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Adenosine triphosphate inhibits melatonin synthesis in the rat pineal gland

Abstract: Adenosine triphosphate (ATP) is released onto the pinealocyte, along with noradrenaline, from sympathetic neurons and triggers $P2Y_1$ receptors that enhance β-adrenergic-induced N-acetylserotonin (NAS) synthesis. Nevertheless, the biotransformation of NAS into melatonin, which occurs due to the subsequent methylation by acetylserotonin O-methyltransferase (ASMT; EC 2.1.1.4), has not yet been evaluated in the presence of purinergic stimulation. We therefore evaluated the effects of purinergic signaling on melatonin synthesis induced by β-adrenergic stimulation. ATP increased NAS levels, but, surprisingly, inhibited melatonin synthesis in an inverse, concentration-dependent manner. Our results demonstrate that enhanced NAS levels, which depend on phospholipase C (PLC) activity (but not the induction of gene transcription), are a post-translational effect. By contrast, melatonin reduction is related to an ASMT inhibition of expression at both the gene transcription and protein levels. These results were independent of nuclear factor-kappa B (NF-kB) translocation. Neither the $P2Y_1$ receptor activation nor the PLC-mediated pathway was involved in the decrease in melatonin, indicating that ATP regulates pineal metabolism through different mechanisms. Taken together, our data demonstrate that purinergic signaling differentially modulates NAS and melatonin synthesis and point to a regulatory role for ATP as a cotransmitter in the control of ASMT, the rate-limiting enzyme in melatonin synthesis. The endogenous production of melatonin regulates defense responses; therefore, understanding the mechanisms involving ASMT regulation might provide novel insights into the development and progression of neurological disorders since melatonin presents anti-inflammatory, neuroprotective, and neurogenic effects.

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Introduction

The sympathetic input to the rat pineal gland, driven by hypothalamic suprachiasmatic nuclei (SCN), promotes melatonin synthesis [1]. Activation of the β_1-adrenoceptor triggers the following cascade: cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/phosphorylated cAMP response element-binding protein (pCREB) [2]. In rodents, the binding of pCREB to CRE motifs induces the gene transcription of the enzyme aralkylamine N-acetyltransferase (AA-NAT; EC 2.1.3.87), which converts serotonin into N-acetylserotonin (NAS). In addition, this enzyme is activated by phosphorylation by PKA [3, 4]. NAS synthesis is circadianly regulated, while its conversion into melatonin by acetylserotonin methyltransferase (ASMT; EC 2.1.1.4; previously known as hydroxyindole-O-methyltransferase, HIOMT) is regulated by changes in photoperiod [5]. Both indoleamines (NAS and melatonin) are readily released into the circulation [6, 7].

Several transduction pathways modulate the β_1-adrenergic-induced AA-NAT gene transcription and enzyme activation. The α_1-adrenoceptors promote an increase of both gene transcription and enzyme activity by a mechanism dependent on phospholipase C (PLC) [8, 9]. Nuclear factor-kappa B (NF-kB), which is pivotal for initiating innate immune responses [10] and the growth and differentiation of neurons [11, 12], translocates to the nucleus and interacts with kB motifs present in the *Aa-nat* gene [13]. This interaction impairs *Aa-nat* transcription in pinealocytes under both physiological [14] and pathophysiological conditions [15, 16].

ATP serves as a co-transmitter of noradrenaline in the rat pineal gland [17] and potentiates β_1-adrenergic-induced NAS synthesis [18, 19]. Adenine nucleotides act on P2X ligand-gated ionotropic receptors (P2X1–7) and the G-protein-coupled P2Y receptors ($P2Y_{1, 2, 4, 6, 11\text{-}14}$) [20]. In cultured rat pineal glands, ATP, ADP, and their less hydrolysable analogs [e.g., adenyl-imidodiphosphate, 2-methylthioATP (2MeSATP), 2-chloroATP, and adenosine 5-O-2-thiodiphosphate], but not UTP, enhance β_1-adrenergic-induced NAS synthesis [18, 19]. Suramin, a nonselective P2 receptor antagonist [21]; pyridoxalphosphate-6-azophenyl-2, 4-dissulphonic acid (PPADS), an

inhibitor of PLC-coupled P2Y receptors [21, 22]; and 1-(6-((17β-3-methoxyestr-1,3,5(10)-trien-17-yl)amino)-hexyl)-1H-pyrrole-2,5-dione (U73122), an inhibitor of PLC [23, 24] reversed this purinergic enhancement of NAS synthesis [18, 19]. The pharmacological profile indicated an ATP response mediated by $P2Y_1$ receptor activation, followed by activation of PLC and an increase in intracellular calcium in pinealocytes [25].

This previous work focused on the synthesis of NAS, as the goal was to evaluate the functional expression of AA-NAT activation. However, emerging data show no direct correlation between enhancing AA-NAT activity and melatonin synthesis [26]. For instance, in some experimental conditions, even α_1-adrenergic potentiation of β_1-adrenergic-induced AA-NAT activation was not followed by an increase in melatonin content [27]. Another interesting example of differential regulation of AA-NAT and ASMT was described for L-glutamate, where the G-protein coupled class II glutamate receptors induced a reduction in AA-NAT activity, whereas the reduction in ASMT was mediated by an as yet unidentified mechanism [28].

In this study, we confirmed the ATP/$P2Y_1$ receptor-mediated potentiation of the β_1-adrenoceptor-induced synthesis of NAS, and we report a decrease in melatonin levels modulated by nonidentified purinergic signaling. The P2Y1 receptor-mediated response does not involve *Aa-nat* transcription. Increases in intracellular calcium concentration mediate a PLC-induced enhance in NAS synthesis. The reduction in melatonin synthesis, on the other hand, is mediated by inhibition of the transcription *Asmt* and the expression of the protein. These effects are not mediated by PLC. In summary, we have disclosed a new mechanism for understanding the responsiveness of the rat pineal gland to ATP and provide strong evidence for an opposite regulation of AA-NAT and ASMT activities.

Materials and methods

Animals

Male and female Wistar rats (45 days old) were housed under a 12/12-h light/dark cycle (lights on at 6:00 a.m.) with water and food ad libitum. The animals were euthanized by decapitation in the light phase (10 hr after lights on) and the pineal glands were immediately removed and placed in culture or stored at −80°C until processing. All experiments were performed in accordance with the Ethics Committee of the Biosciences Institute of the University of São Paulo under protocol 195/2013.

Pineal gland culture

Rat pineal glands were incubated (37°C; 95% O_2; 5% CO_2; 48 hr) in BGJb medium with glutamine (2 mM), penicillin (100 U/mL), and streptomycin (10 μg/mL) in a 24-multiwell plate (1 gland per well, 200 μL per well). The medium was replaced after 24 hr. This denervated culture [29] was stimulated with ISO (0.1 μM) for 5 hr in the presence or absence of other treatments (0.01–3 mM ATP; 1 μM melatonin). Antagonists and inhibitors (100 μM suramin; 30 μM PPADS; 1 μM U73122) were incubated for 1 hr before the isoprenaline-ATP costimulation. PDTC (12.5 μM) was incubated for 48 hr. For RNAm assays, the glands were stimulated with ISO (0.1 μM) for 90 min in the presence or absence of ATP (1 mM).

Pinealocyte culture

Pinealocytes were cultured according to previous studies [25]. The glands were removed, cut into small pieces, and dissociated with trypsin (0.25%, 37°C, 15 min), followed by mechanical dispersion in the presence of 0.3% trypsin inhibitor in a solution containing 120 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 12 mM glucose, and 0.1% w/v bovine serum albumin. After centrifugation (1000 g, 15 min, 25°C), the supernatants were resuspended in DMEM containing fetal bovine serum (10% heat inactivated) and penicillin (100 U/mL). Cell viability was estimated by trypan blue exclusion. The pinealocytes were seeded in poly l-lysine-coated wells (0.5 to 1 × 10^5 cells/well) and maintained at 37°C in 5% CO_2 for 18 hr before beginning the treatments. Pinealocytes were stimulated with ISO (0.1 μM) for 4 hr in the presence or absence of ATP (1 mM).

Determination of NAS and melatonin by high-performance liquid chromatography (HPLC)

NAS and melatonin contents in the incubation medium were measured by HPLC as previously described [17, 18]. The chromatographic system (Waters, Milford, MA, USA) was isocratically operated with a mobile phase consisting of sodium acetate (0.1 M), citric acid (0.1 M), EDTA (0.15 mM), and 10% methanol for N-acetylserotonin (30% methanol for melatonin), pH 3.7, flowed at a rate of 0.5 mL/min through a 5 μm Resolve C18 reversed-phase column (150 × 3.9 mm i.d.; Waters, Milford, MA, USA). The detector potential was adjusted to + 0.90V versus an Ag/AgCl reference electrode.

RT-PCR real time

Pineal glands were processed for total RNA extraction using TRIzol reagent following the manufacturer's instructions. The cDNA was generated from 1 μg of total RNA using SuperScript III reverse transcriptase. Pineal cDNA was used to quantify the relative mRNA expression of the *Aa-nat* and *Asmt* genes, normalized by the expression of *Gapdh* and *18S* housekeeping genes, by real-time RT-PCR using SYBR Green PCR mix reagent. The sequences of forward (F) and reverse (R) primers were as follows: 5′-AGCGCGAAGCCTTTATCTCA-3′ (*Aa-nat,* F); 5′-AAGTGCCGGATCTCATCCAA-3′ (*Aa-nat,* R); 5′-AGCGCCTGCTGTTCATGAG-3′ (*Asmt,* F); 5′-GGAAGCGTGAGAGGTCAAAGG-3′ (*Asmt,* R); 5′-TTCTTGTGCAGTGCCAGCC-3′;, 5′-CGGCTACCACATCCAAGGAA-3′ (*18S*, F); 5′-CTGGAATTACCGCGGCT- 3′ (*18S*, R); and – TTCTTGTGCAGTGCCAGCC-3′ (*Gapdh*, F); and 5′-GTAACCAGGCGTCCGATACG-3′ (*Gapdh*, R).

Immunocytochemistry

After stimulation, pinealocytes were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4), fixed in 4% cold methanol acetone for 15 min, and permeabilized with PBS supplemented with saponin (0.5%) at room temperature. The nonspecific binding sites were blocked with solution containing 0.3 M glycine for 60 min. The preparation was then incubated with primary rabbit polyclonal anti-ASMT antibody (dilution 1:200, IM-0441; Imuny, São Paulo, SP, Brazil) for 18 hr at 4°C, followed by secondary polyclonal anti-rabbit conjugated with FITC C (1:200, SIGMA F7512) for 1 hr at room temperature. Nuclei were stained with 4′, 6-diamidino-2 phenylindole (DAPI, 300 μM, 5 min) at room temperature. Primary and secondary antibodies were diluted in blocking buffer. For negative controls, no primary antibodies were incubated. Immunopositive cells were observed by fluorescence microscopy with a 40× objective and Zeiss Axio Scope A1 instrument (Zeiss Axio Vision 4.8 software, Berlin, Germany). Fluorescence of three randomly chosen fields per well (20 cells) from three independent experiments in duplicate was quantified using ImageJ software (http://rsb.info.nih.gov/ij). The mean fluorescence measured per well was used in the statistical analysis. The results were normalized to untreated control cells. The validation assay of ASMT antibody was performed by Western blot and is presented as supplementary data (Fig. S1).

Drugs

Isoprenaline (ISO) hydrochloride, N-acetylserotonin, melatonin, BGJb medium, DMEM medium, bovine albumin fraction V, poly l-lysine, trypsin, trypsin inhibitor, ATP, pyrrolidine dithiocarbamate (PDTC), and secondary polyclonal anti-rabbit conjugated with FITC C (SIGMA F7512) were purchased from Sigma (St Louis, MO, USA). Suramin was obtained from Bayer (Leverkusen, Germany), ascorbic acid from Hoechst (São Paulo, SP, Brazil), and fetal bovine serum, penicillin/streptomycin, glutamine, Deoxyribonucleotide triphosphate mix, DNase, primers, SuperScript III enzyme, SYBR Green PCR mix, and TRIzol reagent were purchased from Invitrogen (Grand Island, NY, USA and Eugene, OR, USA). Citric acid, ethylenediaminetetraacetic acid (EDTA), sodium acetate, sodium bisulfite, methanol, perchloric acid, and acetic acid were from Merck (Rio de Janeiro, RJ, Brazil) and PPADS and U73122 were from RBI (Natick, MA, USA). Trypan blue solution was purchased from Mediatech Inc (Herndon, VA, USA), 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) from Santa Cruz Biotechnology (Dallas, Texas, USA), and primary rabbit polyclonal antibody anti-ASMT (IM-0441), from Imuny (São Paulo, SP, Brazil).

Statistical analysis

All data are presented as mean ± S.E.M. The difference between two means was compared by Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

ATP (0.01–3 mM) modulated the β-adrenergic (0.1 μM isoprenaline)-induced synthesis of NAS and melatonin in a concentration-dependent manner, although in opposite directions. NAS (control = 29.13 ± 2.23 ng/well) was increased up to 130%, while melatonin synthesis (control = 62.31 ± 5.71 ng/well) was decreased up to 70% (Fig. 1). Notably, the values of EC_{50} for ATP potentiating NAS (280 μM, 95% confidence intervals 230–350 μM) and for impairing melatonin synthesis (225 μM, 95% confidence intervals 31–1618 μM) were not significantly different.

We tested whether ATP activation accelerates the degradation of melatonin by incubating the pineal glands with melatonin itself (1 μM) in the presence or absence of ATP (1 mM). After 5 hr, the melatonin content was not significantly different between control (72.43 ± 3.35 ng/well, n = 6) and stimulated glands (69.47 ± 4.94 ng/well, n = 5), indicating no increase in degradation.

Since $P2Y_1$ receptor activation enhances NAS synthesis [19], we evaluated whether these receptors were also responsible for the effects observed on melatonin synthesis. Suramin (100 μM) and PPADS (30 μM) blocked ATP-induced enhancement of NAS synthesis, but did not reverse the inhibition of melatonin synthesis (Fig. 2). Likewise, the ATP potentiation of isoprenaline-induced NAS synthesis was reversed by the PLC inhibitor U73122 (1 μM), while the melatonin content was persistently diminished. Therefore, different pathways appeared to mediate the ATP effects on NAS and melatonin.

The gene that encodes the enzyme responsible for the conversion of serotonin to N-acetylserotonin is regulated by NF-kB [13, 30]. In the pineal gland, nuclear translocation of NF-kB impairs the gene activation of *Aa-nat* [14–16, 31]. NF-kB is also regulated by ATP in different cell types [32, 33]. Here, we evaluated the effect of the NF-kB inhibitor, PDTC (12.5 μM). As expected, isoprenaline-induced NAS and melatonin synthesis was potentiated in the presence of PDTC (Fig. 3). Otherwise, blocking the NF-kB pathway had no effect on the ATP-induced changes in NAS and melatonin synthesis. In addition, ATP did not modify *Aa-nat* mRNA transcription. Therefore, we excluded any participation of NF-kB in the response to ATP in our model.

Despite the NAS enhancement and melatonin reduction, ATP displayed the same affinity regarding its ability to modulate pineal hormones (Fig. 1). Therefore, we tested whether ATP would impair ASMT. As expected, ATP impaired gene transcription and protein expression of ASMT (Fig. 4). Taken together, the results indicated that the ATP-dependent decrease in melatonin synthesis was dependent on ASMT inhibition.

Discussion

The sympathetic innervation of the rat pineal gland is responsible for transducing environmental light information into a hormonal signal: melatonin, the darkness hormone. As in other regions innervated by sympathetic nervous system, ATP is a cotransmitter of noradrenaline

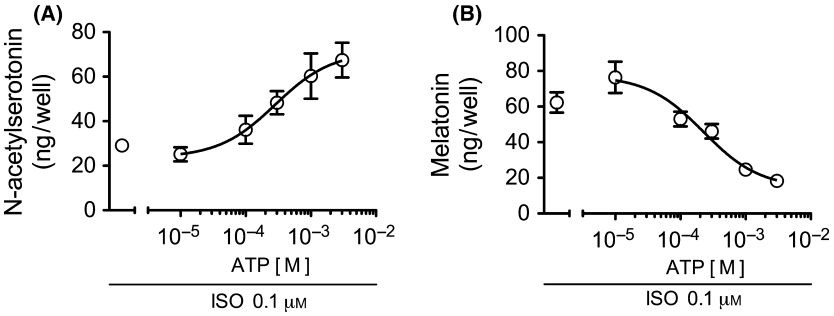

Fig. 1. Dual ATP effects on NAS and melatonin synthesis. (A) N-acetylserotonin and (B) melatonin contents induced by ISO (0.1 μM, 5-hr exposure) in the presence or in the absence of ATP and measured in the pineal gland incubation media. Data represent the mean ± S.E.M. of 7–8 glands per point.

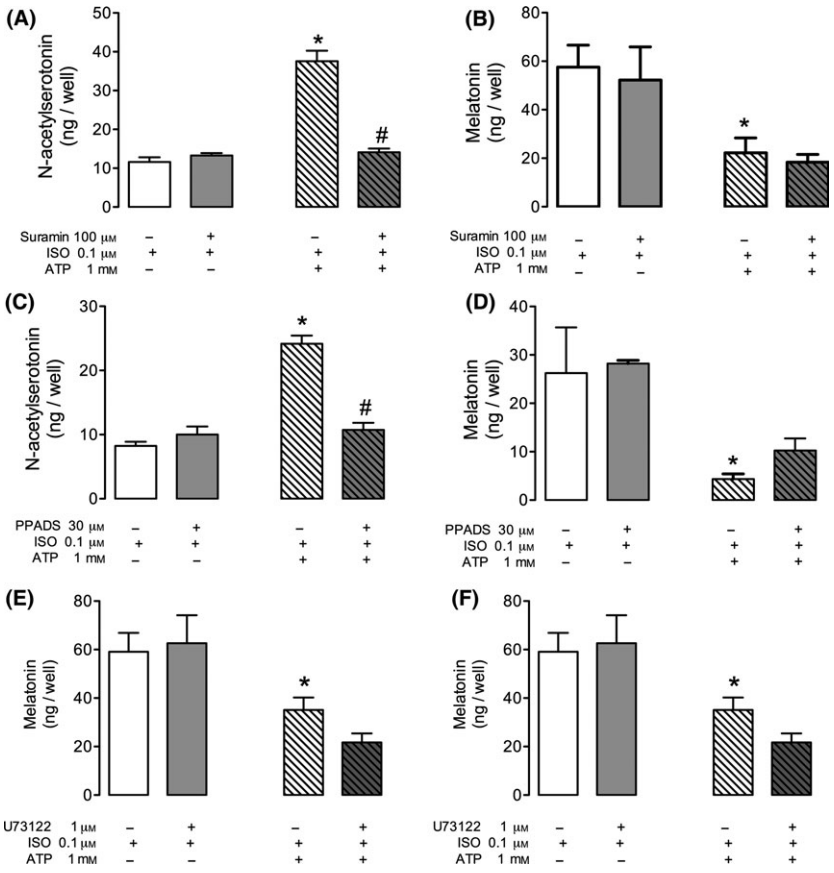

Fig. 2. Purinergic receptor involvement in the ATP effect. Antagonistic assay of purinergic receptors with suramin (100 μM, 1-hr pretreatment) on the contents of N-acetylserotonin (A) and melatonin (B). Antagonistic assay of purinergic receptors with PPADS (30 μM, 1-hr pretreatment) on the contents of N-acetylserotonin (C) and melatonin (D). Inhibition of the PLC pathway (U73122 1 μM, 1-hr pretreatment) on the contents of N-acetylserotonin (E) and melatonin (F). For every assay, pineal glands were concomitantly stimulated with ISO (0.1 μM) and ATP (1 mM) for 5 hr when appropriate. Data represent mean ± S.E.M. of 4–6 glands per treatment. $^{*}P < 0.05$ versus ISO, and $^{\#}P < 0.05$ versus ISO + ATP.

in the pineal gland [17] and is able to potentiate noradrenaline-induced NAS synthesis by triggering $P2Y_1$ receptors [19]. We therefore investigated the effects of ATP upon melatonin synthesis. We confirmed the $P2Y_1$ receptor mediated NAS enhancement, whereas, unexpectedly, ATP reduced melatonin content. This effect was dependent on a reduction in ASMT gene transcription and protein expression, which was not mediated by $P2Y_1$ receptors. Therefore, ATP promoted opposite effects on the final contents of NAS and melatonin, resulting in an increase in NAS and a reduction in melatonin levels, in agreement with ASMT as the limiting step in melatonin synthesis [5, 26, 27, 34, 35]. Interestingly, a different modulation between NAS and melatonin was previously described in the literature, although with a reduction in NAS and a potentiation of melatonin, in response to stressful conditions [36].

Besides acting as a cotransmitter [37], ATP is also a damage-associated molecular pattern (DAMP) molecule [38, 39]. High levels of ATP are released during stress conditions [40] and injury [41], which might trigger P2X7 receptors and activate NF-kB [32, 33, 41]. Moreover, *Aa-nat* transcription is regulated by kB motifs [13]. In fact, data show that NF-kB is constitutively expressed in the pineal gland and its expression is inversely related to melatonin synthesis [14], as the translocation of NF-kB inhibits the melatonin biosynthetic pathway under physiological [14, 31] and pathophysiological [15, 16, 42] conditions. Our data, however, showed that ATP effects did not involve either the NF-kB pathway or changes in *Aa-nat* transcription. This finding was expected, since ATP, although it decreases the melatonin levels, increases NAS content.

The triggering of $P2Y_1$ receptors could activate the regulator of G-protein signaling 2 (RGS2) [43], which exerts

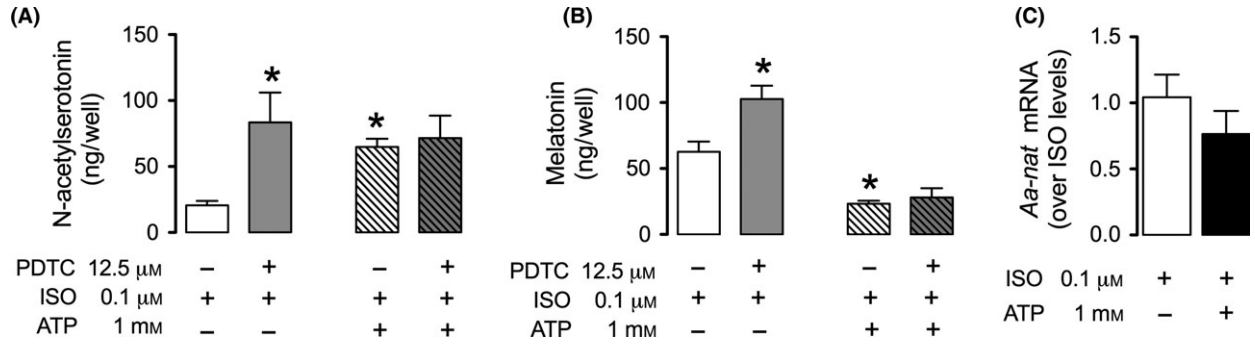

Fig. 3. Putative control of *Aa-nat* by ATP. Inhibition of NF-kB translocation with PDTC (12.5 μM, 48-hr incubation) on the contents of N-acetylserotonin (A) and melatonin (B). (C) *Aa-nat* mRNA in pineal glands stimulated with ISO (0.1 μM) in the presence or absence of ATP (1 mM) for 90 min. *Gapdh* and *18S* were used as normalizers. Data represent mean ± S.E.M. of 4–6 glands. *$P < 0.05$ versus ISO.

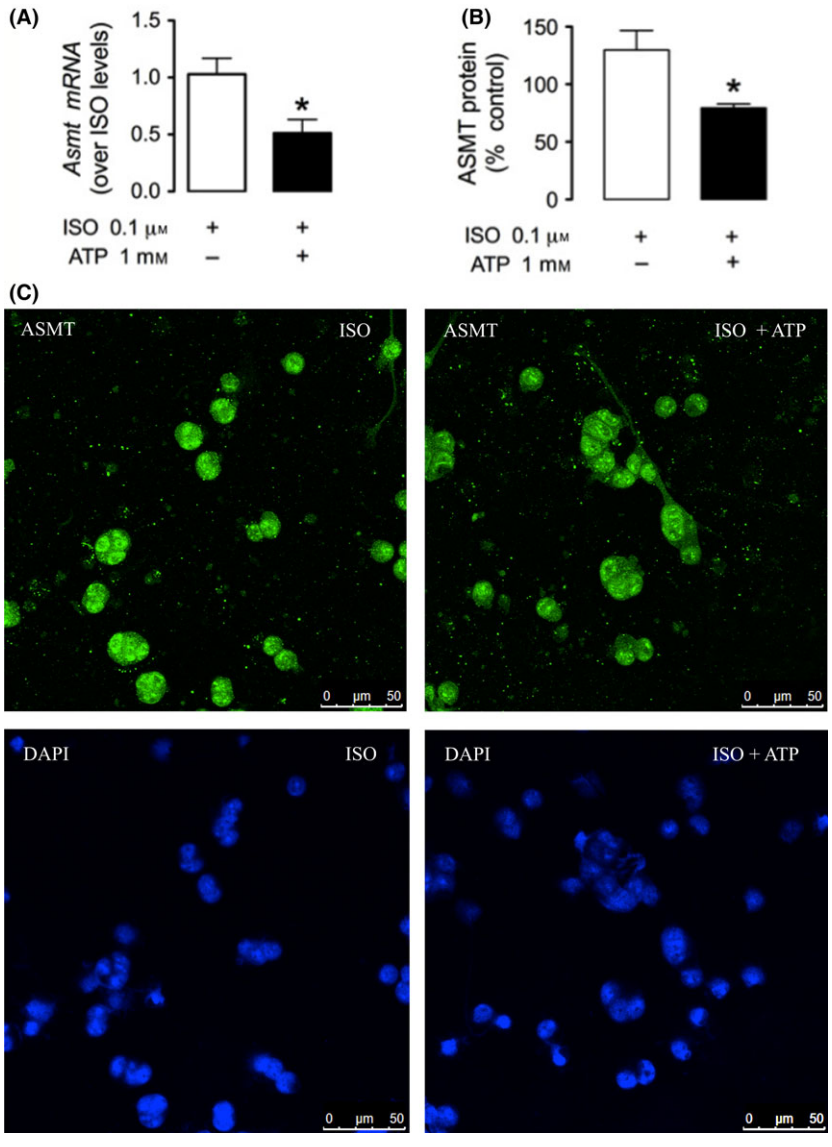

Fig. 4. Putative control of ASMT by ATP. (A) *Asmt* mRNA in pineal glands stimulated with ISO (0.1 μM) in the presence or absence of ATP (1 mM) for 90 min. *Gapdh* and *18S* were used as normalizers. Data represent mean ± S.E.M. of 4–6 glands per treatment. *$P<0.05$ versus ISO. (B) Immunofluorescence quantification of ASMT labeling in pinealocytes treated with ISO (0.1 μM) in the presence or absence of ATP (1 mM) for 4 hr. Data are normalized to the untreated control cells and represent mean ± S.E.M. of three independent experiments. *$P < 0.05$ versus ISO. (C) Representative fluorescence image of ASMT immunostaining in dispersed pinealocytes stimulated with ISO (0.1 μM) in the presence or absence of ATP (1 mM) for 4 hr. Nuclei were labeled with DAPI.

a negative feedback signal on the melatonin biosynthetic pathway and impairs noradrenaline-induced *Aa-nat* transcription in pinealocytes [44]. In our case, however, ATP did not induce changes in *Aa-nat* transcription. These purinergic receptors also increase intracellular calcium [31], which could activate protein kinase C (PKC) and

enhance AA-NAT phosphorylation [45]. The NAS enhancement is dependent on PLC activation [19; present study] and does not involve gene transcription, so our data strongly suggest a post-translational effect of ATP that leads to AA-NAT phosphorylation.

The last hypothesis we tested was whether ATP reduces melatonin synthesis by impairing ASMT activity. Notably, ASMT regulation by neurotransmitters has already been shown; for instance, neuropeptide Y (NPY) is released to the pineal gland in a circadian rhythm [46] and increases ASMT availability [47]. In fact, suprachiasmatic nuclei also control extracellular levels of ATP, which present a circadian rhythm and reach a peak during the dark phase [48]. Nonetheless, ATP has an opposite effect to NPY and inhibits ASMT availability.

The fact that ATP regulates NAS and melatonin in opposite directions suggests that both these serotonin metabolites play independent functional roles, and might indicate that, in some conditions, NAS synthesis is preferred over melatonin synthesis. Corroborating this hypothesis, NAS, but not melatonin, was previously shown to activate brain derived neurotrophic factor (BNDF) and tyrosine kinase receptors type 2 (TRK-B) [49, 50]. A NAS/TRK-B interaction ameliorates depressive behavior in a swim test [49, 51], promotes proliferation of progenitor neuronal cells in sleep-deprived animals [52], and shows neuroprotective properties by reducing caspase 3 activation in the brain in response to a neurotoxic glutamate analog [53].

There are data showing that NAS distribution throughout the central nervous system differs from that of melatonin [54]. In the cortex and hypothalamus, but not in the hippocampus, an increase in *Aa-nat* transcription is not followed by potentiated melatonin synthesis [55, 56]. Emphasizing the importance of NAS itself in some scenarios, primate retina displays *Aa-nat* but barely expresses ASMT gene or enzyme activity [57]. Some clinical evidence supports the relevance of ASMT control; for example, low expression of mRNA and protein was correlated with depression and cognitive impairment [58], reinforcing the association of ASMT with decreased levels of melatonin in severely depressed patients [59]. Autism spectrum disorders, which present different rates of sleep disturbance [60], also show a positive correlation between the increase/reduction of NAS/melatonin and the severity of the disorder [61], suggesting a reduction in ASMT activity. Interestingly, antipurinergic therapy corrects autism-like features in the Fragile X mouse model [62]. Thus, taking together the effects of antipurinergic drugs and the changes in NAS and melatonin in autistic patients, we might speculate that changes in the quantity of ATP in the pineal gland could be relevant in this syndrome. Emphasizing this hypothesis, ATP released by astrocytes has been shown to follow a circadian rhythm [48], and autism spectrum disorder is associated with an activation of white matter astrocytes [63].

In summary, to the best of our knowledge, this is the first study to demonstrate that purinergic signaling regulates NAS and melatonin, in opposite directions and through independent mechanisms in mammals. Melatonin deficit is a result of impaired transcription and protein expression of ASMT, emphasizing the role of this enzyme in the modulation of melatonin synthesis regardless the NAS availability. We also started to uncover the mechanisms underlying ASMT regulation, which might reveal novel insights about the development and progression of various disorders.

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Authors' contribution

Souza-Teodoro LH, Dargenio-Garcia L, Markus RP, and Ferreira ZS involved in concept/design of the study; Souza-Teodoro LH, Dargenio-Garcia L, Petrilli-Lapa CL, and Souza ES performed the acquisition of data; Souza-Teodoro LH, Dargenio-Garcia L, Petrilli-Lapa CL, Fernandes PACM, Markus RP, and Ferreira ZS took part in data analysis/interpretation; Souza-Teodoro LH, and Ferreira ZS drafted the manuscript; Fernandes PACM and Markus RP did the critical revision of the manuscript; all authors involved in the approval of the article submitted.

Conflict of interest

The authors declare none.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. ASMT detection by Western blot analysis in protein extracts from rat pineal gland.