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

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Melatonin controls 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. Male Wistar rats were administered an intracerebroventricular (icv) injection of melatonin and one of following: an icv injection of a phosphatidylinositol 3-kinase (PI3K) inhibitor, an icv injection of a melatonin receptor (MT) antagonist, or an intraperitoneal (ip) injection of a muscarinic receptor antagonist. Anesthetized rats were subjected to pyruvate tolerance test to estimate in vivo glucose clearance after pyruvate load and in situ liver perfusion to assess hepatic gluconeogenesis. The hypothalamic was removed to determine Akt phosphorylation. Melatonin injections in the central nervous system suppressed hepatic gluconeogenesis and increased hypothalamic Akt phosphorylation. These effects of melatonin were suppressed either by icv injections of PI3K inhibitors and MT antagonists and by ip injection of a muscarinic receptor antagonist. We conclude that melatonin activates hypothalamic-liver communication that may contribute to circadian adjustments of gluconeogenesis. These data further suggest a physiopathological relationship between the circadian disruptions in metabolism and reduced levels of melatonin found in type 2 diabetes patients.

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 transmit environmental information (i.e., the length of the dark period) to the living organism, thereby synchronizing the circadian clock in the hypothalamic suprachiasmatic nucleus (22). In vivo and in vitro experiments have demonstrated that melatonin also plays a role in energy homeostasis by regulating body mass and adiposity and leptin expression by adipocytes (1, 40). Glucose homeostasis is also altered by the absence of melatonin in such a way that pinealectomized rats display glucose intolerance and desynchronized circadian pattern of gluconeogenesis, hallmarking by increased nighttime glucose levels (17, 18, 23). Moreover, chronic melatonin administration has been shown to improve glucose homeostasis not only in pinealectomized rats but also in rats rendered insulin resistant by diet manipulation (16, 33, 34).

Although it has been demonstrated that melatonin stimulates glucose uptake in adipocytes and skeletal muscle cells in vitro (10, 19), the precise mechanism by which this hormone reduces whole body glucose intolerance has not been determined precisely. In mammals, the effects of melatonin are mediated in part by specific high-affinity G protein-coupled receptors known as melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2) (31). We have demonstrated previously that melatonin acts locally in the hypothalamus to activate the phosphatidylinositol 3-kinase/insulin-stimulated RAC-α serine/threonine-protein kinase (PI3K/Akt) pathway through a mechanism that is dependent on MT1/MT2 receptors (2). However, the metabolic relevance of this signal triggered by melatonin remains unknown. Importantly, it is noteworthy that melatonin concentrations in the cerebrospinal fluid is ~20 times higher than in blood since melatonin reaches the third and forth ventricles from the pineal recess (36, 38).

Studies over the past decade have demonstrated the pivotal role of hypothalamic insulin signaling in the control of hepatic glucose production. The insulin-activated PI3K/Akt pathway in the hypothalamus has been shown to suppress hepatic glucose production irrespective of changes in peripheral insulin and other glucoregulatory hormones (24, 25). The pathways underlying this interorgan 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 suppression of gluconeogenesis (28, 37).

In the present study, we sought to demonstrate whether melatonin-induced Akt activation in the hypothalamus would control hepatic gluconeogenesis. We also aimed to determine whether this action of melatonin would depend on hypothalamic MT1/MT2 receptors and muscarinic receptors out of the central nervous system.

MATERIALS AND METHODS

Surgical procedures and treatments. Male Wistar rats weighing ~180 g were obtained from the Animal Breeding Center at the University of Campinas (Campinas, Sao Paulo, Brazil) and were housed under a 12:12-h light-dark cycle (lights on at 0700) with free access to food and water. Rats were anesthetized with diazepam and ketamine (2 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 localization of the cannula was tested by evaluating the drinking response to intracerebroventricular (icv) angiotensin II injection 1 wk after surgery (13). Cannulas were also implanted in the mediodorsal hypothalamus (MBH) using the following the coordinates: 3.1 mm posterior to bregma, 0.4 mm lateral, and 9.6 mm deep. Localization of the cannula was confirmed previously by staining of the hypothalamic region after an injection with bromophenol blue (15). Angiotensin-responsive rats received their icv injections at 9 AM after a 13-h fast (fasting starting at 8 PM on the days prior to the experiments). Rats remained fasted after icv injections.

Melatonin (cat. no. A9525; Sigma-Aldrich, St. Louis, MO) concentrations in the injected solutions were 2.8, 14, 28, and 140 nM. The final injected volume was 2 μl. In all cases, the melatonin dilutions were prepared from a 71.8 mM stock solution (100% ethanol). The final concentration of melatonin was set to 0.2% in all melatonin dilutions, and 0.2% ethanol was given to control (CTL) rats. LY-294002 (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 with either 28 nM melatonin or 0.2% ethanol at a ratio of 1:3. Three microliters of either of these mixtures was injected through the cannula implanted in the lateral ventricle.

Wortmannin (cat. no. 1232; Tocris) was diluted in 100% DMSO to a final concentration of 300 μM. This solution or its vehicle (100% DMSO) was mixed with either 28 nM melatonin or 0.2% ethanol at a ratio of 1:3. Three microliters of either of these mixtures was injected through the cannula implanted in the lateral ventricle.

Luzindole and 4-phenyl-2-propionamidotetraline (4-PDPT; cat. no. 0877 and 1034, respectively; Tocris) 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 at a ratio of 1:3. Three microliters of either of these mixtures was injected through the cannula implanted in the lateral ventricle.

Ipratropium bromide (IpBr; 50 mg/kg) (cat. no. I1637; Sigma-Aldrich) or its vehicle (0.9% NaCl) was injected intraperitoneally (ip) 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.

Intrapitoneal pyruvate tolerance test and intrapitoneal glucose tolerance test. For intrapitoneal pyruvate tolerance test (IPPTT), a sodium pyruvate solution (250 mg/ml) was injected ip at a dosage of 2 g/kg 2 h after the icv injections. Glucose levels were determined in blood extracted from the tail before (0 min) and 15, 30, 60, 90, 120, and 150 min after ip pyruvate injection. The area under the curve (AUC) of glycemia vs. time was calculated using each individual baseline (basal glycemia) to estimate glucose clearance after pyruvate load. Reduced AUC values were thereafter interpreted as increased glucose clearance after pyruvate load. Blood was also collected 5, 15, 30, and 60 min after pyruvate load for insulin measurements.

Intrapitoneal glucose tolerance test (IPGTT) was performed after an ip injection with a 20% (wt/vol) d-glucose solution (2 g/kg). IPGTT was performed 2 h after icv injections. Glucose levels were determined in blood extracted from the tail before (0 min) and 10, 15, 30, 60, and 120 after an ip injection. The AUC of glycemia vs. time was calculated using each individual baseline (basal glycemia) to estimate glucose tolerance.

In situ liver perfusion with pyruvate. Anesthetized rats (ip injection of thiopental sodium, 40 mg/kg) were subjected to perfusion experiments 120 min after icv injections. Rats were submitted to a laparotomy, and the livers were perfused in situ with a constant flux (4 ml·min⁻¹·g⁻¹). The weight of the liver was estimated as 4% of body weight. The perfusion buffer was pumped into a controlled temperature (37°C) membrane oxygenator prior to entering the liver via the portal vein. The collection of the effluent perfusion fluid was provided by a cannula inserted into the infrahepatic segment of the cava vein. The perfusion was performed in an open system without recirculation of the perfusate. Every animal was subjected to a preperfusion (20 min) with perfusion buffer [Krebs-Henseleit bicarbonate buffer without glucose saturated with O2/CO2 (95:5%), pH 7.4], during which samples of the effluent perfusion fluid were discarded. After this procedure, livers were perfused for 10 min with perfusion buffer, and samples of the effluent fluid were collected at time 0 and after 2, 5, 8, and 10 min. After this interval the perfusion buffer was added with sodium pyruvate (5 mM), and livers were perfused for additional 20 min. During this interval, samples of the effluent perfusion fluid were collected at time 0 and 2, 5, 10, 15, 18, and 20 min after the beginning of the pyruvate perfusion. Next, livers were subjected to a postperfusion for 20 min with perfusion buffer without sodium pyruvate. A sample of the effluent perfusion fluid was collected at the end of this period.

Livers were removed and weighted after the experiments. Glucose concentration of the samples was measured using a kit based on the glucose oxidase method (Vitro Diagnostica). The glucose production rate was expressed per liver mass (μmol·min⁻¹·g⁻¹) and plotted vs. time. The AUC during the perfusion with sodium pyruvate was calculated (μmol/g) to estimate the amount of glucose synthesized from pyruvate relative to liver mass.

Protein extraction and immunoblotting. The anesthetized rats were decapitated at the intervals after icv injections, as indicated in the figure legends. The hypothalamus was removed and processed for Western blotting, as described previously (2). The primary antibodies anti-Akt1/2/3 and anti-p-Akt1/2/3 (Ser473) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-FoxO1 was from Cell Signaling Technology (Beverly, MA), and anti-b-actin was from Abcam (Cambridge, UK). Secondary antibodies conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA) were used, followed by chemiluminescent detection of the bands on X-ray-sensitive films. Optical densitometry of the films was performed using the Scion Image analysis software (Scion, Frederick, MD).

Immunofluorescent staining. The central nervous systems were removed 90 min after icv injection with either 28 nM melatonin or 0.2% ethanol and processed for immunofluorescent staining, as described previously (30). We used the anti-p-Akt (Ser473), anti-MT1, or anti-MT2 antibodies (Santa Cruz Biotechnology). Secondary FITC-conjugated antibody was used to visualize p-Akt staining, and secondary rhodamine-conjugated antibody was used to visualize either MT1 or MT2. A separate set of sections from the central nervous systems was stained with only the secondary antibodies (omitting the primary antibody) to ensure specificity of the fluorescent signals. Images were acquired either under high (×400) or low (×100) magnification.

Hormone measurements. The levels of 6-sulfatoxymelatonin (aMT6S) were determined in urine samples using an ELISA kit according to the manufacturer’s instructions (IBL-International, Hamburg, Germany). Two protocols were performed to sample collection. 1) Overnight aMT6S production was accessed by placing the rats in metabolic cages 30 min prior lights off. One half of the rats had their food removed 1 h after lights off, and the other half remained with free access to food during the night. Urine was allowed to accumulate in a glass tube placed under the cages from 7 PM to 7 AM. Total volume was determined, and samples were stored at −20°C until assay. 2) Rats were anesthetized, and an intravesical puncture was performed 2 h after icv injections containing either vehicle or 28 or 140 nM melatonin. Total urine volume was collected and determined. Samples were stored at −20°C until assay.

After determination, the concentrations of aMT6S in the urine samples were multiplied by the volumes to estimate the mass of flux.
Fig. 1. Intracerebroventricularly (icv) injected melatonin activates hypothalamic Akt and increases glucose clearance after pyruvate load. Rats received icv injections containing melatonin (2.8, 14, 28, or 140 nM) or vehicle (0.2% ethanol; control). The rats were decapitated 90 min after icv injections, and the hypothalamuses were removed and processed for Western blot detection of Akt and phosphorylated Akt (p-Akt). A: p-Akt levels were normalized to Akt. An independent set of rats received icv injections containing melatonin (28 nM) or vehicle (0.2% ethanol). Control rats were decapitated 120 min after icv injection. Rats that received icv melatonin were decapitated 30, 60, 90, and 120 min after injections. The hypothalamuses were removed and processed for Western blot detection of Akt, p-Akt, p-FoxO1, and β-actin. B and C: p-Akt levels were normalized to Akt (B), and p-FoxO1 levels were normalized to β-actin (C). Another set of animals was subjected to pyruvate tolerance tests 2 h after icv injections. Glycemia was measured before and 15, 30, 60, 90, 120, and 150 min after pyruvate injection. D: dotted line with gray triangles, 2.8 nM melatonin; dotted line with gray diamonds, 14 nM melatonin; solid line with closed circles, 28 nM melatonin; dotted line with gray squares, 140 nM melatonin; solid lines with open circles, control. E: the area under the curve (AUC) was calculated for each individual animal within each group. F and G: fragments of liver were removed 2 h after icv injection and processed for measurements of pck1 (F) and g6pc (G) mRNAs. The results are shown as means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. 30 min after melatonin injection (n = 4–5 for Western blots and real-time PCR, and n = 4 for pyruvate tolerance tests).
aMT6S produced during either the 12-h interval of the dark period or the 2-h interval after icv injections. Trunk blood was collected 120 min after the icv injections. Serum and plasma with heparin were stored. Insulin (cat. no. EZRMI-13K; Merck Millipore, Billerica, MA) and glucagon (cat. no. 297-57101; Wako Pure Chemical Industries, Osaka, Japan) were quantified in serum and plasma, respectively, by ELISA according to the manufacturers’ instructions. Trunk blood was also collected 60 min after the icv injections. Plasma was stored with EDTA for epinephrine and norepinephrine determination. Serum was also collected for corticosterone determination. Catecholamines (cat. no. E-6500; Rocky Mountains, Colorado Springs, CO) were quantified by ELISA according to the manufacturer’s instructions. Corticosterone was measured with the MILLIPLEX Map Rat Stress Hormone Panel (cat. no. RSH69K; Merck Millipore) using the Milliplex Analyzer System (Merck Millipore) according to the manufacturer’s instructions.

Total RNA extraction and real-time PCR. Anesthetized rats were decapitated 120 min after icv injections. Total RNA was extracted from ~100 mg of liver using Trizol reagent. Total RNA was reverse transcribed, as described previously (23). Real-time PCR was performed to detect pck1, g6pc, and gapdh mRNAs using the KAPA Sybr FAST quantitative PCR kit (KAPA Biosystems, Woburn, MA) in Mx3005P equipment (Agilent Technologies, Santa Clara, CA). Primer sequences and fragment sizes were pck1 (NM_198780) sense 5’-TGGTCTGGACTTCTGCAAG-3’ and antisense 5’-AATGATGACCGTCTTTGCTTCG-3’, 264 bp; g6pc (NM_013098) sense 5’-ACCTTTCTCTGTGTTTGCACC-3’ and antisense 5’-CGGTACATGCTGGAGTTGGGG-3’, 205 bp; and gapdh (NM_017008) sense 5’-GAACATCATCCCTGCATCC-3’ and antisense 5’-CCTGCTTCACCACCTTCTT-3’, 162 bp.

Statistical analysis. The results are presented as means ± SE. Comparisons were performed using an unpaired Student t-test or a one-way ANOVA, followed by Tukey-Kramer post hoc testing when appropriate (INStat; GraphPad Software, San Diego, CA). P values <0.05 indicate a significant difference.

RESULTS

Intracerebroventricular melatonin activates Akt in the hypothalamus and increases glucose clearance after pyruvate load. 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 phos-

Fig. 2. Intracerebroventricularly injected melatonin does not change glucose tolerance after glucose load. Rats received icv injections containing melatonin (28 nM) or vehicle [control (CTL)]. A: a 1st set of animals was subjected to pyruvate tolerance tests 2 h after icv injections. Insulinemia was measured before and 5, 15, 30, and 60 min after pyruvate injection. B: a 2nd set of animals was subjected to glucose tolerance tests 2 h after icv injections. Glycemia was measured before and 10, 15, 30, 60, and 120 min after glucose injection. C: the AUC was calculated for each individual animal within each group. The results are shown as means ± SE. *P < 0.05, CTL compared with with melatonin within the same time point (n = 4).
phorylation. 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 (Fig. 1A). Time course experiments revealed that icv melatonin (28 nM) induced maximal Akt phosphorylation 60 and 90 min postinjection. However, a significant increase was already detected 30 min after injection.

(Fig. 1B). Melatonin-induced FoxO1 phosphorylation was also assessed. Although an increase in FoxO1 phosphorylation was already detected 30 and 60 min postinjection, maximal signal was observed only 90 min after icv melatonin (28 nM) stimulus (Fig. 1C).

In vivo glucose clearance after a pyruvate load was assessed to verify a putative effect of icv melatonin on glu-
coneogenesis. Changes in glycemia vs. time were dissected into four different graphs (Fig. 1D). AUC values obtained during the pyruvate tolerance test for the rats that received 14 and 28 nM melatonin, but not for the rats that received 2.8 and 140 nM, were 70 and 62% lower, respectively, than the AUC values for the CTL (P < 0.05; Fig. 1E). Accordingly, hepatic levels of pck1 and g6pc mRNAs, two step-limiting enzymes for gluconeogenesis, were reduced 120 min after icv injections.

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Fig. 4. Intracerebroventricularly injected melatonin increases glucose clearance after pyruvate load through a mechanism dependent on melatonin receptor (MT1/MT2). Rats received icv injections containing melatonin (28 nM), luzindole, luzindole combined with melatonin, 4-phenyl-2-propionamidotetraline (4P-PDOT) or 4P-PDOT combined with melatonin. CTL rats received the vehicle. A and B: a 1st set of animals was decapitated 90 min after icv injections, and the hypothalamuses were removed and processed for Western blot detection of Akt and p-Akt. p-Akt levels were normalized to Akt, and samples were arranged in gels containing the samples with luzindole (A) or 4P-PDOT (B). C: a 2nd set of animals divided in 4 groups (receiving icv vehicle, melatonin, luzindole, or melatonin + luzindole) was subjected to pyruvate tolerance tests 2 h after icv injections. Glycemia was measured before and 15, 30, 60, 90, 120, and 150 min after pyruvate injection. Solid line with о, CTL; solid line with □, luzindole; solid line with ●, melatonin; solid line with ■, melatonin plus luzindole. D: the AUC was calculated for each individual animal within each group. E: a 3rd set of animals divided in 4 groups (receiving icv vehicle, melatonin, 4P-PDOT, or melatonin + 4P-PDOT) was subjected to pyruvate tolerance tests 2 h after icv injections. Glycemia was measured before and 15, 30, 60, 90, and 120 min after pyruvate injection. Solid line with о, CTL; solid line with □, 4P-PDOT; solid line with ●, melatonin; solid line with ■, melatonin plus 4P-PDOT. F: the AUC was calculated for each individual animal within each group. The results are shown as means ± SE. *P < 0.05 vs. CTL; &P < 0.05 vs. melatonin; #P < 0.05 comparing CTL to melatonin within the same time point (n = 4 for pyruvate tolerance tests and n = 5 for Western blots).
containing 28 nM melatonin ($P < 0.05$; Fig. 1, F and G, respectively).

To better understand the mechanism underlying the increased glucose clearance after pyruvate load induced by icv melatonin, we next assessed circulating insulin levels along with the initial phase of IPPTT. Insulin levels were found to increase 15 min after exogenous pyruvate load in rats that received icv vehicle. However, at this time point, no increase was observed in rats that received icv melatonin (28 nM; Fig. 2A). We have also performed IPGTT in rats receiving either vehicle or 28 nM melatonin icv. Timeline changes in glucose levels after challenge with exogenous glucose as well as the AUC were similar when comparing rats that received icv vehicle with those that received icv melatonin (Fig. 2, B and C, respectively).

Given that no changes in glucose clearance after a challenge with exogenous glucose were induced by icv melatonin, it is not likely that the action of this hormone in the central nervous system might either disrupt in vivo glucose-induced insulin secretion or alter whole body insulin sensitivity. Thus, reduced levels of insulin that were induced by icv melatonin during the PTT are probably secondary to reduced conversion of pyruvate into glucose. As we understand, reduced gluconeogenesis resulting in lower glucose levels would by extension reduce the stimulation of insulin secretion along with the PTT.

**Intracerebroventricular melatonin-induced increased glucose clearance after pyruvate load depends on the hypothalamic PI3K/Akt pathway.** 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 LY-294002, a classical inhibitor 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 not by melatonin combined with LY-294002 (Fig. 3A). Likewise, increased glucose clearance after pyruvate load was induced by icv-injected melatonin (as observed by a 64% reduction in the AUC obtained during the PTT, $P < 0.05$) but not by melatonin combined with LY-294002 (Fig. 3C).

To exclude an unspecific effect of LY-294002, we have also combined melatonin with wortmannin, another well-recognized inhibitor of the PI3K/Akt pathway. As an example of LY-294002, the combined use of wortmannin blunted the ability of icv melatonin to increase glucose clearance after pyruvate load (Fig. 3, D and E).

**Melatonin acts through membrane MT1/MT2 receptors to activate hypothalamic Akt and increase glucose clearance after pyruvate load.** To assess whether melatonin-induced hypothalamic Akt activation would depend on MT1/MT2 receptors, we used luzindole (a MT1/MT2 antagonist) and 4P-PDOT (a specific MT2 antagonist).

In this set of experiments, melatonin-induced Akt phosphorylation (3.0-fold higher than in the CTL, $P < 0.05$) was completely suppressed by the combined use of melatonin and luzindole (Fig. 4A). The ability of icv-injected melatonin to modulate in vivo glucose clearance after pyruvate load was also blunted by luzindole. Thus, the reduction induced by icv

![Fig. 5. Intracerebroventricular melatonin reduces hepatic gluconeogenesis through a mechanism dependent on hypothalamic MT1/MT2 and PI3K/Akt.](http://ajpendo.physiology.org/)

**Fig. 5.** Intracerebroventricular melatonin reduces hepatic gluconeogenesis through a mechanism dependent on hypothalamic MT1/MT2 and PI3K/Akt. Rats received icv injections containing melatonin (28 nM) or vehicle (0.02% ethanol) combined or not with LY-294002, luzindole, or 4P-PDOT. Anaesthetized rats were subjected to perfusion experiments 120 min after icv injection. The perfusion was performed in an open system without recirculation of perfusate. Livers were perfused with perfusion buffer added with sodium pyruvate (5 mM) for 20 min. During this interval, samples of the effluent perfusion fluid were collected at time 0 and 2, 5, 10, 15, 18, and 20 min after beginning of the pyruvate perfusion. At the end of the experiments, glucose concentration of the samples was measured, and livers were weighted for calculations of glucose production rate. A–C: graphs illustrating the glucose production rate vs. time are shown. D: the AUC (µmol/g) was calculated to estimate the amount of glucose synthesized from pyruvate relative to liver mass. The results are shown as means ± SE. *$P < 0.05$ vs. CTL ($n = 4$).
melatonin in the AUC values obtained during the PTT (58% lower than in the CTL, \( P < 0.05 \)) was not observed when icv melatonin was combined with luzindole (Fig. 4D).

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 coinjection of 4P-PDOT (Fig. 4B). Changes in in vivo glucose clearance after pyruvate load induced by icv melatonin were also suppressed by the combined use of 4P-PDOT. Intracerebroventricular melatonin-induced decrease in the AUC values obtained during the PTT (74% lower than in the CTL, \( P < 0.05 \)) was blunted by the combination with 4P-PDOT (Fig. 4F).

**Intracerebroventricular melatonin reduces hepatic gluconeogenesis through a mechanism dependent on MT1/MT2 and PI3K/Akt.** To assess whether hepatic gluconeogenesis would be modulated by icv injection containing melatonin, we next performed in situ liver perfusion with pyruvate after injecting melatonin icv. Changes in glucose production rate vs. time are shown in Fig. 5, A–C. Intracerebroventricularly injected melatonin reduced the total amount of glucose synthesized from pyruvate (32% lower than CTL, \( P < 0.05 \)). The combined use of LY-294002, luzindole, or 4P-PDOT with icv-injected melatonin blunted the ability of this hormone to suppress hepatic gluconeogenesis (Fig. 5D).

**Melatonin-induced Akt phosphorylation in the MBH correlates with increased glucose clearance after pyruvate load.** It has been reported previously that impaired insulin-induced Akt activation in regions of the MBH is related to an increase in hepatic glucose production (28). Our immunofluorescent analysis demonstrated that MT1 colocalized with phosphorylated Akt in distinct regions of the MBH after icv melatonin injections. However, merged high-magnification images showed that MT1-positive cells became stained for phosphorylated Akt mainly in the arcuate nucleus (ARC) but slightly in the lateral hypothalamus and paraventricular nucleus (PVN) after icv melatonin injection (Fig. 6, A–C, respectively). We have also performed a negative control omitting the primary antibodies. In that case, no perinuclear staining can be seen in the images.

![Fig. 6. Intracerebroventricular injections with melatonin results in the colocalization of hypothalamic p-Akt with MT1.](http://ajpendo.physiology.org/)

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Independent set of animals was used for the control, omitting the primary antibodies. Five-micrometer paraffin sections were obtained (Bregma –3.0) and stained with FITC-conjugated secondary antibody (left) or rhodamine-conjugated secondary antibody (middle). Nuclear structures were visualized by DAPI probing (blue). Large magnification (\( \times 400 \)) images are shown for the arcuate nucleus (ARC; A), lateral hypothalamus (LH; B), and paraventricular nucleus (PVN; C). D: an independent set of animals was used for the control, omitting the primary antibodies. Five-micrometer paraffin sections were obtained (Bregma –3.0) and stained with FITC-conjugated secondary antibody (left) or rhodamine-conjugated secondary antibody (middle). Nuclear structures were visualized by DAPI probing (blue) in the merged image (right). Images are shown in low magnification (\( \times 100; n = 3 \)).
of low magnification (Fig. 6D). Phosphorylated Akt also colocalized with MT2 after icv melatonin injection in a pattern similar to that found for MT1 (Fig. 7, A–C, respectively).

We also showed that an injection of melatonin (28 nM) directly into the MBH increased glucose clearance after pyruvate load. 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. 7D). The AUC values obtained during the PTT for rats that received melatonin in the MBH were 48% lower than for the CTL (\( P < 0.05 \); Fig. 7E).

Intracerebroventricular melatonin transmits information to the liver through activation of peripheral muscarinic receptors. Our next step was to investigate the mechanism underlying the transmission of information from the central nervous system to the liver. Overnight fasting by itself increased the total amount of aMT6S that accumulated in the urine of untreated rats from the moment of lights off until the moment of lights on (282.1 \( \pm \) 47.7 vs. 139.7 \( \pm \) 34.8 ng in the urine of ad libitum-fed rats, \( P < 0.05 \); \( n = 5 \)). However, 2 h after icv injections (4 h after lights on), the amounts of urinary aMT6S in overnight-fasted rats were reduced dramatically (1.8 \( \pm \) 0.6 in rats that received icv vehicle; \( n = 4 \)), suggesting that the upregulation of melatonin levels induced by fasting were not sustained until the moment that the metabolic assays were performed.

We also measured urinary aMT6S levels 2 h after icv injections containing either melatonin (28 or 140 nM) or vehicle. We found no differences in the content of aMT6S when comparing rats that received icv vehicle to those that received 28 nM melatonin. Thus, we assumed that no significant amounts of melatonin reached the bloodstream during the 2-h interval after icv injections containing 28 nM melatonin, and therefore, we discarded the possibility of a direct hepatic effect of melatonin to suppress gluconeogenesis in these animals. On the other hand, aMT6S was increased in the urine of rats that received 140 nM melatonin icv, suggesting that a fraction of the hormone reached the bloodstream in this particular group of rats (Fig. 8A). Insulin levels were not changed 2 h after icv injections with melatonin (28 and 140 nM;
Those that received ip injections with IpBr (Fig. 9C).

tions with vehicle (53% lower than CTL, cose synthesized from pyruvate in rats that received ip injections. Intracerebroventricularly injected melatonin reduced the total amount of glucagon in rats that received icv injections. Therefore, we have considered the hypothalamus.

We next investigated the participation of the parasympathetic nervous system using ip injections containing IpBr before the administration of icv injections of melatonin. IpBr is an M2/M3 muscarinic receptor antagonist that does not cross the blood-brain barrier (39). Intraperitoneal IpBr injections did not alter melatonin-induced hypothalamic Akt phosphorylation (Fig. 9A), but they blunted the suppression of hepatic gluconeogenesis induced by melatonin (Fig. 9B). Intracerebroventriculantly injected melatonin reduced the total amount of glucose synthesized from pyruvate in rats that received ip injections with vehicle (53% lower than CTL, \( P < 0.05 \)) but not in those that received ip injections with IpBr (Fig. 9C).

**DISCUSSION**

The present study shows that melatonin activates interorgan communication between the hypothalamus and the liver, leading to the suppression of hepatic gluconeogenesis. The description of this mechanism has a pivotal role in the understanding of the previously described relationship between melatonin and energy metabolism in humans. For instance, recent studies suggested that a disruption in rhythmic melatonin production is relevant to the etiology of diabetes. Nocturnal melatonin levels were reported to be significantly reduced in obese patients with type 2 diabetes compared with weight-matched controls (20). Reduced nocturnal melatonin secretion was also reported to correlate with increased gluconeogenesis during the first hours of the morning in patients with type 2 diabetes (29). In agreement with these studies, rare variants of the MT2 receptor that cause total or partial loss of function were described to be associated with increased risk of developing type 2 diabetes (5). Therapeutically, the potential of melatonin was highlighted by studies demonstrating that its use, either alone or combined with other agents, can improve glycemic control (8, 12).

Studies with rodents have also collected compelling data favoring the proposition that melatonin controls glucose homeostasis. Rats that become obese and spontaneously insulin resistant show a progressive decline in nocturnal melatonin production as they become glucose intolerant (3). On the other hand, melatonin supplementation can improve the glucose intolerance induced by high-fat and high-fructose diets (16, 33). Previously, we have reported the ability of melatonin to control gluconeogenesis in experiments that showed that surgical ablation of the pineal gland reduced glucose clearance after pyruvate load (23). Corroborating 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 hyperglycemic response to the intracranial injection of 2-deoxy-D-glucose (35). The experimental finding showing that melatonin injected directly in the MBH also increased glucose clearance after pyruvate load further corroborates the hypothesis that this hormone might suppress gluconeogenesis due to a primary action in the hypothalamus.

The mechanism through which the signal generated by melatonin in the hypothalamus is transmitted to the peripheral metabolic targets is also clarified by our data. First, we could exclude that melatonin is primarily modulating the secretion of glucoregulatory hormones, since no differences in glucagon, catecholamines, corticosterone, or insulin levels were found in rats injected with 28 nM melatonin icv. We also discarded a direct action of melatonin in peripheral targets because no changes in urinary aMT6S were observed after 28 nM melatonin icv injections. Therefore, we have considered the hypothesis that the signal generated by melatonin was being transmitted from the central nervous system through the autonomic nervous system.

To identify the peripheral target of the melatonin-induced Akt activation in the hypothalamus, we next performed IPGTT in rats that received 28 nM melatonin icv. A putative modulation of insulin secretory capacity by icv melatonin would impact on glucose tolerance during an IPGTT. Since we found no changes in glucose tolerance induced by icv melatonin, we
assumed that increased glucose clearance after a pyruvate load was probably due to decreased conversion of pyruvate into glucose rather than changes in glucose-induced insulin secretion. Accordingly, we believe that lower levels of insulin found during the IPPTT in rats that received icv melatonin might be secondary to reduced amounts of newly synthesized glucose available to stimulate insulin secretion.

Conversion of pyruvate into glucose first involves mitochondrial oxalacetate synthesis that is further reduced into malate. Therefore, in this case malate assumes the role of carrying reduced equivalents to cytosol. In the cytosol, malate is transformed into oxalacetate that is subjected to phosphoenolpyruvate (PEPCK)-mediated carboxylation and forming PEPCK. In rats, few oxalacetate can be carboxylated within the mitochondria due to reduced amounts of mitochondrial PEPCK expression in this specie (11). Because icv melatonin reduces both pck1 expression and hepatic gluconeogenesis, we can speculate that there is a consequent accumulation of oxalacetate during the IPPTT. Accumulated oxalacetate within the mitochondria might react with acetyl-CoA to generate citrate. Citrate, in turn, can follow two putative routes; it can enter the Krebs cycle to be oxidized, or it can be transported to the cytosol. In the cytosol, citrate can be metabolized by citrate lyase, which results in increased cytosolic levels of acetyl CoA. Thus, we do not discard that the carbons from pyruvate are ending up in fatty acids in the rats that received melatonin icv.

We next designed a set of experiments to demonstrate that the liver is the primary target of the melatonin-induced activation of the hypothalamic PI3K/Akt pathway. These experiments were based on in situ liver perfusion after icv injection containing melatonin either alone or in combination with PI3K/Akt and MT1/MT2 inhibitors. Such experiments allowed the hepatic tissue to synthesize glucose irrespective of concomitant changes in blood insulin levels. The data obtained from these experiments showed that icv melatonin reduced hepatic glucose synthesis from pyruvate.

Corroborating the present hypothesis, a study by Cailotto et al. (7) demonstrated that rats exposed to light during the night have increased hepatic levels of the gluconeogenic enzymes PEPCK and glucose-6-phosphatase, with reduced levels of melatonin-synthesizing enzyme in the pineal gland. Exposure to short periods of light during the night is classically known to suppress melatonin production (14). Cailotto et al. (7) 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. Accordingly, our data reveal that the suppression of gluconeogenesis...
induced by icv melatonin is abrogated by the peripheral blockade of muscarinic receptors.

The present results also point to a physiological relevance for this interorgan communication activated by melatonin. Considering that the total volume of rat cerebrospinal fluid is ~400 µl (21), 2 µl of the 28 nM icv-injected melatonin (which efficiently suppressed hepatic gluconeogenesis) might have yielded a final concentration of ~0.14 nM. This concentration is very similar to that observed in the cerebrospinal fluid of healthy volunteers (32). Interestingly, higher doses of icv-injected melatonin (140 nM) failed to increase glucose clearance after pyruvate load, although it still induced Akt phosphorylation. Such absence of metabolic modulation might be a consequence of increased glucacon levels observed exclusively in rats that received 140 nM melatonin. Our data suggest that the mechanism through which the higher dose of melatonin increases glucagon levels is probably connected to a direct action of melatonin in pancreatic α-cells. We are presently considering this hypothesis because, in opposition to that observed in rats that received 28 nM melatonin, rats receiving 140 nM melatonin through icv injection displayed higher levels of urinary aMT6S. Furthermore, melatonin was described previously to act directly in pancreatic α-cells to increase glucagon secretion in vivo and in vitro (4).

As our data further indicate, the hypothalamic signal triggered by melatonin starts with the MT1/MT2-dependent Akt activation mainly in the ARC located in the MBH. The deoxyribose-Akt activation associated with increased hepatic glucose production (26). 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 suppression of hepatic gluconeogenesis through peripheral muscarinic receptors. This interorgan communication clarifies the beneficial effects of melatonin in rodent models of insulin resistance. In addition, this biological action of melatonin may contribute the understanding of previous reports linking diabetic patients poorly controlled with metformin.

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DISCLOSURES

The authors declare no conflicts of interest, financial or otherwise, associated with this article. The authors are responsible for the writing and content of the article.

AUTHOR CONTRIBUTIONS


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