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Brain Damage-linked ATP Promotes P2X7 Receptors Mediated Pineal N-acetylserotonin Release

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Abstract—The pineal gland is a key player in surveillance and defense responses. In healthy conditions, nocturnal circulating melatonin (MEL) impairs the rolling and adhesion of leukocytes to the endothelial layer. Fungi, bacteria, and pro-inflammatory cytokines block nocturnal pineal MEL synthesis, facilitating leukocyte migration to injured areas. ATP is a cotransmitter of the noradrenergic signal and potentiates noradrenaline (NAd)-induced MEL synthesis via $P2Y_1$ receptor ($P2Y_1R$) activation. Otherwise, ATP low-affinity P2X7 receptor (P2X7R) activation impairs N-acetylserotonin (NAS) into MEL conversion in NAd incubated pineals. Here we mimicked a focal increase of ATP by injecting low (0.3 and 1.0 μg) and high (3.0 μg) ATP in the right lateral ventricle of adult rats. Nocturnal pineal activity mimicked the in culture data. Low ATP doses increased MEL output, while high ATP dose and the P2X7R agonist BzATP (15.0–50.0 ng) increased NAS pineal and blood content. In the brain, the response was structure-dependent. There was an increase in cortical and no change in cerebellar MEL. These effects were mediated by changes in the expression of coding genes to synthetic and metabolizing melatonergic enzymes. Thus, the pineal gland plays a role as a first-line structure to respond to the death of cells inside the brain by turning NAS into the darkness hormone.

Keywords: N-acetylserotonin, MEL, Purinergic signaling, BzATP, serotonin-N-acetyltransferase (SNAT), acetylserotonin N-methyltransferase (ASMT).

INTRODUCTION

The pineal gland is the only gland formed by secretory cells of ectodermic origin (pinealocytes), microglia, and astroglia (da Silveira Cruz-Machado et al., 2012; Rodriguez et al., 2016). Nocturnal sympathetic input, mediated by the neurotransmitter noradrenaline (NAd) and the cotransmitter ATP, triggers melatonin (MEL) synthesis (Mortani-Barbosa et al., 2000; Ferreira and Markus, 2001). ATP interacts with two subclasses of P2 purinergic receptors: G-protein coupled receptors (P2Y) and the ligand-gated ion channels (P2X) (Ralevic and Burnstock, 1998). Pinealocytes express the ATP high-affinity $P2Y_1$ receptor ($P2Y_1R$) and the ATP low-affinity P2X7 receptor (P2X7R) (Ferreira and Markus, 2001; Souza-Teodoro et al., 2016).

In a physiological state, ATP acts as a cotransmitter of NAd, potentiating sympathetic-induced N-acetylserotonin (NAS) synthesis via activation of $P2Y_1R$ (Mortani-Barbosa et al., 2000; Ferreira and Markus, 2001). Sympathetic-induced transcription of coding-gene and the activation of the enzyme serotonin-N-acetyltransferase (SNAT, also known as arylalkylamine N-acetyltransferase, EC 2.3.1.87), leads to the conversion of serotonin into NAS. NAd and ATP effects on pinealocytes are mediated by β1-adrenergic receptors (β1-AR) and $P2Y_1R$ (Ferreira and Markus, 2001; Ferreira et al., 2003). In contrast, high extracellular ATP concentration triggers pinealocytes P2X7R, leading to the inhibition of acetylserotonin O-methyltransferase (ASMT, also known as hydroxy-indole O-methyltransferase, EC 2.1.1.4 19) coding gene transcription and protein expression (Simonneaux and Ribelayga, 2003). Thus, high extracellular ATP concentration blocks NAS into MEL conversion (Falzoni et al., 2013; Souza-Teodoro et al., 2016). Finally, MEL in the brain is metabolized to 6-hydroxy MEL (6-HMEL) by CYP1B1 (cytochrome p450 family) (Simonneaux and Ribelayga, 2003).

As a circumventricular organ, the pineal gland can detect pathogen- and danger/damage-associated molecular patterns (PAMPs and DAMPs) circulating in the blood and the cerebrospinal fluid (CSF) (Veening

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Abbreviations: ASMT, acetylserotonin N-methyltransferase; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid; HSP, heat shock protein; HMGB, high-mobility group box; ISO, Isoprenaline; MEL, melatonin; NAd, noradrenaline; NAS, N-acetylserotonin; NF-kB, nuclear factor-kappa B; P2Y1R, P2Y1 receptor; P2X7R, P2X7 receptor; SAA, serum amyloid A; SNAT, serotonin-N-acetyltransferase.

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and Barendregt, 2010). Viruses, fungi, bacteria, and pro-inflammatory cytokines block the conversion of serotonin into NAS, impairing MEL synthesis (Markus et al., 2018). Parasites that depend on mammal daily MEL rhythm to improve host cell survival (Leishmania sp. and Plasmodium sp.) do not alter the nocturnal MEL synthesis (Laranjeira-Silva et al., 2015). All the stimuli that inhibit pinealocyte NAd (sympathetic)-induced MEL synthesis to transduce via the nuclear translocation of NF-kB (nuclear factor-kappa B) p50/p50, which blocks SNAT transcription impairing serotonin to NAS conversion (Markus et al., 2018). Suppression of nocturnal NAS and MEL pineal output blocks the expression of endothelial adhesion molecules necessary for the migration of leukocytes from blood to tissues. Thus, pineal-mediated activation of the NF-kB canonical pathway allows for an effective defense response at any hour of the day.

High CSF-circulating ATP originated from brain trauma (Wang et al., 2004; Choo et al., 2013), hypoxia/ischemia episodes (Lutz and Kabler, 1997; Melani et al., 2005), or epilepsy-associated seizures (Dale and Frenguelli, 2009) acts as a DAMP. In cultured pineal glands and pinealocytes activation of the ATP low-affinity P2X7R with mM ATP decreases *Asmt* expression, reducing MEL and increasing NAS output (Souza-Teodoro et al., 2016). Notably, the reaction of pinealocytes to high-ATP is different from other damage signals, as NAS blocks the emigration of leukocytes from bloodstream, avoiding the mounting of spurious acute inflammatory reactions and present neuroprotective effects (Tosini et al., 2012a; Michael Iuvone et al., 2014). Thus, high-CSF circulating ATP should protect brain areas far from a focal lesion.

Here, we investigated whether the injection of ATP in a lateral ventricle modifies pineal MEL synthesis and plasma, cortex, and cerebellum NAS and MEL levels. Our data show for the first time that high intracerebral ATP activates P2X7R pineal receptors by blocking the conversion of NAS into MEL. In addition, changes in the expression of the MEL biosynthetic enzymes in the cortex and cerebellum point to favoring neuronal protection. Regarding plasma output, a peak of NAS, with no change of MEL level, was observed at nighttime. Thus, high intracerebral ATP released from a focal disturbance will protect far located structures and turn NAS into the darkness hormone.

EXPERIMENTAL PROCEDURE

Animals

Wistar rats (45–60 days old, 180–250 g), obtained from the animal facility of the Department of Physiology (Biosciences Institute, University of São Paulo, Brazil), maintained under 12/12 h light/dark cycle (lights on at 06:00 hours, Zeitgeber time zero, ZT 0) received water and food ad libitum.

Animals were euthanized at ZT 18 by decapitation. Pineal glands and plasma were rapidly removed and maintained at −80 °C. Procedures were approved by the IB-USP Ethical Committee (license number 106/2010, 324/2018) and carried out in compliance with the recommendations of the National Council on Experimental Animal Control (CONCEA).

Drugs

N-acetylserotonin, MEL, ATP, Isoprenaline (ISO), (2′(3′)-O-(4-benzoylbenzoyl)-ATP (BzATP), MRS2179 and A438079 were purchased from Sigma (St Louis, MO, USA). 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).

Surgical procedure

The animals were anesthetized (ketamine 100 mg/kg and xylazine 40 mg/kg, i.p.) and submitted to the surgical procedure as described before (Pinato et al., 2015). Briefly, a guide cannula was introduced (*Plastics One*, Roanoke, VA, USA) to a depth of 3.5 mm from the dura mater into the right lateral ventricle (1.4 mm lateral and 0.4 mm posterior to the Bregman) via a stereotaxic arm, permanently affixed to the cranium using anchor screws (*Plastics One*, Roanoke, VA, USA) and dental acrylic (Jet Clássico, SP, Brazil), covered with a dummy cannula (*Plastics One*, Roanoke, VA, USA). The rats were placed in the home cages and maintained from 7 to 10 days before experimental procedures.

Purinergic treatments

ATP (0.3–3.0 μg) or the most potent P2X7R agonist BzATP (15.0–50.0 ng; Donnelly-Roberts et al., 2009) were injected in the right lateral ventricle (i.c.v.) 30 min before darkness (ZT11.5) or, at daytime experiments, 30 min before ISO (10^{-4} M) at ZT6. The selective $P2Y_1R$ (MRS2179; 2.7 ng/5 μL) and P2X7R (A438079, 1.7 ng/5 μL) antagonists were injected 1 h before ATP, at ZT 10.5. Animals were euthanized 6 h later (ZT 18) at nighttime experiments and 1 min after ISO injection in daytime experiments. The pineal gland, cortex, and cerebellum were collected for determining NAS and MEL content, as well as the expression of *Snat*, *Asmt,* and *Cyp1b1* mRNA, which codify the enzymes that convert serotonin to NAS, NAS to MEL and MEL to its metabolite 6-hydroxi MEL (Simonneaux and Ribelayga, 2003).

Indolamine determination

N-acetylserotonin and MEL content in the pineal gland and blood were determined by high-performance liquid chromatography (HPLC) through electrochemical detection according to a previously described methodology (Mortani-Barbosa et al., 2000). Each gland was homogenized in 0.1 M perchloric acid (130 μL) and centrifugated at 18.000 g (10 min, 4 °C), and 20 μL of the supernatant was injected into the chromatographic system (Waters, Milford, MA, USA). Blood samples were prepared for NAS analysis by mixing a 0.2–1 mL volume of plasma with a 20 μL volume of methanol and water

(1:1). The tubes were briefly vortexed, chilled on ice for 10 min, and centrifuged (10,000 *g*, 15 min). The supernatant of each sample (200 or 500 μL) was solid-phase extracted (SPE) through a Sep-Pak reversed-phase C18 cartridge (Waters, Milford, MA, USA) with methanol 10%, dried by speed-vac drier (Eppendorf, São Paulo, Brazil), reconstituted with 0.1 M perchloric acid (130 μL) and then injected into the chromatographic system (Waters, Milford, MA, USA).

The mobile phase (0.1 M sodium acetate, 0.1 M citric acid, 0.15 mM EDTA, pH3.7, methanol 4%, and 25%, for NAS and MEL respectively) was isocratically operated at flow rate of 0.5 mL min^{-1} through a 5-mm Resolve C 18 reversed-phase column (Waters, Milford, MA, USA). The detector potential was adjusted to + 0.90 V (vs. Ag/AgCl reference electrode).

The brain tissue was homogenized in 200 μL of ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2HPO_4, pH 7.4). MEL concentration in the plasma and the tissues was determined using an ELISA method according to the manufacturer's specifications (rat MEL ELISA kit, IBL, Germany). Protein concentration was determined by directed measurement of absorbance at 280 nm (A280) on NanoDrop1000 UV–VIS Spectrophotometer Software 3.8.1.

RNA extraction

The pineal glands, cortex, and cerebellum were processed for total RNA extraction using trizol reagent and chloroform–isopropanol, according to the manufacturer's instructions. The cDNA was generated starting from 0.5 μg (pineal) and 2 μg (cortex and cerebellum) total RNA using 1 pg of random hexamer primer and SuperScript III Reverse Transcriptase (200U; 25 °C for 5 min, 50 °C for 55 min, 70 °C for 15 min).

RT-qPCR

The RT-qPCR was performed using 2X SYBR Green PCR Master Mix, 200 nM of each primer pair, 5 μL of cDNA (10-fold diluted) and RNase-free water to a final volume of 25 μL. The reactions were incubated for 10-min denaturation step at 95 °C, following by 40 cycles of 15 seg at 95 °C and 1 min at 60 °C. The reactions were performed in StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Primer sequences and the corresponding base sites used for rat mRNA analysis were as follows: *Snat/Aa-nat* sense, 5′-CTCAG GAGTCGCTGACACTA-3′ and *Snat/Aa-nat* antisense, 5′-TCCCCAGGTGGTGAAGGTAT-3′; *Asmt* sense, 5′-A GCGCCTGCTGTTCATGAG-3′ and *Asmt* antisense, 5′-GGAAGCGTGAGAGGTCAAAGG-3′; *Cyp1b1* sense, 5′-GACATCTTTGGAGCCAGCCA-3′ and *Cyp1b1* antisense, 5′-AAAGCCATGACGTATGGTAAGTT-3′ and *Gapdh* sense, 5′-GGAACACGGAAGGCCATG-3′ and *Gapdh* antisense, 5′-GCCCAGAACATCATCCCT-3′. The 2-ΔΔCt formula was applied to calculate the relative expression of *Snat/Aa-nat*, *Asmt* and *Cyp1b1* normalized by *Gapdh*. The mean ΔCt of the control group was used as the calibrator.

RT-qPCR validation assays

The specificity of primer was carried by sequencing the PCR amplicon. In brief, the conventional PCR reaction was performed using with 2x DreamTaq Green Polymerase (ThermoScientific), 0.2 μM of each corresponding primer pair, and 5 μL of cDNA (diluted 5-fold, cortex and pineal samples), and RNAse-free water was added to a final volume of 100 μL. The PCR reaction consisted of 1 cycle of 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, by 30 s at 60 °C and 1 min at 72 °C and final extension step of 5 min at 72 °C. PCR was performed in an MasterCycler Nexus Thermal Cycler (Eppendorf). PCR amplification was analyzed in 2% agarose gel to confirming the length of fragment and specific amplification. PCR amplicon was purified using Accuprep® PCR Purification Kit (Bionner), as manufacturer's instructions. The DNA sequencing was performed with 5 μL of purified PCR amplicon (10 ng/ μL) and 5 μM of each corresponding primer in 7.5 μL by Sanger technology using the facility service from the Human Genome and Stem Cell Research Center (HUG-CELL, Bioscience Institute, University of São Paulo). The alignment of DNA sequences with target genes using BLAST tool from NCBI webpage (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and SnapGene showed an identity of 97% for *Snat*, 93% for *Asmt*, 93% for *Cyp1b1* and 95% for *Gapdh* gen, as showed in Fig. S1**A**–**D**, respectively.

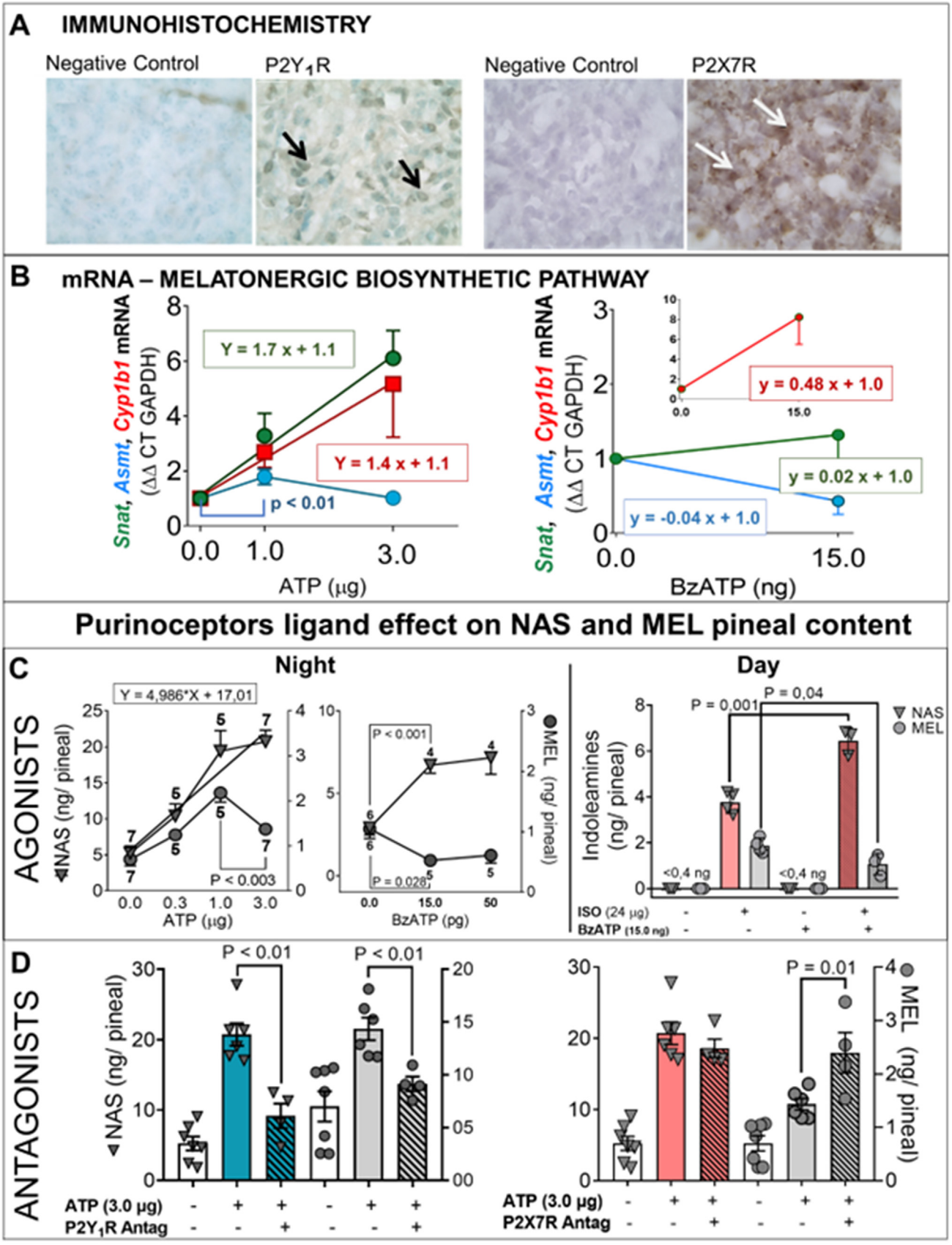
The efficiency of RT-qPCR reaction was analyzed based on a standard curve prepared from a ten-fold serial dilution of a purified and quantified PCR amplicon containing the target DNA, showing efficiency higher than 95%, as showed in Fig. S2.

Immunostaining

Pineal cryosections (20 μm) from animals sacrificed at ZT6 were used for immunohistochemistry. The sections were blocked (BSA 1%, 60 min) and incubated with anti-goat polyclonal antibody anti-P2X7R (2 μg/ml, Santa Cruz Biotechnology, sc31500) or anti-rabbit anti-$P2Y_1R$ (2 μg/ml, Imgenex) for 48 h at 4 °C, followed by secondary biotinylated anti-goat or anti-rabbit IgG antibody (1:200) respectively, for an additional 90 min at room temperature. Negative controls were performed in the absence of the primary antibody. The Avidin-Biotin-Complex (Vectastain Elite kit) staining system was used to localize biotinylated antibody. Peroxidase activity was revealed with 3, 3-diaminobenzidine (DAB). The sections were counterstained with eosin or methylene blue for P2X7R or $P2Y_1R$, respectively.

Statistical analysis

All the data were initially tested for normality using the Shapiro-Wilk test. The null-hypothesis is that the population is normally distributed. All the *samples except for two did not deviate from normality.* Differences between independent variables were evaluated by ANOVA/Turkey's test. Two means were compared by the Student "t" test (parametric data) or


A
IMMUNOHISTOCHEMISTRY
Negative Control
P2Y1R
Negative Control
P2X7R
B
mRNA – MELATONERGIC BIOSYNTHETIC PATHWAY
Snat, Asmt, Cyp1b1 mRNA (ΔΔ CT GAPDH)
Y = 1.7 x + 1.1
Y = 1.4 x + 1.1
p < 0.01
ATP (μg)
y = 0.48 x + 1.0
y = 0.02 x + 1.0
y = -0.04 x + 1.0
BzATP (ng)
Purinoceptors ligand effect on NAS and MEL pineal content
C
AGONISTS
Night
Y = 4,986*X + 17,01
NAS (ng/ pineal)
P < 0.003
ATP (μg)
P < 0.001
P = 0.028
MEL (ng/ pineal)
BzATP (pg)
Day
Indoleamines (ng/ pineal)
P = 0,001
P = 0,04
NAS
MEL
<0,4 ng
ISO (24 μg)
BzATP (15.0 ng)
D
ANTAGONISTS
NAS (ng/ pineal)
P < 0.01
P < 0.01
ATP (3.0 μg)
P2Y1R Antag
P = 0.01
MEL (ng/ pineal)
P2X7R Antag

the Mann-Whitney test (non-parametric data). The number of independent measures (3–5) was not supposed to introduce a size-effect bias. Dose-effect curves were tested for regression from zero (minimum square method) and comparison of slopes and intercepts (elevation). Two-way ANOVA test for parallel lines evaluated significance in slopes and intercepts. Statistical analysis and graph plots were performed with Graph Pad Prism 9. The level of significance was $p < 0.05$.

RESULTS

Purinergic control of the genes coding enzymes for pineal MEL synthesis and degradation

As detected by immunohistochemistry, rat pineal glands express $P2Y_1R$ and P2X7R (Fig. 1**A**). Injection of purinergic agonists, just before lights off, modifies the expression of genes that code SNAT, ASMT, and CYP1B1 (Fig. 1**B**). ATP, but not BzATP, potentiates nocturnal *Snat* transcription ($R^2 = 0.75$, $F_{(1, 13)} = 39$, $p = < 0.0001$). *Asmt*-transcription modulation, induced by ATP, followed a bell-shaped curve. The ATP dose of 1.0 μg potentiated the *Asmt*-transcription, while the higher dose (3.0 μg) had no significant effect. BzATP (15 ng) reduced *Asmt* transcription ($R^2 = 0.63$, $F_{(1, 6)} = 10$, $p = 0.018$). Regarding the gene that codes the metabolizing enzyme CYP1B1, both ATP ($R^2 = 0.48$, $F_{(1, 12)} = 11$, $p = 0.0058$) and BzATP ($R^2 = 0.47$, $F_{(1, 8)} = 7$, $p = 0.029$) increased in a dose-dependent manner its transcription. Thus, the effect of purinergic agonists on the genes coding the enzymes involved in the MEL biosynthetic pathway and metabolization suggests that $P2Y_1R$ increase NAS and MEL synthesis, while P2X7R increase NAS at the expense of reducing MEL output.

Purinergic agonists and antagonists modulate NAS and MEL pineal synthesis

ATP doses (0.3–3.0 μg) injected 30 min before darkness increased in a dose-dependent manner NAS pineal content ($R^2 = 0.65$, $F_{(1, 22)} = 40.8$, $p < 0.0001$; degree of freedom are shown in parenthesis). In contrast, MEL content followed a bell-shaped curve, being the maximal response attained with ATP 1.0 μg (Fig. 1**C**). BzATP (15.0–50.0 ng) increased NAS ($t_{(8)} = 5.68$, $p = 0.0005$) and decreased MEL ($t_{(9)} = 2.84$, $p = 0.028$) pineal content (Fig. 1**C**). At daytime (12:00 h), isoprenaline (ISO), a β-selective agonist, induced NAS and MEL synthesis. In the presence of BzATP, but not in the absence of ISO, increased NAS ($t_{(5)} = 6.88$, $p = 0.001$) and reduced MEL ($t_{(5)} = 2.72$, $p = 0.04$) pineal content (Fig. 1**C**). The selective $P2Y_1R$ antagonist, MRS2179 (2.7 ng), blocked ATP-induced NAS ($t_{(8)} = 4.77$, $p = 0.0014$) and MEL ($t_{(8)} = 3,597$, $p = 0,007$) potentiation, whereas the P2X7R, antagonist A438079 (1.7 ng), blocked high ATP dose-induced reduction in pineal MEL content ($t_{(8)} = 2,93$, $p = 0,019$)(Fig. 1**D**). The present result strongly suggests that $P2Y_1R$ potentiates 5-HT acetylation, whereas P2X7R impairs NAS methylation. Consequently, low ATP doses potentiates pineal MEL synthesis, whereas high ATP dose increases NAS and reduces MEL output.

Plasma and brain

Plasma MEL mirrored the changes in the pineal gland. ATP doses (1.0 and 3.0 μg) increased nocturnal plasma NAS ($F_{(2, 10)} = 2.663$, $p = 0.0003$), whereas BzATP doses (15.0–50.0 ng) reduced plasma MEL content ($R_2 = 0.91$, $F_{(1, 11)} = 109.7$, $p < 0.0001$) (Fig. 2). In the cortex (Fig. 3**A**), but not in the cerebellum (Fig. 3**B**), ATP doses (1.0 and 3.0 μg) increased MEL content ($F_{(2, 11)} = 0.82$, $p = 0.0051$), while BzATP had no effect in both structures. Changes in the expression of genes encoding SNAT, ASMT, and CYP1B1 offer a clue to understanding regional specificity (Fig. 4). In the cortex, ATP induced a reduction in *Snat* expression ($R^2 = 0.61$, $F_{(1, 10)} = 15.68$, $p = 0.0027$) and an increase in *Asmt* ($t_{(6)} = 2.86$, $p = 0.029$), suggesting that NAS originated from the pineal gland is converted into MEL (Fig. 4**A**). As, there is no change in the expression of *Cyp1b1* in the cortex, MEL will accumulate in the tissue. Otherwise, in the cerebellum, a simultaneous reduction in the expression of *Asmt* mRNA ($R^2 = 0.60$, $F_{(1, 10)} = 14.99$, $p = 0.003$) and an increase in *Cyp1b1* mRNA ($R^2 = 0.78$, $F_{(1, 10)} = 36.44$, $p = 0.0001$) should contribute to avoiding changes in MEL content (Fig. 4**B**).

DISCUSSION

An acute brain injury leads to massive release of ATP and other DAMPs, such as serum amyloid A (SAA), high-

Fig. 1. The mechanistic basis for rat pineal gland response to ATP and BzATP injected in the right lateral ventricle. (**A**) $P2Y_1R$ and P2X7R expression in cultured rat pineal glands was determined in immunostained cryosections (20 μm). No primary antibodies were incubated in negative controls. Results represent independent experiments (three rats) and the arrows indicate $P2Y_1R$- and P2X7R-positive cells. (**B**) ATP and BzATP modified "in vivo" expression of *Snat*, *Asmt* and *Cyp1b1* mRNA. The relative expression of the enzymes *Snat*, *Asmt*, and *Cyp1b1* mRNA were normalized by *Gapdh*. Data were fit by the minimum square method and lines validated by ANOVA and the graph expresses the linear regression equation of the form $Y = bX + a$, where X is the explanatory variable, Y is the dependent variable, b is the line slope, and a is the intercept (the value of y when $x = 0$). BzATP did not change the expression of *Snat*, whereas ATP promoted a dose-dependent change in *Snat* ($R^2 = 0.75$, $F_{(1, 13)} = 39$, $p = < 0.0001$) and *Cyp1b1* ($R^2 = 0.48$, $F_{(1, 12)} = 11$, $p = 0.0058$) expression. ATP 1.0, but not 3.0 μg, significantly increased *Asmt* expression ($t_{(6)} = 3.75$, $p = 0,009$). Purinoceptors agonists (**C**) and antagonists (**D**) modulate NAS and MEL pineal content "in vivo". The agonists ATP (0.3–3.0 μg) or BzATP (15.0–50.0 ng) were injected 30 min before lights off (ZT11.5) in darkness or, at daytime experiments, 30 min before ISO (24 μg) at ZT6. The selective $P2Y_1R$ (MRS2179; 2.7 ng) and P2X7R (A438079, 1.7 ng) antagonists were injected 1 h before ATP (3.0 μg) at ZT 10.5. Data were compared by Student "t" test. Animals were killed at ZT18 (night) and ZT06 (day). NAS and MEL content were measured by HPLC. Data are expressed as mean ± SEM. The number of glands is indicated in the figure.

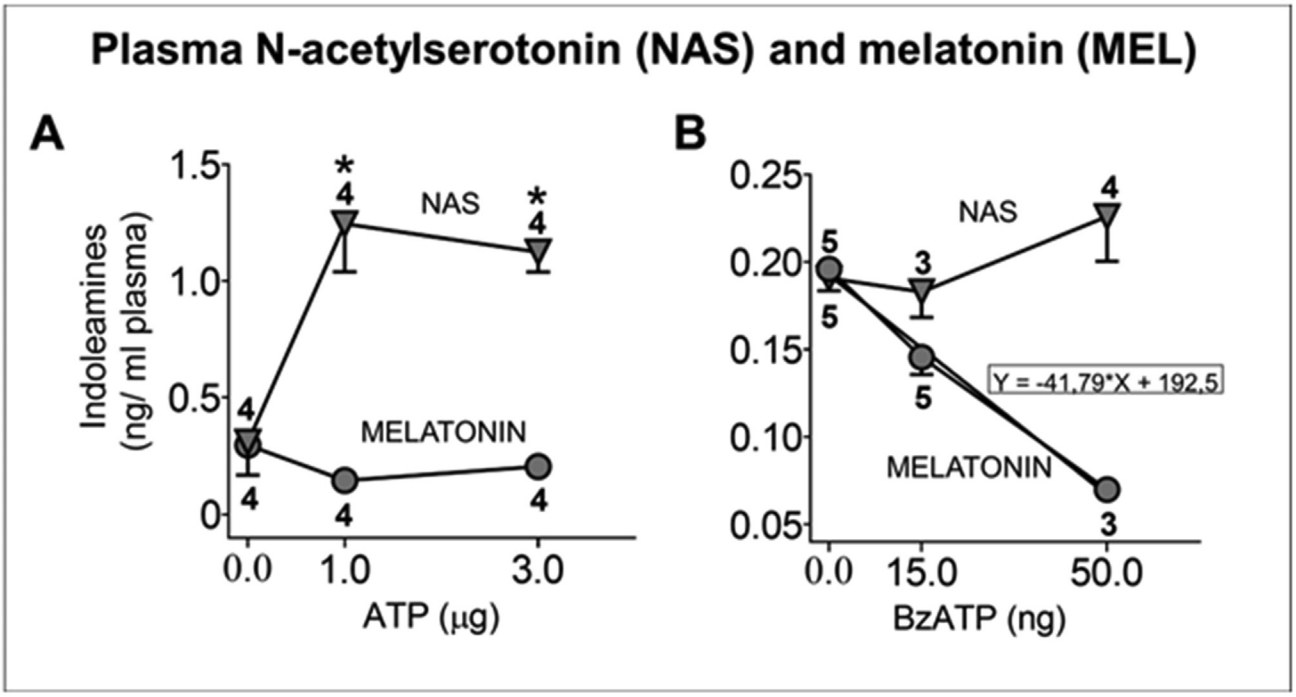


Fig. 2. Effect of purinergic stimulation on plasma indoleamine content. (**A**) – ATP doses 1.0 ($p = 0{,}0005$) and 3.0 μg ($p = 0{,}0015$) increase nocturnal plasma NAS and (**B**) – BzATP doses (15.0–50.0 ng) reduced plasma MEL content. Data were fit by the minimum square method and lines validated by ANOVA and the graph (**B**) expresses the linear regression equation of the form $Y = bX + a$, where X is the explanatory variable, Y is the dependent variable, b is the line slope, and a is the intercept (the value of y when $x = 0$). Both, ATP and BzATP, were injected in the right lateral ventricle just before lights off (ZT11.5), and the animals killed at ZT18. The content of NAS and MEL in the plasma were measured by HPLC and ELISA, respectively. Data are presented as mean ± SEM. Number of rats is shown in the graph.

mobility group box (HMGB)-1 and heat shock protein (HSP)-70 (Denes et al., 2010; Trotta et al., 2014) into the interstitial space and the CSF. ATP increase in the brain extracellular space, once considered a risk factor for neurodegenerative progression, now is known to restrict damage and microglia reaction to areas around the lesion (Burnstock, 2016). Here we show that the pineal gland is highly relevant for transducing the response to high ATP dose injected in a lateral ventricle, mimicking a focal apoptosis or necrosis.

Pineal gland, a sensor for reporting brain injuries

Lower doses of ATP (0.3–1.0 μg) increase simultaneously nocturnal pineal NAS and MEL, while the higher ATP dose (3.0 μg) sustained NAS and reduced MEL output. ATP-induced NAS and MEL changes in the pineal gland followed a classic hyperbolic and a bell-shaped curve, respectively. Accordingly, in cultured pineal glands, the ATP low-affinity P2X7R blocks the conversion of NAS into MEL, and the ATP high-affinity $P2Y_1R$ potentiates the conversion of serotonin into NAS (Ferreira and Markus, 2001; Souza-Teodoro et al., 2016). The currently most potent P2X7R agonist, BzATP, and the selective $P2Y_1R$ and P2X7R antagonists confirmed that CSF-ATP level can be decodified by the pineal gland. It is noteworthy that in physiological condition, nocturnal NAS serum increase is roughly equal to MEL (Lee et al., 2020). However, despite the well-known reduction in nocturnal MEL after a stroke (Ritzenthaler et al., 2009), there is no information about NAS output "in vivo".

N-acetylserotonin, as MEL, were shown to protect the organism against spurious mounting of defense responses and promote cell protection at the peripheral and central level. The switch of nocturnal pineal hormone from MEL to NAS still provides peripheral protection, as besides impairing the mounting of spurious defense response, it reduces oxidative stress (Foster et al., 2000; Jockers et al., 2016). NAS has also a neuroprotective effect in several models of brain injury {striatal dopaminergic system (Aguiar et al., 2005), depression (Jang et al., 2010), hepatic ischemia–reperfusion (Yu et al., 2013), ischemic injury (Zhou et al., 2014), traumatic brain injury (Li et al., 2019), neonatal hypoxic-encephalopathy (Luo et al., 2021)} reducing neuronal death and increasing neural connection. Each indolamine triggers a specific mechanism of action. Inhibition of leukocyte migration to healthy tissue by MEL and NAS is mediated by MT2 and quinone reductase II, respectively (Dubocovich et al., 2003; Lotufo et al., 2006). MEL plays a neuroprotective role via direct scavenger of free radicals, regulation of transcription factors, modulation of mitochondrial activity, and activation of MT1 and MT2 receptors (de Butte and Pappas, 2007; Radogna et al., 2007; Wang, 2009; Cardinali et al., 2010; Galano et al., 2011, 2013; Cary et al., 2012; Fink et al., 2014; Pinato et al., 2015). NAS cytoprotection of neurons (Lotufo et al., 2001; de Butte and Pappas, 2007; Zhou et al.,

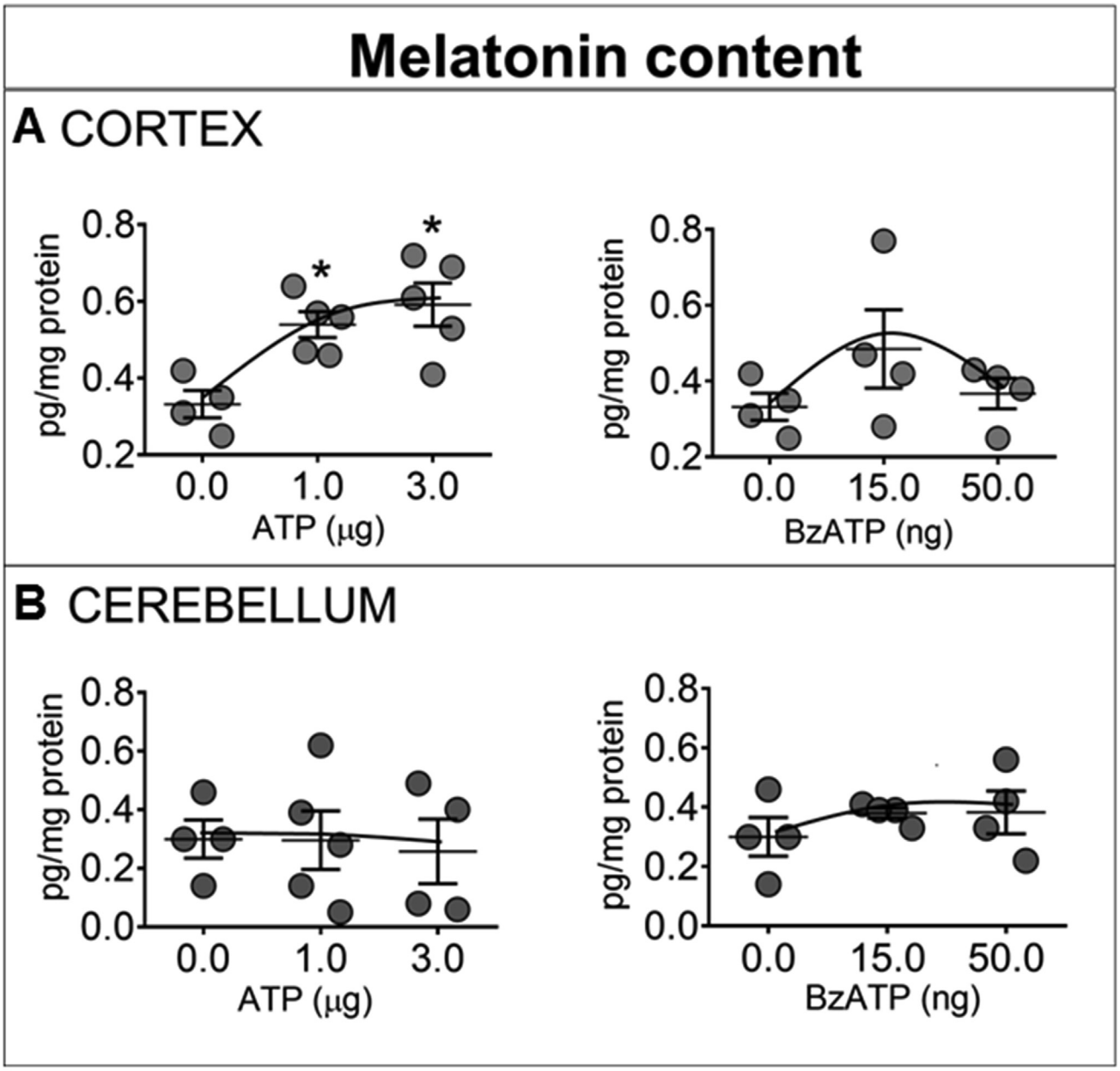


Fig. 3. Effect of purinergic stimulation on MEL content in the brain. (**A**) ATP doses 1.0 ($p = 0.02$) and 3.0 μg ($p = 0.005$) increase MEL content in the cortex, but not in cerebellum (**B**). BzATP doses (A, B) (15.0–50.0 ng) has no effect. Both, ATP and BzATP were injected in the right lateral ventricle just before lights off (ZT11.5). Animals were killed at ZT18. Data were analyzed by ANOVA. Each symbol represents one animal.

2014) and hepatic tissue (Yu et al., 2013) is mediated by tropomyosin kinase B receptors (TrkB), which are not sensitive to MEL (Tosini et al., 2012b). TrkB activation by its classical agonist, BDNF, protects brain areas near-death cells (Tabakman et al., 2004).

Here we show that low and high ATP doses are decoded in a different manner by the pineal gland. Low ATP doses increased, while high ATP dose decreased MEL output. As both indolamines impair leukocyte migration from blood to tissue, this pineal function is preserved. However, as nocturnal MEL output determines only the level of MEL in the cortex, but not in the cerebellum (Pinato et al., 2015), we decided to explore changes in MEL concentration in these structures after low and high ATP doses administration.

Cortex and Cerebellum: ATP modulation of MEL/NAS content

Loops of inducing/repressing gene expression and protein synthesis are the basis for reaching a successful defense response (Cherry et al., 2014; Franco and Markus, 2014). Our data indicate that the cortex, which relies on MEL as a neuroprotector (Pinato et al., 2015),

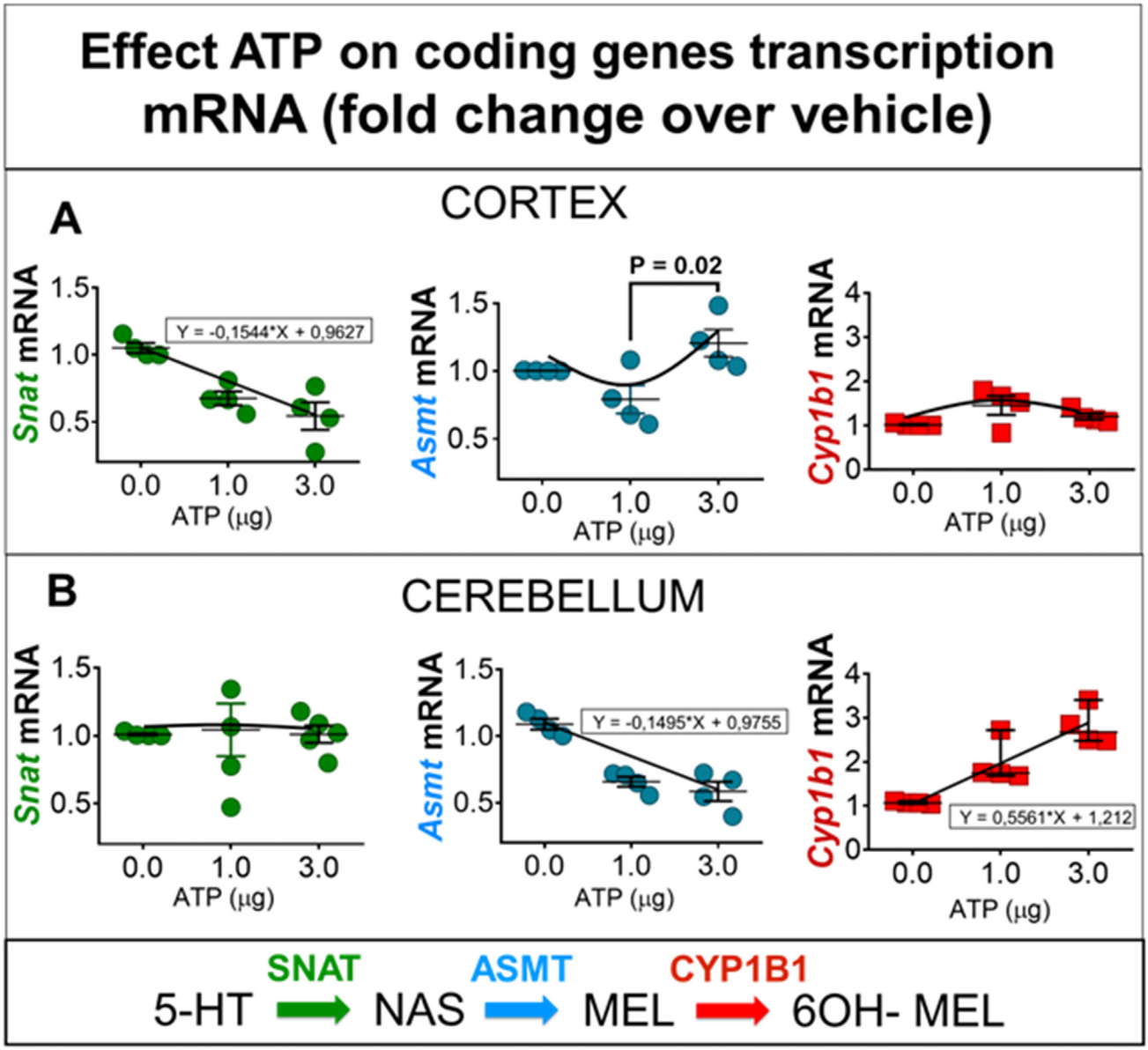


Fig. 4. Effect of purinergic stimulation on melatonergic enzymes coding genes in the cortex and cerebellum. The agonists ATP (1.0 and 3.0 μg) induce a reduction in *Snat* expression and an increase in *Asmt* in the cortex (**A**), while in the cerebellum (**B**) occurs a simultaneous reduction in the expression of *Asmt* mRNA and an increase in *Cyp1b1* mRNA. ATP was injected in the right lateral ventricle just before lights off (ZT11.5). The animals were killed at ZT18. The relative expression of the enzymes *Snat*, *Asmt* and *Cyp1b1* mRNA was normalized by *Gapdh*. Linear regression equations are shown in the graph. $Y = bX + a$, where X is the explanatory variable, Y is the dependent variable, b is the line slope, and a is the intercept (the value of y when $x = 0$). Data are expressed as mean ± SEM from four animals.

converts circulating NAS into MEL. Both low and high ATP doses administration similarly increased MEL content, whilst BzATP had no effect. Even more, both concentrations reduced *Snat* expression, whilst high ATP dose also increased *Asmt* expression, strongly suggesting that the increased pineal NAS output leads to the synthesis of MEL (Fig. 5).

The cerebellum is a unique structure regarding its reactivity to MEL. In the presence of LPS (lipopolysaccharide), MEL reduces oxidative/ nitrergic stress in activated cerebellar cells by blocking the nuclear translocation of NFκB, which leads to an inhibition of LPS-induced transcription of the gene that codifies nitric oxide synthase II (inducible NOS), generating protection (Pinato et al., 2015). However, in basal conditions, the nitric oxide (NO) reduction potentiates the nuclear translocation of NFκB, and the induction of cell death, because NO positively regulates the expression of the inhibitory κB protein, fine-tuning the activation of this transcription factor (Peng et al., 1995). This control is important in brain regions with progenitor cells, and a blockage of NO production leads to sterile acute inflammatory responses, and neurodegeneration (Kaltschmidt and Kaltschmidt, 2010; Franco and Markus, 2014). Here we show a reduction of ASMT and an increase in CYP1B1 coding genes, which predicts a reduction in the

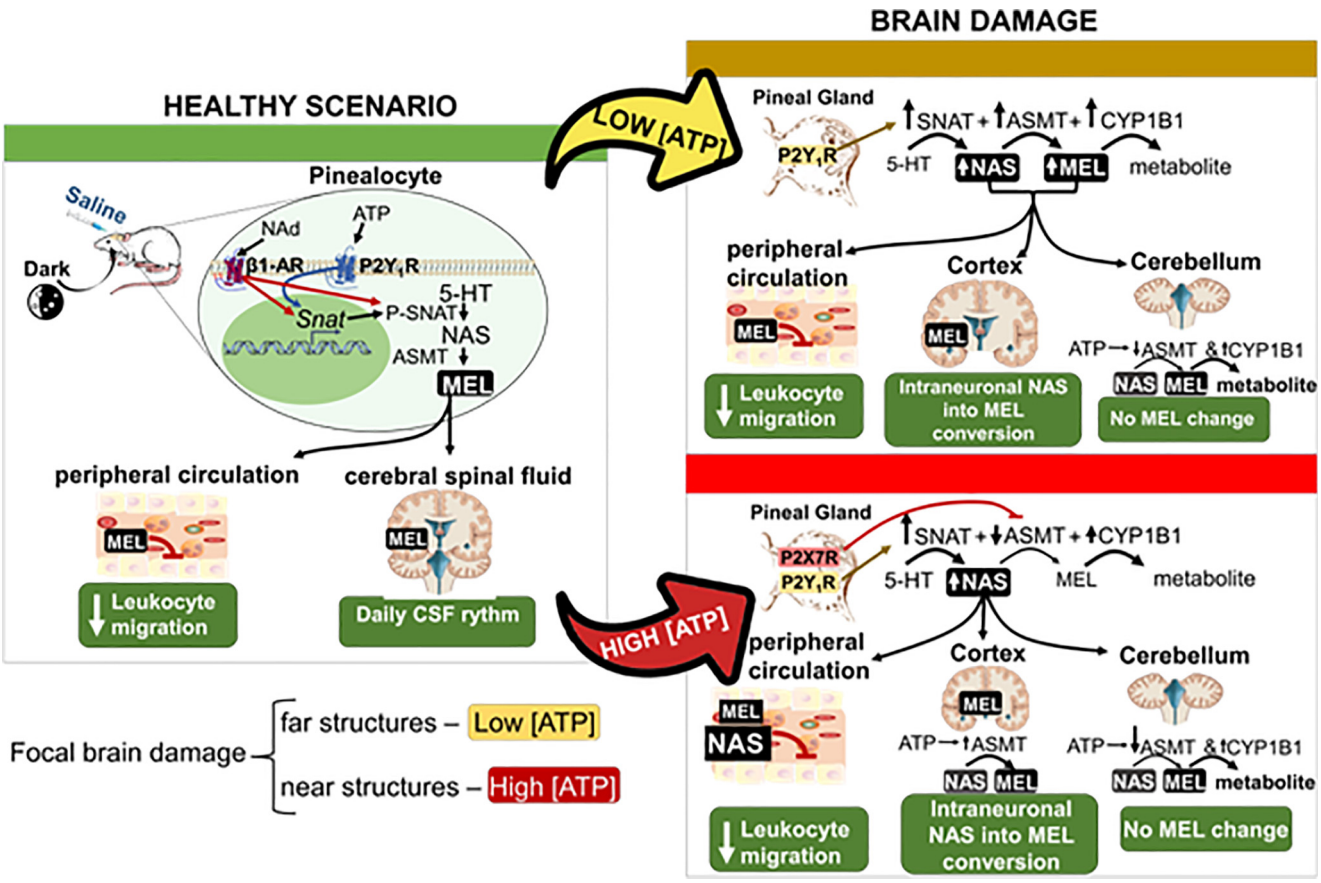


Fig. 5. Brain-Damage Alert increase in CSF-ATP turns NAS into the Darkness Hormone. In healthy conditions, darkness induces melatonin (MEL) synthesis due to noradrenaline (NAd) and ATP release from sympathetic nerve terminals, triggering β1-adrenoceptors (β1-AR) and P2Y1 receptors. The signaling pathway initiated by these receptors induces the transcription of Snat and the phosphorylation of the enzyme (P-SNAT), which converts serotonin (5-HT) into N-acetylserotonin (NAS). In sequence, enzyme acetylserotonin O- methyltransferase (ASMT) converts NAS into MEL. Low and high ATP concentration are decoded differently by the pineal gland. ATP injected at low (1.0 μg) and high (3.0 μg) doses mimic far and near lesions, as only 3.0 μg triggered P2X7R. Low ATP doses increased, while high ATP dose decreased MEL output. As both indoleamines impair leukocyte migration from blood to tissue (Lotufo et al., 2001), this pineal function is preserved. The stimulation of P2X7R, responsible for detecting high ATP concentration in the CSF, induces NAS, but not MEL, pineal gland synthesis. Local regulation of the enzymes involved in converting NAS into MEL and MEL metabolization leads to an increase in MEL in the cortex and no change in the cerebellum. Both low and high ATP doses similarly increased cortical MEL content. Even more, both concentrations reduced *Snat* expression, while high ATP dose also increased *Asmt* expression, strongly suggesting that the increased pineal NAS output leads to the synthesis of MEL. In the cerebellum, we showed a reduction/ increase in A*smt*/ C*yp1b1* expression, which predicts a reduction in the conversion of NAS into MEL and an increase in MEL because in the cerebellum high MEL promotes neuronal death. Thus, the specific regulation of indoleamines metabolism by high ATP concentration protects structures that react differently to MEL.

conversion of NAS into MEL and an increase in MEL degradation. Thus, the effect of increasing pineal NAS output in the brain is structure-dependent.

In conclusion, high ATP concentration intracerebral release turns the precursor of MEL, NAS, into the nocturnal pineal hormone (Fig. 5). The ATP high-affinity $P2Y_1R$ potentiates MEL output, while the ATP low-affinity P2X7R restrain the conversion of NAS into MEL. The idea is not that NAS will cover all the MEL effects spectrum but changing the NAS/MEL ratio will signal focal brain damage to the whole body. Inasmuch, each brain structure will control its level of MEL.

COMPETING INTERESTS

The authors declare no conflicts of interest.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2022.06.039.

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