981).

A transmissão da informação a partir do sistema nervoso central até o fígado é foco atual de investigações por parte de vários grupos. Até presente momento pode-se afirmar dois consensos: (i) a geração da informação em neurônios do hipotálamo ventromedial e do hipotálamo médiobasal requer a transmissão intracelular do sinal da insulina desde as etapas iniciais que compreendem as fosforilações em resíduos de tirosina no IR e no IRS1 (Obici *et al*., 2002), e (ii) a ativação do sistema nervoso autonômico parassimpático, possivelmente o ramo hepático do nervo vagal, compreende a transmissão da sinalização da insulina a partir do sistema nervoso central (SNC) para o território hepático. Esta última afirmação é corroborada por trabalhos que demonstraram que tanto a vagotomia quanto o tratamento prévio com atropina impediram a supressão da produção hepática de glicose induzida pela injeção central de insulina (Szabo *et al*., 1983; Pocai *et al*., 2005).

OBJETIVO

Objetivo Geral

Investigar se a ativação da via da hipotalâmica da PI3K/AKT pela melatonina resulta na modulação da gliconeogênese hepática.

Objetivos Específicos

- Estudar se a injeção intracerebroventricular (icv) de melatonina reduz a gliconeogênese;
- Estudar se a via PI3K/AKT hipotalâmica é importante para o efeito hepático da injeção icv de melatonina;
- Estudar se os receptores MT1/MT2 hipotalâmicos são importante para o efeito hepático da injeção icv de melatonina;
- Estudar se a atividade parassimpática vagal é importante para o efeito hepático da injeção icv de melatonina.

MATERIAIS E MÉTODOS

Animais e tratamentos

Foram utilizados ratos Wistar adultos mantidos em ciclo de iluminação (12h claro/12h escuro) e temperatura controlados, com alimentação e água *ad libitum*. Os animais foram fornecidos pelo Biotério Central da UNICAMP (CEMIB) e submetidos a protocolos experimentais quando completaram 8 semanas de vida.

Para a implantação de uma cânula no ventrículo lateral hipotalâmico, os animais foram previamente anestesiados por via intraperitonial com uma mistura de 1:1 de cloridrato de cetamina e diazepan. Depois de testados os reflexos pedioso, corneano e caudal, os animais foram posicionados no aparelho de estereotaxia e seguiu-se as coordenadas do Atlas Paxinos-Watson (Paxinos & Watson, 1986). Após o período de uma semana de recuperação da cirurgia estereotáxica, os animais receberam diferentes tratamentos através de injeções pelas cânulas. Para a injeção ICV de cada tratamento foi utilizada uma seringa Hamilton (50 µL) acoplada, por meio de uma cânula plástica a uma agulha 30G, de modo que esta fosse capaz de ultrapassar o comprimento da cânula metálica (0,1 – 0,2 mm) garantindo a entrada do líquido no ventrículo lateral.

O tratamento com melatonina foi feito inicialmente em 4 doses (2.8 nM, 14 nM, 28 nM e 140 nM) . Esta doses foram selecionadas a partir de estudos de Anhe *et al*. (2004) e foi avaliado qual delas demonstra ser mais eficiente na ativação aguda da AKT no hipotálamo de ratos. Foram realizados experimentos dose resposta com o intuito de definir a menor dose de melatonina

intracerebroventricular eficiente na promoção dos efeitos hepáticos e hipotalâmicos.

O tratamento proposto consistiu de uma injeção aguda de melatonina duas horas antes do teste de tolerância ao piruvato (PTT) e antes da extração de fígado e hipotálamo para estudo da sinalização. Para o tratamento, melatonina foi diluída em álcool absoluto (solução estoque); desta solução, foi retirada uma alíquota a qual foi adicionada a 5 ml de água, caracterizando a solução tratamento 28 nM, a dose selecionada para a realização dos demais protocolos. Desta solução, 2 microlitros foram injetados agudamente duas horas antes de qualquer protocolo executado. Quando usados, os inibidores de PI3K/AKT (LY 294002), de MT2 (4-P-PDOT) e de MT1/MT2 (Luzindole) foram injetados concomitantemente com a melatonina.

O desafio agudo ICV de melatonina foi inicialmente realizado para investigação da fosforilação da AKT hipotalâmica. Quando ativada a via PI3K/AKT, investigamos se houve repercussão periférica através da fosforilação de STAT3 hepático e sua ligação ao promotor da PEPCK e G6Pase. Neste ensaio também foram investigadas a expressão da PEPCK e G6Pase e a produção hepática de glicose a partir do piruvato de sódio.

Produção de glicose após desafio intraperitoneal de piruvato

O teste de tolerância intraperitoneal ao piruvato foi realizado com a finalidade de estimar a taxa de gliconeogênese em cada animal submetido a um diferente tratamento (Yao & Nyomba, 2008). Os ratos receberam uma injeção

intraperitoneal de piruvato (2 g/kg) dissolvido em uma solução de NaCl 0,9% (m/v). A glicemia foi mensurada anteriormente à injeção de piruvato de sódio e, após a mesma, nos tempos 15, 30, 60, 90, 120 minutos a partir do sangue total colhido de uma pequena incisão na cauda do animal.

Imunoblotting

Fragmentos de aproximadamente 100 mg de tecido hepático e hipotalâmico retirados dos animais foram homogeneizados com um Polytron (Kinematica. Suíça) em 2 mL de tampão de extração (SDS 1%, Tris (pH 7,4) 100mM, pirofosfato de sódio 100mM, fluoreto de sódio 100mM, EDTA 10mM, ortovanadato de sódio 100mM) e incubadas à 96ºC por 10 min. Em seguida, as amostras foram centrifugadas para a remoção do material insolúvel. Após centrifugação, parte do sobrenadante das amostras foi utilizada para determinação do conteúdo protéico por espectrofotometria com reagente Bradford (Biorad, CA, USA) e o restante foi acrescido de tampão Laemmli 5X e incubado à 96ºC por 10 min. A mesma quantidade de proteínas totais de cada amostra tratada com Laemmli foi fracionada em SDS-PAGE (2,6%C e 8-12%T) em aparelho para minigel (*Mini-Protean, Bio-Rad*). Após separação eletroforética, as proteínas foram transferidas para uma membrana de nitrocelulose (Bio-Rad, CA, USA).

As membranas foram então bloqueadas com uma solução contendo BSA 5%, Tris-Base, NaCl e Tween-20 por 2 horas a temperatura ambiente. Após o bloqueio, as membranas foram incubadas com anticorpos específicos contra fosfo-AKT 1/2/3(Ser 473), AKT1/2/3, fosfo-STAT3 (Tyr), STAT3, PEPCK, G6Pase e B-

actina por 4 horas a temperatura ambiente. Após marcação com anticorpo primário, as membranas foram incubadas com anticorpo secundário conjugado à peroxidase por 1 hora à temperatura ambiente (GE Healthcare, USA). Antes da detecção, as membranas foram incubadas por 1 minuto com uma solução contendo luminol, ácido p-cumárico e H_2O_2 e, então, expostas durante tempos variados a filmes de raioX. Depois de expostos, esses filmes foram submetidos a análise por densitometria óptica pelo software Scion Image (Scioncorp, NIH, USA).

Dosagem dos níveis plasmáticos hormonais

O sangue dos animais foi coletado e estocado para determinação dos níveis plasmáticos de corticosterona, insulina, melatonina e catecolaminas.

Chromatin Imuno-Precipitation (ChIP) Assay

Fragmentos de fígado foram submetidos a uma etapa inicial de *cross-link* com formaldeído 1% a temperatura ambiente por 10 minutos, seguida da fragmentação da cromatina por sonicação em fragmentos entre 500 e 1.000 pares de bases. Os fragmentos de DNA ligados aos fatores transcricionais de interesse foram separados por imunoprecipitação com anticorpo específico anti-pSTAT3-Tyr e esferas de sefarose revestidas de Proteína A. O *cross-link* do DNA com o fator de transcrição foi revertido com incubação das amostras a 65°C por 24 horas. No final, o DNA foi purificado com fenol-clorofórmio e submetido à amplificação por PCR com *primers* específicos para a região promotora de genes da PEPCK e G6Pase.

Ensaio de Imunofluorescência

Foram feitos cortes de 5µM do hipotálamo. Foi utilizado DAPI para marcação dos núcleos celulares, anticorpo primário anti-pAkt (Ser473), anticorpo primário anti-MT1, anticorpo primário anti-MT2 e anticorpo secundário conjugado a FITC para marcação da pAkt e anticorpo secundário conjugado a Rodamina para marcação de MT1 ou MT2. Todos os anticorpos utilizados neste ensaio foram fornecidos pela *Santa Cruz biotechnology*®, inc.

Análise estatística

Os resultados foram expressos como média ± erro padrão da média (EPM) e analisados estatisticamente por análise de variância (ANOVA de uma via com pós-teste de Tukey, ou duas vias, quando apropriado). Também foi realizado, quando adequado, o teste de *Student*. Em todos os resultados foram adotados 5% como limite de significância estatística ($p < 0,05$).

CAPÍTULO 1 – ARTIGO

Melatonin acts through MT1/MT2 receptors to activate hypothalamic AKT and suppress hepatic gluconeogenesis in rats

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Word count

Main text: 3710

Abstract: 248

Abstract

Aims/Hypothesis: Melatonin can contribute to glucose homeostasis either by decreasing gluconeogenesis or by counteracting insulin resistance in distinct models of obesity. However, the precise mechanism through which melatonin controls glucose homeostasis is not completely understood.

Methods: Wistar rats received intracerebroventricular (icv) injections containing melatonin combined with icv administration of phosphatidylinositol 3-kinase (PI3K) inhibitors, melatonin receptors (MT) antagonists, or ip administration of a muscarinic receptor antagonist. Melatonin injections were also given in the mediobasal hypothalamus (MBH). Gluconeogenesis was assessed by pyruvate tolerance tests, and the hypothalamus was removed to determine AKT phosphorylation and co-localisation with MT1 and MT2. PEPCK and glucose-6-phosphatase (G6Pase) expression and signal transducer and activator of transcription 3 (STAT3) phosphorylation and binding activity were monitored in the liver.

Results: Melatonin injections, both in the third ventricle and in the MBH, suppressed gluconeogenesis, PEPCK and G6Pase expression and increased hypothalamic AKT phosphorylation in MT1/MT2-positive neurons. Icv-injected melatonin also increased STAT3 binding activity in the liver, a known repressor of PEPCK and G6Pase. These effects of melatonin were suppressed either by icv injections of PI3K inhibitors and MT antagonists or by ip injection of a muscarinic receptor antagonist. Icv-injected melatonin did not change the circulating levels of insulin and other glucoregulatory hormones.

Conclusions/Interpretation: We conclude that melatonin activates hypothalamus-liver communication that may contribute to the circadian adjustments of gluconeogenesis. These

data further suggest a physiopathological relationship between the circadian disruptions in metabolism (hallmarked by morning hyperglycaemia) and reduced levels of melatonin found in type 2 diabetes patients.

Key words: Melatonin, gluconeogenesis, melatonin receptors, liver

Abbreviations

AKT - insulin-stimulated RAC-α serine/threonine-protein kinase

ARC - Arcuate nucleus

CNS - Central nervous system

ChIP - Chromatin immunoprecipitation

GABA - γ-amino butyric acid

GPCR - G protein-coupled receptor

G6Pase - Glucose-6-phosphatase (protein)

G6pc - Glucose 6 phosphatase (gene)

HGP - hepatic glucose production

IpBr - Ipratropium bromide

LH - Lateral hypothalamus

MBH - Mediobasal hypothalamus

MT1 - Melatonin receptor 1

MT2 - Melatonin receptor 2

PVN - Paraventricular nucleus

Pck1 - Phosphoenolpyruvate carboxykinase 1 (gene)

PI3K - Phosphatidylinositol 3-kinase

SCN - Suprachiasmatic nucleus

STAT3 - Signal transducer and activator of transcription 3

Introduction

Melatonin (5-methoxy-N-acetyltryptamine) is produced and secreted by the pineal gland in a circadian fashion, with peak levels during the dark phase of the light/dark cycle. The canonical function of melatonin is to transduce the environmental information (i.e., the length of the dark period) to the living organism, thereby synchronising the circadian clock in the hypothalamic suprachiasmatic nucleus (SCN) [1]. *In vivo* and *in vitro* experiments have demonstrated that melatonin also plays a role in energy homeostasis by regulating body mass and adiposity and the circadian rhythm of leptin expression [2,3]. Glucose homeostasis is also altered by the absence of melatonin in such way that pinealectomised rats display glucose intolerance and desynchronised circadian pattern of gluconeogenesis, hallmarked by increased night-time glucose levels [4,5,6]. Moreover, melatonin administration has been shown to improve glucose homeostasis not only in pinealectomised rats but also in rats rendered insulin resistant by diet manipulation [7,8,9].

Although it has been demonstrated that melatonin stimulates glucose uptake in adipocytes and skeletal muscle cells in vitro [10,11], the precise mechanism by which this hormone reduces whole-body glucose intolerance has not been precisely determined. In mammals, the effects of melatonin are mediated in part by specific high-affinity G protein-coupled receptors (GPCRs), known as melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2) [12]. We have previously demonstrated that melatonin acts locally in the hypothalamus to activate insulin signal transmission through a pathway that is dependent

on MT1/MT2 receptors [13]. However, the metabolic relevance of this signal triggered by melatonin remains unknown.

Studies over the past decade have demonstrated the pivotal role of hypothalamic insulin signalling in the control of hepatic glucose production (HGP). The insulin-activated phosphatidylinositol 3-kinase/insulin-stimulated RAC-α serine/threonine-protein kinase (PI3K/AKT) pathway in the hypothalamus has been shown to suppress HGP, irrespective of changes in peripheral insulin and other glucoregulatory hormones [14,15]. The pathways underlying this inter-organ communication are still being investigated, but data collected so far suggest that hypothalamic activation of PI3K/AKT transmits parasympathetic inputs to the liver that may lead to the activation of the hepatic signal transducer and activator of transcription 3 (STAT3) [16,17,18]. Activated STAT3 in the liver may repress the transcription of glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase (*Pck1*) genes, which has implications for two of the limiting steps of gluconeogenesis [19].

In the present study, we sought to demonstrate whether melatonin-induced AKT activation within specific regions of the mediobasal hypothalamus (MBH) would control hepatic gluconeogenesis. We also aimed to determine whether melatonin action in the central nervous system generates parasympathetic inputs to the liver that modulate STAT3 activity and *G6pc* and *Pck1* repression.

Research design and methods

Surgical procedures and treatments

Male Wistar rats weighing approximately 180 g were obtained from the Animal Breeding Center at the University of Campinas (Campinas, Sao Paulo, Brazil) and were housed under

a 12 h/12 h light dark cycle with free access to food and water. Rats were anaesthetised with diazepam and ketamine (2 mg/kg and 50 mg/kg, respectively) and cannulated using a stereotaxic apparatus to fix a stainless steel cannula into the lateral ventricle. Stereotaxic coordinates were 0.2 mm anteroposterior, 1.5 mm lateral, and 4.0 mm deep. The localisation of the cannula was tested by evaluating the drinking response to intracerebroventricular (icv) angiotensin II injection 1 week after surgery [20]. Cannulas were also implanted in the MBH using the following the coordinates: 3.1 mm posterior to bregma, 0.4 mm lateral, and 9.6 mm deep. Localisation of the cannula was confirmed in euthanised animals by staining of the hypothalamic region (Bregma -3.1) after an injection with bromophenol blue (ESM Fig. 1).

Angiotensin-responsive rats received an icv injection containing 2 μL of a melatonin solution (Cat. No. A9525, Sigma-Aldrich, St. Louis, USA) at 9:00 AM. Melatonin concentrations in the injected solutions were 2.8, 14, 28 and 140 nM. In all cases, the melatonin dilutions were prepared from a 71.8 mM stock solution (100% ethanol). The final concentration of ethanol was adjusted to 0.2% in all melatonin dilutions, and 0.2% ethanol was given to CTL rats.

LY294002 (Cat. No. 1130; Tocris, Bristol, UK) was initially diluted in 100% ethanol to generate a 5 mM stock solution and further diluted to 1.7 mM (33% ethanol). Either this solution or its vehicle (33% ethanol) was mixed either with 28 nM melatonin or 0.2% ethanol at a ratio of 1:3. Three microlitres of either of these mixtures was injected through the cannula implanted in the lateral ventricle.

Luzindole and 4P-PDOT (Cat. No. 0877 and 1034, respectively; Tocris, Bristol, UK) were diluted in 100% ethanol to a concentration of 100 mM and further diluted to 33

mM (33% ethanol). Either one of these solutions or their vehicle (33% ethanol) was mixed with 28 nM melatonin or 0.2% ethanol to the ratio of 1:3. Three microlitres of either of these mixtures was injected through the cannula implanted in the lateral ventricle.

Wortmannin (Cat. No. 1232 from Tocris, Bristol, UK) was diluted in 100% DMSO to a final concentration of 300 nM. This solution or its vehicle (100% DMSO) was mixed either with 28 nM melatonin or 0.2% ethanol to a ratio of 1:3. Three microlitres of either of these mixtures was injected through the cannula implanted in the lateral ventricle.

Ipratropium bromide (50 mg/kg) (Cat No I1637, Sigma-Aldrich, St. Louis, USA) or its vehicle (0.9% NaCl) was ip injected 30 min before the icv injections containing 2 μL of either 28 nM melatonin or 0.2% ethanol.

All experiments were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation and were approved by the State University of Campinas Committee for Ethics in Animal Experimentation.

Intraperitoneal Pyruvate Tolerance Test

Rats were fasted for 13 h, and a sodium pyruvate solution (250 mg/mL) was ip injected at a dosage of 2 g/Kg 2 hours after the icv injections. Glucose levels were determined in blood extracted from the tail before (0 min) and 15, 30, 90 and 120 min after an ip pyruvate injection. The AUC of glycaemia vs. time was calculated using each individual baseline (basal glycaemia) measurement to estimate the total glucose synthesised from pyruvate. We have previously demonstrated that gluconeogenesis accounts for the increase in glucose levels using 3-mercaptopicolinic acid (3-mpa), an inhibitor of gluconeogenesis, 30 min before the pyruvate injection [5].

Protein extraction and immunoblotting

Anaesthetised rats were decapitated either 90 min after the icv injections for the removal of the hypothalamus or 120 min after the icv injections for the removal of liver fragments. The hypothalamus and a fragment of the liver (approximately 100 mg) were removed and processed for Western blotting as previously described [5]. The primary antibodies anti-AKT1/2/3, anti-pAKT1/2/3 (Ser473), anti-STAT3, anti-pSTAT3 (Tyr705), anti-G6Pase and anti-PEPCK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody against β-actin was obtained from Abcam, Cambridge, UK. Secondary antibodies conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA, USA) were applied, and chemiluminescent detection of the bands on X-ray-sensitive films was performed. Optical densitometry of the films was performed using the Scion Image analysis software (Scion Corporation, Frederick, MD, USA).

Immunofluorescent staining

The central nervous systems (CNS) were removed 90 min after icv injection with either 28 nM melatonin or ethanol 0.2% and processed for immunofluorescent staining as previously described [21]. We used the anti-pAKT (Ser473), anti-MT1 or anti-MT2 antibodies (Santa Cruz, CA, USA). Secondary FITC-conjugated antibody was used to visualise pAKT staining, and secondary rhodamine-conjugated antibody was used to visualise either MT1 or MT2. A separate set of sections from were stained with only the secondary antibodies (omitting the primary antibody) to ensure specificity of the fluorescent signals (ESM Fig. 2). Images were acquired under high magnification (400x).

Hormone measurements

Trunk blood was collected 60 min after the icv injections. Plasma was stored with EDTA for epinephrine and norepinephrine determination. Serum was also extracted for corticosterone, melatonin and insulin determinations. Catecholamines (Cat. No. E-6500, Rocky Mountains, Colorado Springs, CO, USA) and insulin (Cat No. EZRMI-13K, Merck Millipore, Billerica, MA, USA) were quantified by ELISA according to the manufacturer's instructions. Corticosterone and melatonin were measured with the MILLIPLEX Map Rat Stress Hormone Panel (Cat. No. RSH69K, Merck Millipore, Billerica, MA, USA) using the MILLIPLEX Analyzer System (Merck Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Chromatin Immunoprecipitation (ChIP) assay

Liver fragments for ChIP assays were removed 120 min after the icv injections and processed as previously described [22]. After DNA shearing, samples were pre-cleared for 1 h at 4°C with protein A-Sepharose saturated with salmon sperm DNA. An aliquot of 10 µL was collected as the "input". The remaining supernatants were immunoprecipitated with protein A-Sepharose and 2 µg of anti-pSTAT3 (Tyr705) antibody (Santa Cruz, CA, USA). In parallel, one sample was incubated with only protein A-Sepharose to generate the negative control (no-AB). DNA extracted from the Sepharose pellets was subjected to crosslinking reversal and purification using phenol-chloroform. DNA samples were amplified for detection of the *Pck1* and the *G6pc* genes. A 179 bp fragment flanking bases 164 to 342 of the rat *Pck1* gene and a 172 bp fragment flanking the bases 63 to 234 of the

rat *G6pc* gene were amplified by real-time PCR. The sequences of the primers were: *Pck1* sense 5'-TGGTCTGGACTTCTCTGCCAAG-3', *Pck1* antisense 5'-GGATGACACCCTCCTCCTGC-3' (annealing at 62°C), *G6pc* sense 5'-GTACCAAGGAGGAAGGATGGAGG-3' and *G6pc* antisense 5'-GTGGAACCAGATGGGAAAGAGG-3' (annealing at 56°C). To check the primer specificity (by estimated product length), reaction products were resolved on an EtBr-agarose gel. pSTAT3 binding was calculated after normalisation to the input of each sample.

Statistical Analysis

The results are presented as the means ± S.E. Comparisons were performed using an unpaired Student's t-test or a one-way ANOVA, followed by Tukey-Kramer post hoc testing when appropriate (INStat - Graph Pad Software, Inc., San Diego, USA). P values <0.05 indicate a significant difference.

Results

Intracerebroventricular injections with melatonin activate AKT in the hypothalamus and suppress gluconeogenesis

The icv melatonin injections stimulated hypothalamic AKT phosphorylation in a dose-dependent manner. Melatonin, at the concentration of 2.8 nM, did not stimulate hypothalamic AKT phosphorylation. However, when used at the concentrations of 14, 28 and 140 nM, icv-injected melatonin induced a similar increase in AKT phosphorylation in

the hypothalamus (4.1-, 3.36- and 3.82-fold higher than CTL, respectively; $P<0.05$) (Fig. 1a).

The intraperitoneal pyruvate load induced a lower increase in blood glucose in rats that received icv melatonin injections at the 14 and 28 nM concentrations but not at the 2.8 and 140 nM concentrations. This effect was best observed 120 and 150 min after the pyruvate ip injection, when glucose levels in the rats that received 14 and 28 nM melatonin were approximately 25% and 17% lower, respectively, than the glucose levels in the CTL ($P<0.05$) (Fig. 1b). The AUC for the rats that received 14 and 28 nM melatonin were 70% and 62% lower, respectively, than the AUCs for the CTL ($P<0.05$) (Fig. 1c).

We also found reduced levels of PEPCK and glucose-6-phosphatase (G6Pase) expression in the livers of the rats that received icv injections with 14 and 28 nM melatonin. The PEPCK and G6Pase levels were 22% and 64% lower, respectively, than the levels in the CTL animals that received 14 nM melatonin, and the levels were 71% and 85% lower, respectively, than the levels in the CTL animals that received 28 nM melatonin (Fig. 1d and e). Simultaneously, icv-injected melatonin at 14 and 28 nM induced a progressive increase in hepatic STAT3 tyrosine phosphorylation (5.71- and 7.64-fold higher, respectively, than in the CTL; $P<0.05$) (Fig. 1f).

Suppression of gluconeogenesis induced by icv melatonin depends on hypothalamic PI3K/AKT activation

To determine the existence of a causal relationship between the melatonin-induced hypothalamic activation of AKT and the suppression of gluconeogenesis, we next combined melatonin with either LY294002 or Wortmannin, two classical inhibitors of

PI3K-dependent AKT activation. In these experiments, hypothalamic AKT phosphorylation was stimulated by melatonin (45% higher than in the CTL; $P<0.05$) but was not stimulated by melatonin combined with LY294002 (Fig. 2a). Likewise, gluconeogenesis and PEPCK and G6Pase hepatic expression were inhibited by icv-injected melatonin (64%, 33% and 49% lower, respectively, than in the CTL; $P<0.05$) but not by melatonin combined with LY294002 (Fig. 2b-c, d and e). In agreement with these findings, the stimulation of hepatic STAT3 tyrosine phosphorylation by icv-injected melatonin (7.3 fold higher than in the CTL; $P<0.05$) was suppressed by the combination of melatonin and LY294002 (Fig. 2f).

Experiments using melatonin combined with Wortmannin yielded results similar to those observed for the combination of melatonin and LY294002 (ESM Fig. 3).

Melatonin acts through membrane MT1/MT2 receptors in the hypothalamus to activate AKT and suppress gluconeogenesis

We have previously demonstrated that melatonin-induced AKT activation in the hypothalamus is dependent on upstream activation of the MT1/MT2 receptors [13]. To assess whether the suppression of gluconeogenesis would also depend on the previous interaction of melatonin with the MT1/MT2 receptors, we then used luzindole (a nonspecific MT1/MT2 antagonist) and 4P-PDOT (a specific MT2 antagonist).

In this set of experiments, melatonin-induced AKT phosphorylation (3.0 fold higher that in the CTL; $P<0.05$) was completely suppressed by the combined use of melatonin and luzindole (Fig. 3a). The suppression of gluconeogenesis and PEPCK and G6Pase expression induced by icv-injected melatonin (58%, 47% and 54% lower, respectively, than in the CTL; $P<0.05$) was not observed in rats that received melatonin plus luzindole (Fig.

3b-c, d and e). Icv melatonin-induced hepatic STAT3 tyrosine phosphorylation (2.8 fold higher than in the CTL; $P<0.05$) was also suppressed by the combination of melatonin and luzindole (Fig. 3f).

The binding activity of STAT3 is directly determined by its tyrosine phosphorylation. Activated STAT3, in turn, has been shown to bind to promoter regions of the *Pck1* and *G6pc* genes and to repress their transcription [19]. To evaluate whether the suppression of gluconeogenesis induced by icv-injected melatonin was causally linked to the stimulation of STAT3 phosphorylation, we then assessed the in vivo binding of STAT3 to *Pck1* and *G6pc* promoters by using a ChIP assay. Icv injections containing melatonin stimulated hepatic STAT3 binding to the *Pck1* and *G6pc* genes (70% and 93% higher, respectively, than in the CTL; $P<0.05$). In accordance with our data on STAT3 phosphorylation, the icv injection of luzindole abrogated the melatonin-induced binding of hepatic STAT3 to the *Pck1* and *G6pc* genes (Fig. 3g and h).

The stimulation of hypothalamic AKT phosphorylation by icv-injected melatonin (2.3 fold higher than in the CTL; $P<0.05$) was also suppressed by the co-injection of 4P-PDOT (Fig. 4a). Accordingly, the melatonin-induced suppression of gluconeogenesis and PEPCK and G6Pase expression (74%, 57% and 55% lower, respectively, than in the CTL; $P<0.05$) was blocked by the combination of melatonin and 4P-PDOT (Fig. 4c, d and e).

The stimulation of STAT3 tyrosine phosphorylation and binding to the *Pck1* and *G6pc* genes induced by icv-injected melatonin (130%, 97% and 91% higher, respectively, than in the CTL; $P<0.05$) were also suppressed by the simultaneous use of melatonin and 4P-PDOT (Fig. 4f, g and h).

Melatonin-induced AKT phosphorylation in the MBH reduces gluconeogenesis

It has been previously reported that impaired insulin-induced PI3K/AKT activation in regions of the MBH is related to the increase in HPG [14]. We proceeded to study the importance of melatonin action in the MBH for the reduction of gluconeogenesis. First, we demonstrated that MT1 co-localises with phosphorylated AKT in distinct regions of the MBH after icv melatonin injections. Merged high-magnification images showed that MT1-positive cells became stained for phosphorylated AKT in the arcuate nucleus (ARC), lateral hypothalamus (LH) and paraventricular nucleus (PVN) after icv melatonin injection (Fig. 5). A similar pattern was observed in sections stained with antibodies against MT2 and phosphorylated AKT. Merged high-magnification images showed that MT2-positive cells became stained for phosphorylated AKT in the ARC, LH and PVN after icv melatonin injection (Fig. 6a).

We also showed that an injection of melatonin (28 nM) directly into the MBH reduced pyruvate conversion into glucose. This result was better observed 90 and 150 min after the pyruvate injection (23% and 32% lower, respectively, than the CTL levels; $P<0.05$) (Fig. 6b). The AUC for rats that received melatonin in the MBH was 48% lower than for the CTL ($P<0.05$) (Fig. 6c).

Melatonin-induced hypothalamic AKT activation transmits information to the liver through activation of peripheral muscarinic receptors

After establishing a causal relationship between melatonin-induced hypothalamic AKT activation and a reduction in gluconeogenesis, we next investigated the mechanism underlying the transmission of information from the central nervous system to the liver. We

measured initial melatonin levels in rats that received icv injections containing either melatonin (28 nM) or vehicle. We found no differences in the circulating melatonin levels, and therefore we discarded the possibility of a direct hepatic melatonin action. Icv injections containing melatonin did not increase circulating insulin nor decreased the levels of the classic glucoregulatory hormones corticosterone, epinephrine and norepinephrine (Table 1).

Given the evidence suggesting the lack of participation of an endocrine signal in the inter-organ communication triggered by the hypothalamic action of melatonin, we decided to investigate the participation of the parasympathetic nervous system. The experimental protocol we used consisted of the use of i.p. injections containing ipratropium bromide (IpBr) before administering icv injections with melatonin. IpBr is an M2/M3 muscarinic receptor antagonist that does not cross the blood-brain barrier [23].

The icv injections with melatonin reduced gluconeogenesis in the rats that received previous i.p. injections with vehicle. This effect was better observed at 90 and 150 min after the pyruvate injection (24% and 20% lower, respectively, than the CTL levels; $P<0.05$). Previous i.p. injections of IpBr blocked this effect of melatonin (Fig. 7a). The AUC values in rats that received icv melatonin and i.p. IpBr were 2.8 fold higher than those of rats that received icv melatonin and i.p. vehicle ($P<0.05$) (Fig. 7b). As proof that IpBr did not affect the hypothalamic action of melatonin, we found that AKT phosphorylation was increased in the rats that received icv melatonin concomitantly with either i.p. vehicle or i.p. IpBr (1.5 and 2.4 fold higher, respectively, than in the CTL; $P<0.05$) (Fig. 7c).

In agreement with the data of the pyruvate tolerance test, the previous i.p. injection of IpBr suppressed the reduction of PEPCK and G6Pase induced by icv melatonin (Fig. 7d

and e). Additionally, the icv melatonin-induced hepatic STAT3 phosphorylation and binding to *Pck1* and *G6pc* genes were abrogated by the previous i.p. injections containing IpBr (Fig. 7f, g and h).

Discussion

Recent studies conducted on humans suggest that a disruption in rhythmic melatonin production is relevant to the aetiology of diabetes. Nocturnal melatonin levels were reported to be significantly reduced in obese patients with type 2 diabetes compared with weight-matched controls [24]. Reduced nocturnal melatonin secretion is also correlated with increased gluconeogenesis during the first hours of the morning in patients with type 2 diabetes [25]. In agreement with these studies, rare variants of the MT2 receptor that cause total or partial loss of function are associated with increased type 2 diabetes risk [26]. The present study shows that melatonin activates inter-organ communication between the hypothalamus and the liver, leading to the suppression of gluconeogenesis. Therapeutically, the potential of melatonin is highlighted by studies demonstrating that its use, either alone or combined with other agents, can improve glycaemic control [27,28].

Studies with rodents have also collected compelling data favouring the proposition that melatonin controls glucose homeostasis. Rats that become spontaneously obese and insulin resistant show a progressive decline in nocturnal melatonin production as they become glucose intolerant [29]. On the other hand, melatonin supplementation can improve the glucose intolerance induced by high-fat and high-fructose diets [7,8]. We have previously reported the ability of melatonin to control gluconeogenesis using experiments that showed that surgical ablation of the pineal gland increased the pyruvate conversion

into glucose and hepatic PEPCK expression [5]. Supporting our present hypothesis that melatonin may suppress gluconeogenesis due to its action in the central nervous system, a previous study demonstrated that intracranial, rather than intraperitoneal, injections of melatonin effectively suppressed the hyperglycaemic response to the intracranial injection of 2-deoxy-D-glucose [30].

The two experimental findings presented here convincingly demonstrate that the melatonin-induced suppression of hepatic PEPCK and G6Pase expression and gluconeogenesis occurs due to melatonin's primary action in the hypothalamus: (i) no changes in circulating melatonin levels were found in rats that received icv injections containing the hormone and (ii) melatonin injection directly into the MBH can reduce whole-body gluconeogenesis. Considering that the total volume of cerebrospinal fluid is approximately 400 μL [31], the 2 μL of the 28 nM icv-injected melatonin yielded a final concentration of approximately 0.14 nM, which efficiently suppressed gluconeogenesis. This concentration is very similar to that observed in the cerebrospinal fluid of healthy volunteers [32].

Corroborating the present hypothesis, a study by Cailotto et al. showed that rats exposed to light during the night have increased hepatic levels of PEPCK and G6Pase, with reduced levels of melatonin-synthesising enzyme in the pineal gland [33]. Exposure to short periods of light during the night is classically known to suppress melatonin production [34]. Cailotto et al. also showed that the autonomic inputs to the liver mediate the effects of nocturnal light over hepatic PEPCK expression, given that the above-mentioned effects were suppressed by sympathetic and parasympathetic hepatic denervation [33]. Our data reveal that the suppression of gluconeogenesis and hepatic

PEPCK and G6Pase induced by icv melatonin is abrogated by the peripheral blockade of muscarinic receptors. The description of a parasympathetic transmission to the liver arising from melatonin-induced hypothalamic AKT activation has already been reported to be activated by leptin and insulin [16,35].

As our data reveal, the hypothalamic signal triggered by melatonin starts with the MT1/MT2-dependent AKT activation in distinct regions of the MBH hypothalamus. Accordingly, melatonin activates AKT in peripheral organs through the previous interaction with MT1/MT2 receptors [11,13].

Our study further demonstrates that melatonin-mediated parasympathetic transmission to the liver suppresses gluconeogenesis due to STAT3 activation. This conclusion arose from our data showing that icv melatonin stimulated the in vivo binding of STAT3 to *Pck1* and *G6pc* genes. In turn, this response was inhibited by the central blockade of melatonin receptors and the peripheral blockade of muscarinic receptors. Hepatic STAT3 was demonstrated to bind to promoter regions of the *Pck1* and *G6pc* genes, inhibiting their transcription [19]. Similarly, insulin-induced AKT activation in the hypothalamus was also demonstrated to inhibit PEPCK and G6Pase expression through a mechanism dependent on hepatic STAT3 activation [18].

In summary, the present data show that melatonin activates a brain-liver communication that is triggered by MT1/MT2-dependent hypothalamic AKT activation, thus leading to the parasympathetic stimulation of hepatic STAT3. STAT3 activation in the liver, in turn, is likely to repress PEPCK and G6Pase expression, thereby suppressing gluconeogenesis. This inter-organ communication clarifies the beneficial effects of melatonin in rodent models of insulin resistance. In addition, this biological action of

melatonin may help us understand the observations linking disruptions in the light-dark cycle to glucose intolerance in humans [39,40].

Acknowledgements

The authors would like to thank Thyelle Cipriano (State University of Campinas) for technical assistance and Dr. José Cipolla-Neto and Dr. Fernanda G. Amaral for the melatonin measurements.

Funding

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Pesquisa (CNPq).

Conflicts of Interest

The authors declare that there are no conflicts of interest associated with this manuscript.

Contributing statement

The authors JAF, AK, LMI-S, TM de A, DSR, LBP and CL-S researched the data, and LAV participated in the analysis and interpretation of data and in the revision of manuscript. The authors SB and GFA conceived and designed the experiments and wrote and reviewed the manuscript. All authors approved the version to be published.

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Table 1 Circulating levels of melatonin, insulin and other glucoregulatory hormones after icv injection containing either vehicle or melatonin

	CTL	icv Melatonin
Melatonin (pg/mL)	20.97 ± 5.53	22.57 ± 1.56
Insulin (μU/mL)	65.67 ± 11.05	57.25 ± 6.21
Corticosterone (ng/mL)	184.80 ± 46.17	180.61 ± 38.83
Epinephrine (nM)	12.34 ± 1.23	13.88 ± 1.14
Norepinephrine (nM)	5.98 ± 0.08	5.93 ± 0.52

Rats were decapitated 60 min after receiving icv injections containing either vehicle (CTL) or melatonin (28 nM). Serum and plasma were collected and used for melatonin, insulin, corticosterone, epinephrine and norepinephrine measurements. Data are presented as the mean ± SEM (n=5).

Legends for Figures

Fig. 1 Icv-injected melatonin activates hypothalamic AKT and decreases gluconeogenesis, hepatic PEPCK and G6Pase. Three sets of 13 hour-fasted wistar rats received icv injections containing melatonin (2.8, 14, 28 or 140 nM) or vehicle (0,2% ethanol) (CTL). The first set of animals was decapitated 90 min after icv injections and the hypothalamus were removed and processed for western blot detection of AKT and pAKT. pAKT levels were normalized to AKT **(A)**. The second set of animals was subjected to pyruvate tolerance tests 2h after icv injections. Glycemia was measured before and 15, 30, 60, 90, 120 and 150 min after pyruvate injection. The full line with grey circles represents the CTL, dotted line with grey triangles represents 2.8 nM melatonin, the dotted line with grey lozenges represents 14 nM melatonin, the full line with black circles represents 28 nM melatonin and dotted line with grey squares represents 140 nM melatonin **(B)**. The AUC was calculated for each individual animal within each group **(C).** The third set of animals were decapitated 120 min after icv injections and a fragment of the liver was removed for western blot detection of PEPCK, G6Pase, β actin, pSTAT3 and STAT3 **(D)**. PEPCK and G6Pase levels were normalized to β actin **(**respectively **E** and **F)** and pSTAT3 was normalized to STAT3 **(G)**. The results are shown as the mean ±SE. #P<0.05 comparing CTL to 14 and 28 nM melatonin within the same time point; &P<0.05 vs. 14 nM melatonin; *P<0.05 vs. CTL (n=5 for western blots and n=4 for pyruvate tolerance tests).

Fig. 2 Icv-injected melatonin reduces gluconeogenesis, hepatic PEPCK and G6Pase through a mechanism dependent on hypothalamic PI3K/AKT pathway. Three sets of 13 hour-fasted wistar rats received icv injections containing melatonin (28nM),

LY294002 or the combination of both. CTL rats received the vehicle. The first set of animals was decapitated 90 min after icv injections and the hypothalamus were removed and processed for western blot detection of AKT and pAKT. pAKT levels were normalized to AKT **(A)**. The second set of animals was subjected to pyruvate tolerance tests 2h after icv injections. Glycemia was measured before and 15, 30, 60, 90, 120 and 150 min after pyruvate injection. The full line with open circles represents the CTL, the full line with open squares represents LY294002, the full line with black circles represents melatonin and the full line with black squares represents melatonin plus LY294002 **(B)**. The AUC was calculated for each individual animal within each group **(C).** The third set of animals were decapitated 120 min after icv injections and a fragment of the liver was removed for western blot detection of PEPCK, G6Pase, β actin, pSTAT3 and STAT3 **(D)**. PEPCK and G6Pase levels were normalized to β actin **(**respectively **E** and **F)** and pSTAT3 was normalized to STAT3 **(G)**. The results are shown as the mean ±SE. #P<0.05 comparing CTL to melatonin within the same time point; *P<0.05 vs. CTL (n=5).

Fig. 3 Icv-injected melatonin reduces gluconeogenesis, hepatic PEPCK and G6Pase and increases hypothalamic AKT phosphorylation through a mechanism dependent on MT1/MT2. Three sets of 13 hour-fasted wistar rats received icv injections containing melatonin (28nM), luzindole or the combination of both. CTL rats received the vehicle. The first set of animals was decapitated 90 min after icv injections and the hypothalamus were removed and processed for western blot detection of AKT and pAKT. pAKT levels were normalized to AKT **(A)**. The second set of animals was subjected to pyruvate tolerance tests 2h after icv injections. Glycemia was measured before and 15, 30, 60, 90, 120 and 150 min after pyruvate injection. The full line with

open circles represents the CTL, the full line with open squares represents the luzindole, the full line with black circles represents melatonin and the full line with black squares represents melatonin plus luzindole **(B)**. The AUC was calculated for each individual animal within each group **(C).** The third set of animals were decapitated 120 min after icv injections and a fragment of the liver was removed for western blot detection of PEPCK, G6Pase, β actin, pSTAT3 and STAT3 **(D)**. PEPCK and G6Pase levels were normalized to β actin **(**respectively **E** and **F)** and pSTAT3 was normalized to STAT3 **(G)**. A second fragment of the liver was removed and processed for chromatin immunoprecipitation using an anti-pSTAT3 antibody. The *Pck1* and *G6pc* genes were amplified from ChIP samples and normalized to the respective inputs **(**respectively **H** and **I)**. The results are shown as the mean ±SE. #$P<0.05$ comparing CTL to melatonin within the same time point; *$P<0.05$ vs. CTL; &$P<0.05$ vs. melatonin (n=4 for chromatin immunoprecipitations and n=5 for western blots and pyruvate tolerance tests).

Fig. 4 Icv-injected melatonin reduces gluconeogenesis, hepatic PEPCK and G6Pase and increases hypothalamic AKT phosphorylation through a mechanism dependent on MT2. Three sets of 13 hour-fasted wistar rats received icv injections containing melatonin (28nM), 4P-PDOT or the combination of both. CTL rats received the vehicle. The first set of animals was decapitated 90 min after icv injections and the hypothalamus were removed and processed for western blot detection of AKT and pAKT. pAKT levels were normalized to AKT **(A)**. The second set of animals was subjected to pyruvate tolerance tests 2h after icv injections. Glycemia was measured before and 15, 30, 60, 90, 120 and 150 min after pyruvate injection. The full line with open circles represents the CTL, the full line with open squares represents the 4P-

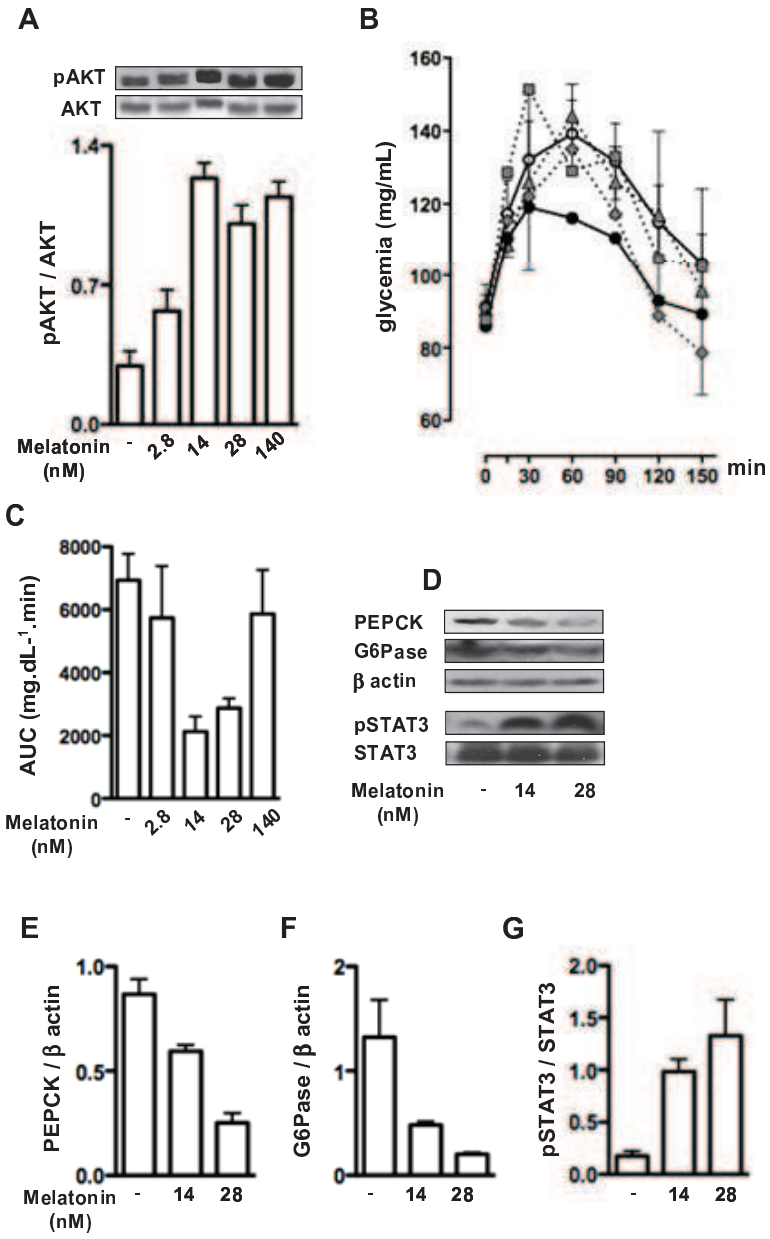
PDOT, the full line with black circles represents melatonin and the full line with black squares represents melatonin plus 4P-PDOT **(B)**. The AUC was calculated for each individual animal within each group **(C).** The third set of animals were decapitated 120 min after icv injections and a fragment of the liver was removed for western blot detection of PEPCK, G6Pase, β actin, pSTAT3 and STAT3 **(D)**. PEPCK and G6Pase levels were normalized to β actin **(**respectively **E** and **F)** and pSTAT3 was normalized to STAT3 **(G)**. A second fragment of the liver was removed and processed for chromatin immunoprecipitation using an anti-pSTAT3 antibody. The *Pck1* and *G6pc* genes were amplified from ChIP samples and normalized to the respective inputs **(**respectively **H** and **I)**. The results are shown as the mean ±SE. #$P<0.05$ comparing CTL to melatonin within the same time point; *$P<0.05$ vs. CTL; &$P<0.05$ vs. melatonin (n=5).

Fig. 5 Direct action of melatonin in the MBH results in co-localisation of hypothalamic pAKT with MT1. 13 hour-fasted wistar rats received icv injections containing either melatonin (28nM) or vehicle (0,02% ethanol) (CTL). The animals were decapitated 90 min after icv injections and the central nervous systems were removed and processed for immunefluorescent staining. Five-micrometer sections were stained with an anti-pAKT antibody followed by a FITC-conjugated secondary antibody (Green) or with an anti-MT1 antibody followed by a rhodamine-conjugated secondary antibody (Red). Nuclear structures were visualized by DAPI probing (Blue). Large magnification (400x) images are shown for the arcuate nucleus (ARC) **(A)**, lateral hypothalamus (LH) **(B)** and paraventricular nucleus (PVN) **(C)** (n=3).

Fig. 6 Direct action of melatonin in the MBH results in co-localisation of hypothalamic pAKT with MT2 and reduction gluconeogenesis. 13 hour-fasted wistar rats received icv injections containing either melatonin (28nM) or vehicle (0,02% ethanol) (CTL). The animals were decapitated 90 min after icv injections and the central nervous systems were removed and processed for immunofluorescent staining. Five-micrometer sections were stained with an anti-pAKT antibody followed by a FITC-conjugated secondary antibody (Green) or with an anti-MT2 antibody followed by a rhodamine-conjugated secondary antibody (Red). Nuclear structures were visualized by DAPI probing (Blue). Large magnification (400x) images are shown for the arcuate nucleus (ARC) **(A)**, lateral hypothalamus (LH) **(B)** and paraventricular nucleus (PVN) **(C)**. Another set of 13 hour-fasted wistar rats were injected with either melatonin (28nM) or vehicle (0,02% ethanol) (CTL) directly in the MBH and subjected to pyruvate tolerance tests 2h after injections. Glycemia was measured before and 15, 30, 60, 90, 120 and 150 min after pyruvate injection. The full line with open circles represents the CTL and the full line with black circles represents melatonin **(D)**. The AUC was calculated for each individual animal within each group **(E).** The results are shown as the mean ±SE. #$P<0.05$ comparing CTL to melatonin within the same time point; *$P<0.05$ vs. CTL (n=3 for immunofluorescent staining and n=4 for pyruvate tolerance tests).

Fig. 7 Icv-injected melatonin reduces gluconeogenesis, hepatic PEPCK and G6Pase through a mechanism dependent on peripheral Muscarininc receptors. Three sets of 13 hour-fasted wistar rats received icv injections containing melatonin (28nM)or vehicle (0.2% ethanol). Ipratropium bromide or its vehicle was ip injected 30 min before the icv injections. The first set of animals was decapitated 90 min after icv

injections and the hypothalamus were removed and processed for western blot detection of AKT and pAKT. pAKT levels were normalized to AKT **(A)**. The second set of animals was subjected to pyruvate tolerance tests 2h after icv injections. Glycemia was measured before and 15, 30, 60, 90, 120 and 150 min after pyruvate injection. The full line with open circles represents the CTL, the full line with open squares represents the Ipratropium bromide, the full line with black circles represents melatonin and the full line with black squares represents melatonin plus Ipratropium bromide **(B)**. The AUC was calculated for each individual animal within each group **(C).** The third set of animals were decapitated 120 min after icv injections and a fragment of the liver was removed for western blot detection of PEPCK, G6Pase, β actin, pSTAT3 and STAT3 **(D)**. PEPCK and G6Pase levels were normalized to β actin **(**respectively **E** and **F)** and pSTAT3 was normalized to STAT3 **(G)**. A second fragment of the liver was removed and processed for chromatin immunoprecipitation using an anti-pSTAT3 antibody. The *Pck1* and *G6pc* genes were amplified from ChIP samples and normalized to the respective inputs **(**respectively **H** and **I)**. The results are shown as the mean ±SE. #$P<0.05$ comparing CTL to melatonin within the same time point; *$P<0.05$ vs. CTL; &$P<0.05$ vs. melatonin (n=4 for western blots and chromatin immunoprecipitations and n=5 for pyruvate tolerance tests).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pAKT / AKT
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B
glycemia (mg/mL)
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140
120
100
80
60
0 30 60 90 120 150 min
C
AUC (mg.dL-1.min)
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6000
4000
2000
0
Melatonin (nM)
- 2.8 14 28 140
D
PEPCK
G6Pase
β actin
pSTAT3
STAT3
Melatonin (nM)
- 14 28
E
PEPCK / β actin
1.0
0.5
0.0
Melatonin (nM)
- 14 28
F
G6Pase / β actin
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1
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G
pSTAT3 / STAT3
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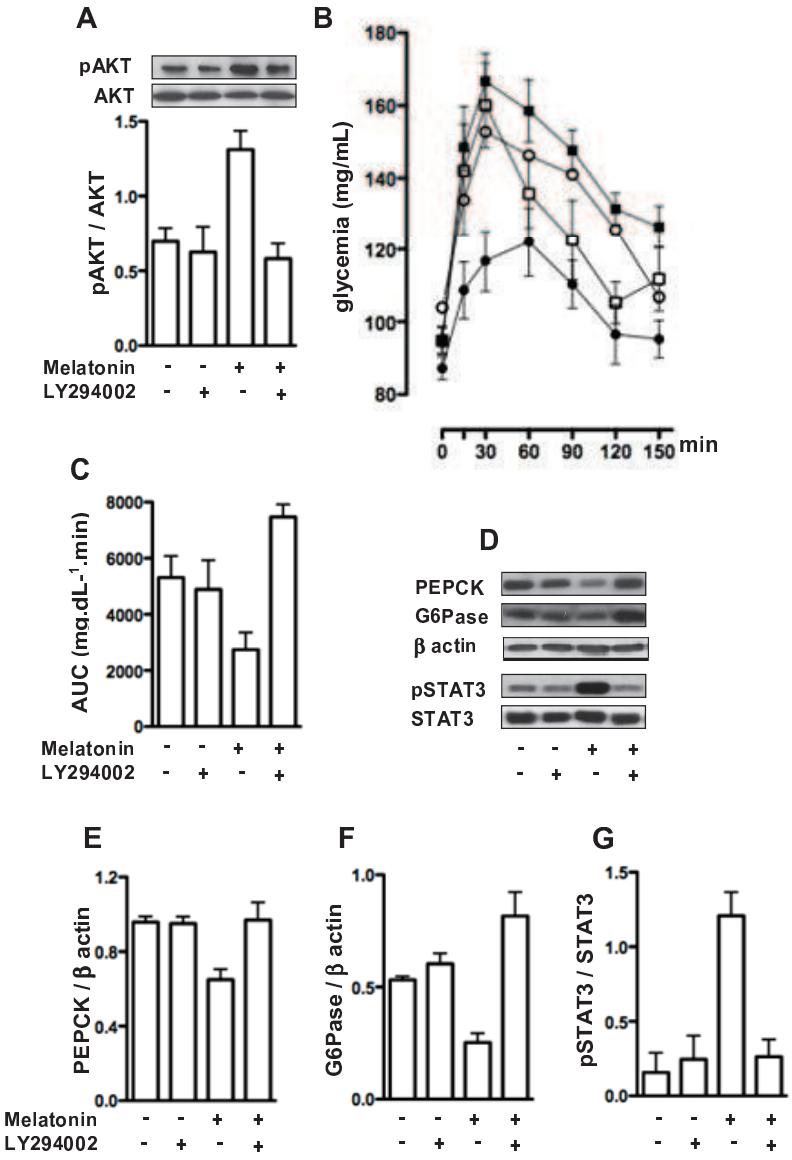
Figure 1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pAKT / AKT
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LY294002 - + - +
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glycemia (mg/mL)
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C
AUC (mg.dL-1.min)
8000
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Melatonin - - + +
LY294002 - + - +
D
PEPCK
G6Pase
β actin
pSTAT3
STAT3
- - + +
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PEPCK / β actin
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0.8
0.4
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Melatonin - - + +
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F
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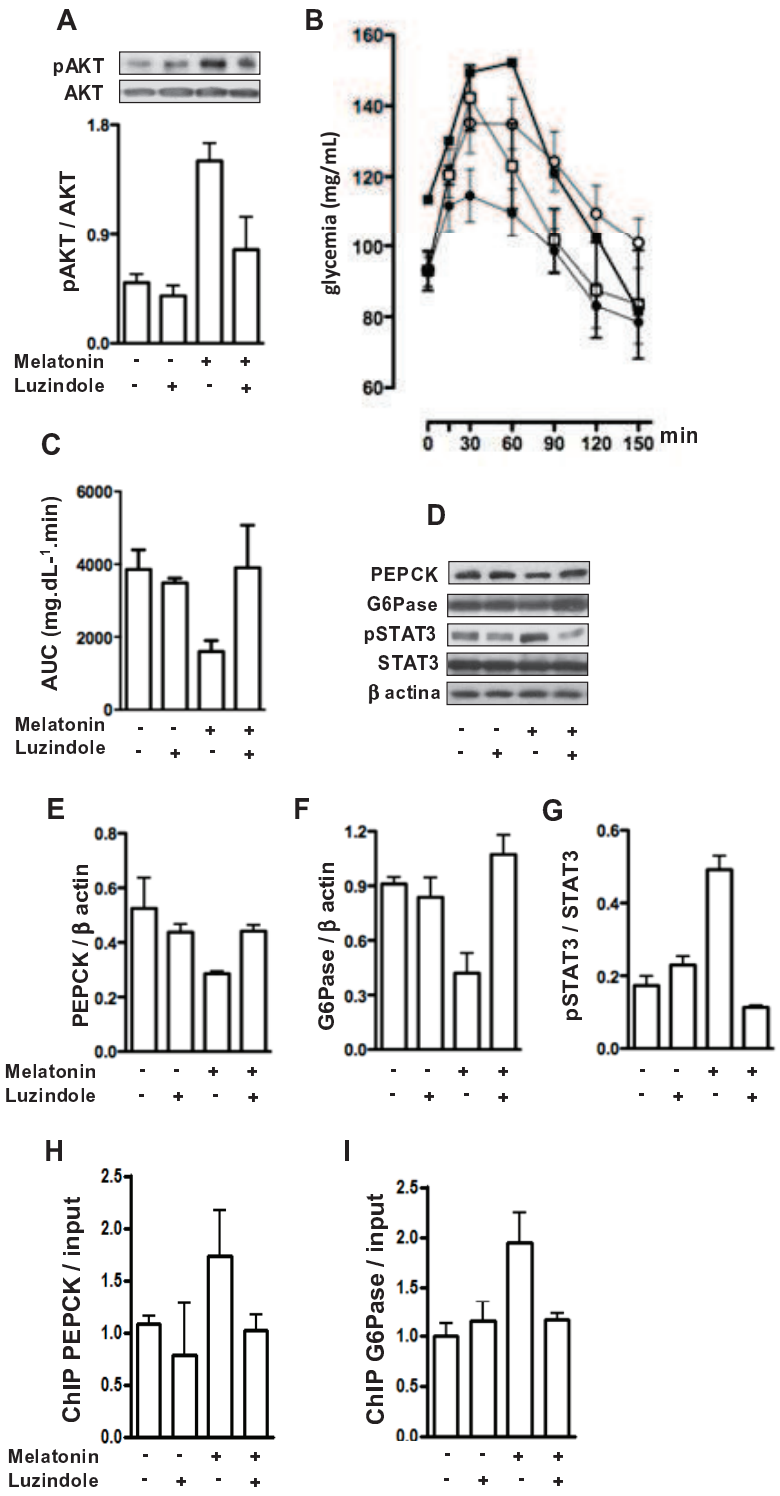
Figure 2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Melatonin - - + +
Luzindole - + - +
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glycemia (mg/mL)
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C
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4000
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Luzindole - + - +
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PEPCK
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PEPCK / β actin
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0.2
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G6Pase / β actin
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0.9
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pSTAT3 / STAT3
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Melatonin - - + +
Luzindole - + - +
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2.5
2.0
1.5
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I
ChIP G6Pase / input
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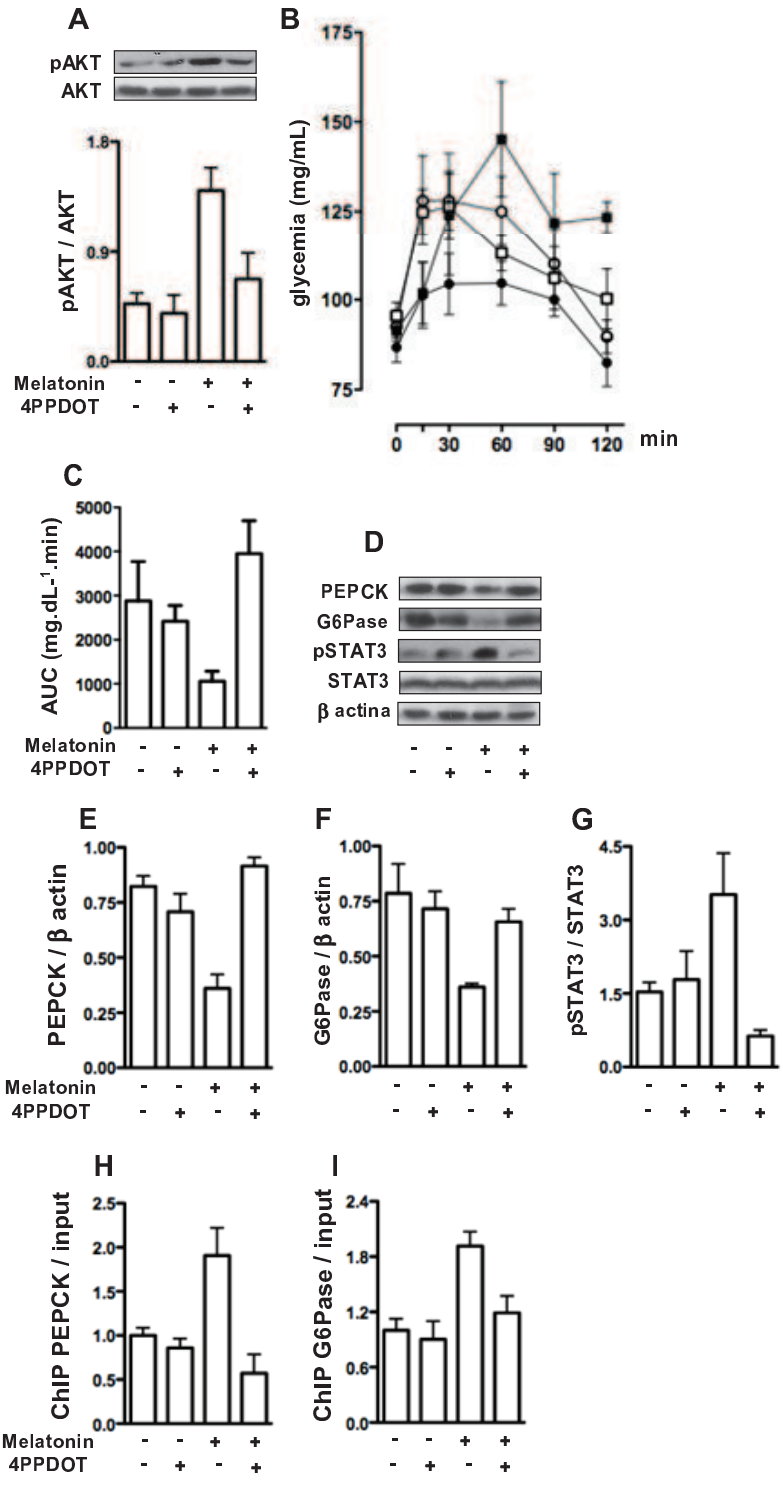
Luzindole - + - +

Figure 3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175
150
125
100
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C
AUC (mg.dL-1.min)
5000
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3000
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Melatonin - - + +
4PPDOT - + - +
D
PEPCK
G6Pase
pSTAT3
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β actina
E
PEPCK / β actin
1.00
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Melatonin - - + +
4PPDOT - + - +
F
G6Pase / β actin
1.00
0.75
0.50
0.25
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pSTAT3 / STAT3
4.5
3.0
1.5
0.0
H
ChIP PEPCK / input
2.5
2.0
1.5
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Melatonin - - + +
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ChIP G6Pase / input
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1.8
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0.6
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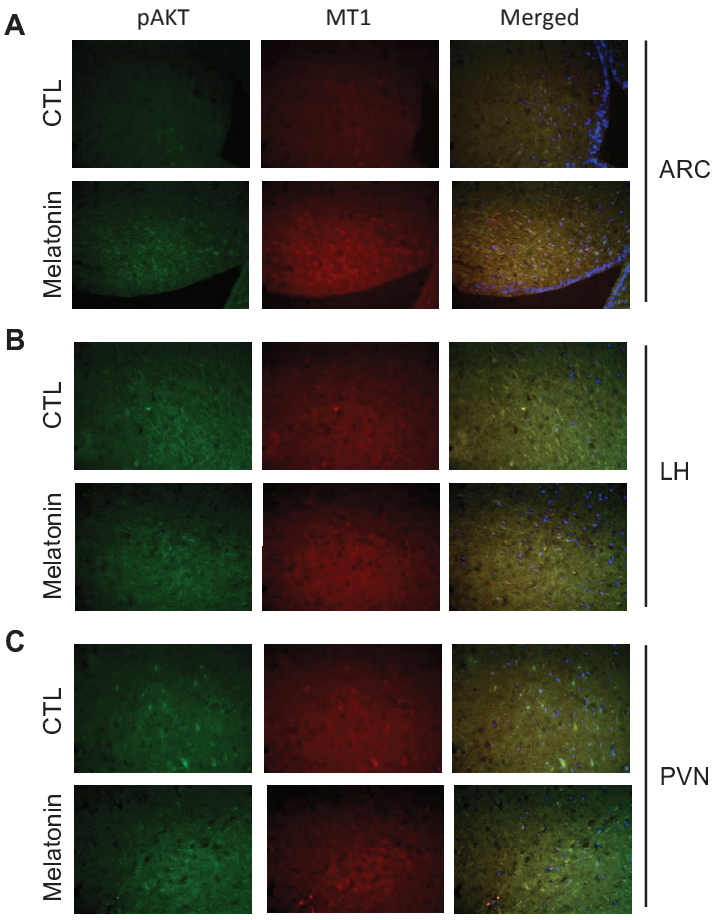
Figure 4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Melatonin
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CTL
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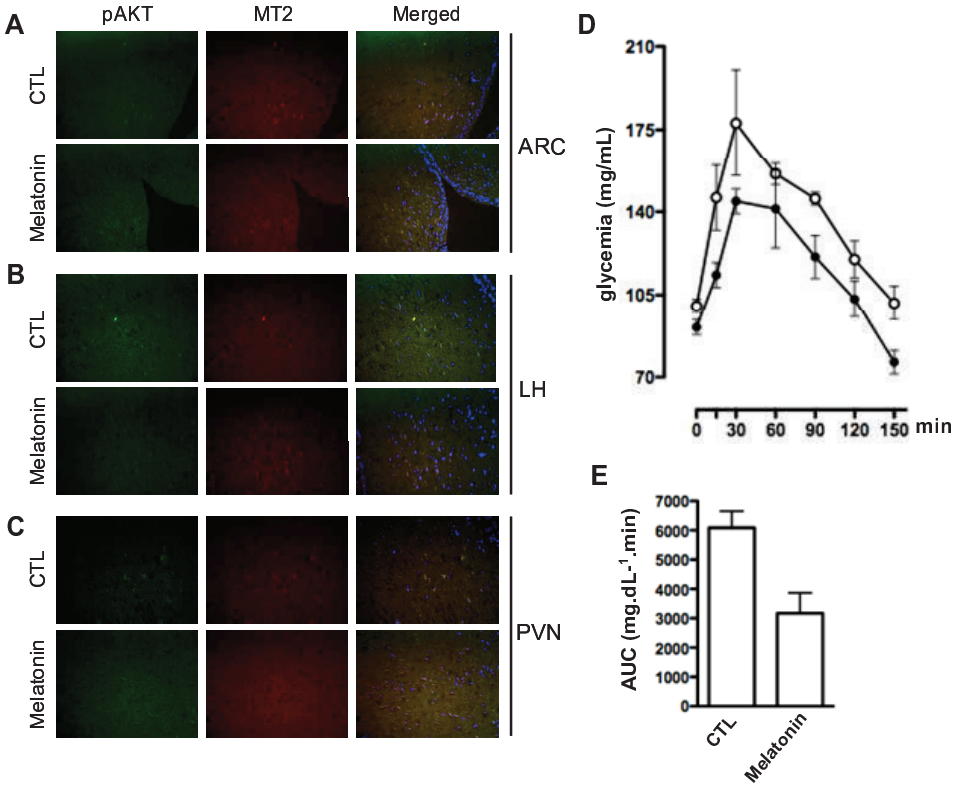
LH
C
CTL
Melatonin
PVN

Figure 5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pAKT
MT2
Merged
CTL
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ARC
B
CTL
Melatonin
LH
C
CTL
Melatonin
PVN
D
glycemia (mg/mL)
210
175
140
105
70
0
30
60
90
120
150
min
E
AUC (mg.dL-1.min)
7000
6000
5000
4000
3000
2000
1000
0
CTL
Melatonin

Figure 6

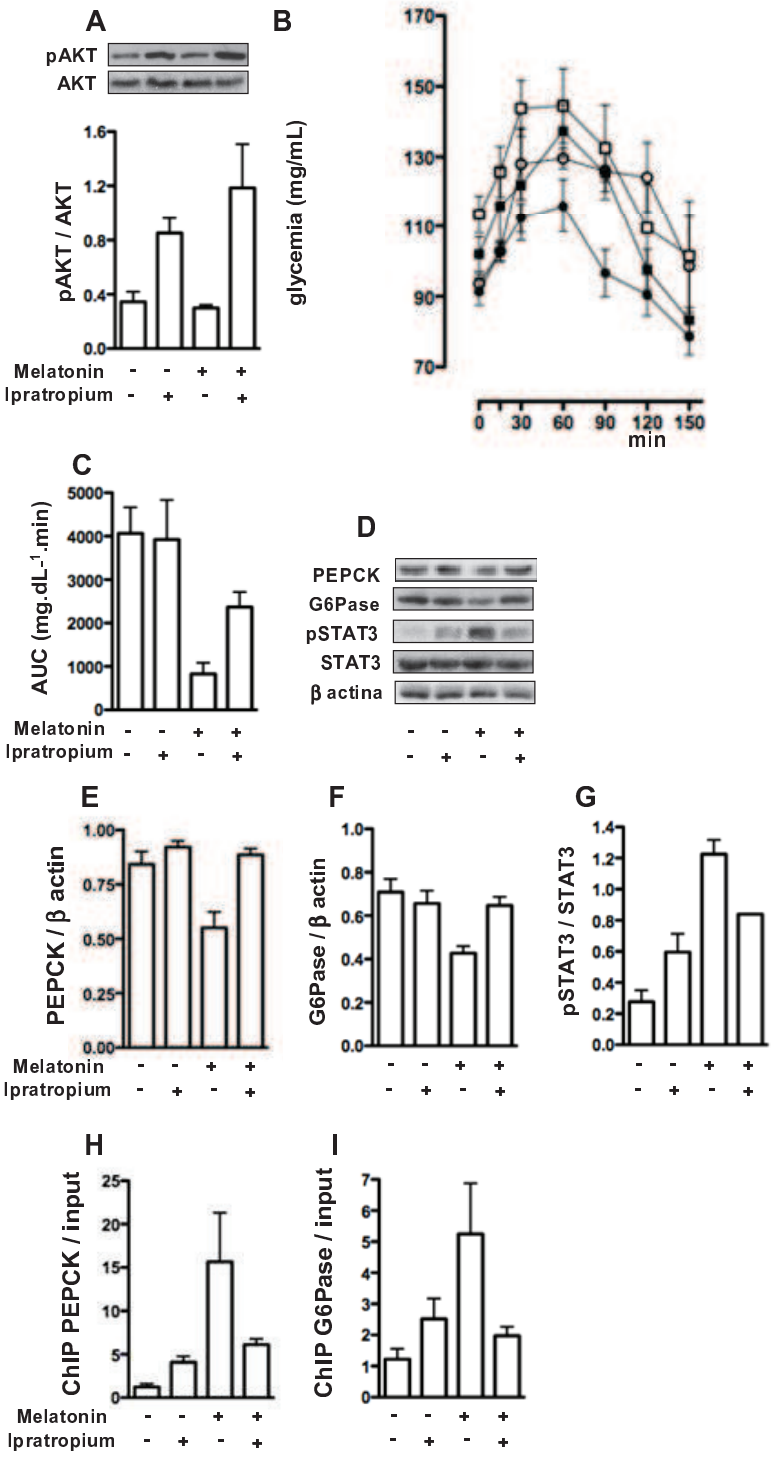
A
pAKT
AKT
pAKT / AKT
Melatonin
Ipratropium
B
glycemia (mg/mL)
min
C
AUC (mg.dL-1.min)
D
PEPCK
G6Pase
pSTAT3
STAT3
β actina
E
PEPCK / β actin
F
G6Pase / β actin
G
pSTAT3 / STAT3
H
ChIP PEPCK / input
I
ChIP G6Pase / input

Figure 7

CONCLUSÃO

O tratamento intracerebroventricular com melatonina é capaz de ativar a fosforilação da Akt no hipotálamo, aumentar a fosforilação do Stat3 hepático, diminuir a expresão da PEPCK e da G6Pase e, portanto, reduzir a gliconeogênese no fígado de maneira dose-dependente. A diminuição da gliconeogênese é dependente da ativação dos receptores MT1/ MT2 pela melatonina.

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