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Pineal arylalkylamine *N-acetyltransferase (Aanat)* gene expression as a target of inflammatory mediators in the chicken

Aneta Piesiewicz [a], Urszula Kedzierska [a], Iwona Adamska [a], Michal Usarek [b], Michal Zeman [c], Krystyna Skwarlo-Sonta [a], Pawel Marek Majewski [a,*]

[a] Department of Animal Physiology, Faculty of Biology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland
[b] Department of Metabolic Regulation, Faculty of Biology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland
[c] Department of Animal Physiology and Ethology, Faculty of Natural Sciences, Comenius University in Bratislava, Mlynska Dolina, 842 15 Bratislava 4, Slovakia

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ABSTRACT

Previously, we demonstrated that experimental peritonitis in chickens was attenuated by treatment with exogenous melatonin, while the developing inflammation decreased pineal AANAT activity. This suggested the existence of a bidirectional relationship between the activated immune system and pineal gland function. The aim of the present study was to identify the step(s) in the chicken pineal melatonin biosynthetic pathway that are affected by inflammation. Peritonitis was evoked by i.p. injection of thioglycollate solution, either 2 h after the start, or 2 h before the end of the light period, and the animals were sacrificed 4 h later. The effect of inflammation on the expression of genes encoding enzymes participating in melatonin biosynthesis in the pineal gland, i.e. *tryptophan hydroxylase 1* (*Tph1*), *dopa decarboxylase* (*Ddc*), *arylalkylamine N-acetyltransferase (Aanat)* and *acetylserotonin O-methyltransferase* (*Asmt*), was evaluated by qPCR. The pineal and serum melatonin concentration as well as the content of its precursors in the pineal gland were measured, along with the activity of the relevant biosynthetic enzymes. Developing peritonitis caused an increase in the pineal levels of the *Tph1* mRNA during the night and the *Asmt* mRNA during the day, while nocturnal *Aanat* transcription was reduced. Both the pineal and serum melatonin level and the pineal content of *N*-acetylserotonin (NAS) were decreased during the night in birds with peritonitis. The amount and activity of pineal AANAT were significantly reduced, while the activity of HIOMT was increased under these experimental conditions. These results indicate that the observed decrease in MEL biosynthesis in chickens with developing inflammation is a result of transcriptional downregulation of the *Aanat* gene, followed by reduced synthesis and activity of the encoded enzyme.

1. Introduction

Biosynthesis of melatonin (MEL) is a well characterized multistep sequence of reactions [50] starting with the hydroxylation of the essential amino acid tryptophan (TRP) to 5-hydroxytryptophan (5-HTP), catalyzed by tryptophan hydroxylase (TPH; E.C.1.14.16.4), encoded in chickens by the *tryptophan hydroxylase 1* (*Tph1*) gene. The next enzyme, aromatic amino acid decarboxylase (AADC; E.C.4.1.1.28), encoded in chickens by the *dopa decarboxylase* (*Ddc*) gene, converts 5-HTP to 5-hydroxytryptamine (serotonin, 5-HT). Then, 5-HT is transformed to *N*-acetylserotonin (NAS) by arylalkylamine-*N*-acetyltransferase (AANAT; E.C.2.3.1.87), encoded by the *arylalkylamine-N-acetyltransferase* (*Aanat*) gene. The final enzyme, hydroxyindole-*O*-methyltransferase (HIOMT; E.C.2.1.1.4), encoded in chickens by the *acetylserotonin O-methyltransferase* (*Asmt*) gene, converts NAS to MEL. The biosynthesis of MEL is controlled by local factors, such as the availability of MEL substrates, as well as by central regulatory agents including the release of neurotransmitters from the autonomic nervous system and plasticity of the SCN-pineal circuit [7]. The activity of AANAT, the rhythm-generating enzyme in the MEL biosynthetic pathway, is regulated by 14-3-3 proteins [1], especially by their 14-3-3 ζ and ε isoforms. The 14-3-3 subunits comprise a family of proteins that are able to bind other proteins, forming complexes which play crucial roles in signal transduction and cell cycle control. The 14-3-3 proteins are also

Abbreviations: TRP, tryptophan; 5-HTP, 5-hydroxytryptophan; 5-HT, 5-hydroxytryptamine (serotonin); NAS, *N*-acetylserotonin; MEL, melatonin; TPH, tryptophan hydroxylase; AADC, aromatic amino acid decarboxylase; AANAT, arylalkylamine-*N*-acetyltransferase; HIOMT, hydroxyindole-*O*-methyltransferase; *Tph1*, *tryptophan hydroxylase 1* gene; *Ddc*, *dopa decarboxylase* gene; *Aanat*, *arylalkylamine N-acetyltransferase* gene; *Asmt*, *acetylserotonin O-methyltransferase* gene; *Tbp*, *TATA-binding protein* gene; ZT, zeitgeber time.

* Corresponding author. Fax: +48 22 554 12 03.
E-mail addresses: apiesiewicz@biol.uw.edu.pl (A. Piesiewicz), u.kedzierska@biol.uw.edu.pl (U. Kedzierska), iadamska@biol.uw.edu.pl (I. Adamska), musarek@biol.uw.edu.pl (M. Usarek), mzeman@fns.uniba.sk (M. Zeman), kss25@biol.uw.edu.pl (K. Skwarlo-Sonta), pmajew@biol.uw.edu.pl (P.M. Majewski).

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allosteric cofactors that modulate the catalytic activity of their binding partners [1,42]. The molecular details of the rhythmic regulation of MEL biosynthesis vary between species [10,61]. The pineal gland is innervated mainly by postganglionic sympathetic fibers releasing noradrenaline (NA), which activates β1- and α1-adrenergic receptors during darkness in mammals, whereas the α2-adrenergic receptors present in bird pinealocytes are stimulated during the light phase [53]. Furthermore, in contrast to mammals, avian pinealocytes are directly photosensitive. Studies conducted on avian pineal glands cultured *in vitro* have confirmed the nocturnal MEL biosynthesis generated by its internal oscillator, which works similarly to the mammalian and avian SCN, according to the periodic changes in clock gene expression [6,45]. This means that the avian pineal gland or pinealocytes may be cultured without NA supplementation, which is indispensable for the stimulation of MEL biosynthesis in mammalian cultures. Therefore, the avian pineal gland may represent a very useful model to examine the regulation of MEL biosynthesis during immune system activation in vertebrates.

MEL is known to regulate many physiological processes [59], such as seasonal reproduction [44], locomotor activity [56], body temperature [8] and immunity [24,51]. The immunomodulatory activity of MEL produced in the vertebrate pineal gland has been confirmed by several experimental approaches [13,32,52], while the effect on pineal MEL biosynthesis exerted by the activated immune system is less well documented. In an early study in this area, we demonstrated that the immunization of chickens with sheep erythrocytes (SRBC) modified the activity of pineal AANAT in a season- and sex-related way [37]. Similarly, the treatment of rats with the inflammatory cytokine interleukin 1β (IL-1β) [40] or LPS [28,54] caused a decrease in MEL concentration in the serum. A decreased level of MEL has also been observed in humans, e.g. women with mastitis [47], in the colostrum of women after cesarean section [48], in patients with ischemic stroke [2], and after various surgical interventions [14,25,30,41]. On the other hand, an increased level of MEL was observed in patients with end-stage renal disease [33]. Furthermore, MEL secretion from the human pineal gland gradually diminishes with age, and this decrease seems to correlate with the increased incidence of various age-related inflammatory diseases [57]. These data support the notion of cross-talk between the pineal gland and the immune system [53], and of a functional immune–pineal axis [39]. Accordingly, it has been postulated that during the early phase of inflammation, proinflammatory cytokines inhibit MEL biosynthesis, allowing the mounting of an inflammatory response, and then MEL production is increased to shut down this response [48].

Recently published data concerning the NF-κB signaling pathway, derived from experiments on mammalian pineal glands conducted mainly *in vitro*, suggest its involvement in the modulation of pineal activity [38]. The existence of membrane receptors for TNF and the inhibitory effect of this cytokine on MEL biosynthesis [9,15], *via* activation of the NF-κB pathway and transcriptional regulation of the *Aanat* gene, indicate that this molecule may be the primary messenger responsible for immune–pineal interaction observed during the early phase of inflammation in mammals and perhaps also in birds. However, it is still unclear whether this cytokine or its homolog [26] exists in birds, although the TNF receptor has been identified in chicken [29]. Therefore, it is difficult to corroborate the findings of the mammalian studies in an avian model.

Other important immunomodulatory properties of MEL are connected with its ability to cross biological barriers and its free radical scavenging capacity [55]. These properties were observed in our experiments on chickens with peritonitis, where pre-treatment with exogenous MEL attenuated the development of inflammation due to its anti-inflammatory and free radical scavenging activities [35]. Moreover, experimentally evoked inflammation caused a decrease in pineal biosynthetic activity, leading to the disappearance of the nocturnal peak of pineal AANAT activity [34]. These findings confirm the existence of bidirectional communication between the pineal gland and the activated immune system.

For many years, pineal AANAT has been thought of as the key enzyme and most important regulatory factor of MEL biosynthesis. However, recent data have indicated that the genes encoding enzymes involved in this process are expressed rhythmically and may be important in the regulation of MEL biosynthesis [3,7,45]. To date, there have been no comprehensive studies focused on the enzymes and intermediate compounds involved in MEL biosynthesis in relation to activation of the immune system. Therefore, we decided to identify the particular steps in the MEL biosynthetic pathway that are affected by developing peripheral inflammation in chickens at two time points, during the day (ZT6) and night (ZT14), when the level of MEL biosynthesis is almost in antiphase [45]. To achieve this goal, we evaluated the transcription of genes encoding each of the enzymes participating in pineal MEL biosynthesis, determined the *ex vivo* activity of these enzymes and quantified the endogenous level of their substrates. To investigate the mechanisms regulating pineal function during peritonitis we also measured the level of pineal 14-3-3 proteins.

2. Materials and methods

2.1. Animals

Experiments were performed on 16-day-old male Hy-Line chickens hatched and reared under controlled conditions from the day of hatch. The chicks were transported from a commercial hatchery to the animal facility of the Faculty of Biology, University of Warsaw on the day of hatch, and kept under controlled light (L:D 12:12, lights on at 4:00 am) and temperature (32 ± 2 °C during first week and 24 ± 2 °C thereafter) conditions, with free access to the standard food and water.

All procedures were performed in accordance with the regulations of the Polish Ethical Council for the care and use of laboratory animals, and the European Community Directive for the ethical use of experimental animals.

2.2. Peritonitis induction, pineal gland and blood collection

Peritonitis was evoked in 16-day-old birds as described previously [52,35,36] by i.p. injection of thioglycollate (TG) solution 2 h after the start of the light phase (ZT2) or 2 h before the beginning of darkness (ZT10). Control (INT) and TG-treated animals were sacrificed 4 h after the induction of inflammation, i.e. at ZT6 or ZT14, respectively. The trunk blood and the pineal glands were isolated under dim red light. The pineal glands were immediately frozen in liquid nitrogen and stored at −80 °C prior to analysis. The blood samples were used to prepare serum, which was stored at −20 °C prior to analysis. The development of peritonitis was verified by evaluating the number of leukocytes in the peritoneum (PTL) [34,36], and only the pineal glands and blood from chickens with an elevated PTL number, not present in intact control birds, were used in subsequent analyses.

2.3. Isolation of pineal gland RNA and RT-qPCR

Total RNA was isolated from pineal glands using Ron's Tissue RNA Mini Kit (Bioron), the concentration of RNA was assessed spectrophotometrically (NanoDrop) and its quality was tested by gel electrophoresis. Additionally DNase treatment was also performed (RQ1-Rnase-Free Dnase, Promega). Reverse transcription (RT) reaction mixtures contained 650 ng total RNA, 200 U M-MuLV