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The Immune-Pineal Axis: the Role of Pineal and Extra-Pineal Melatonin in Modulating Inflammation

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Abstract. Participation of the pineal gland and melatonin in the innate immune response is part of a dynamic and intricate network that acts throughout the inflammatory response, integrating signaling pathways and regulatory processes at the molecular, cellular and organism levels. The pineal gland is a target for pathogen-associated molecular patterns and for inflammatory mediators, and melatonin, which may be produced by the pineal gland or by activated immune-competent cells, plays a role in modulating inflammatory responses. Nocturnal melatonin surge is suppressed at the beginning of an inflammatory response in order to allow a full mounting of an innate immune response at any hour of the day. Melatonin produced at the site of a lesion by activated immune-competent cells favors phagocytosis and reduces inflammatory reactions that could promote tissue lesion. Here, we discuss the mechanism underneath this crosstalk between the pineal gland and the innate immune response and extend the concept of an Immune-Pineal Axis.

Keywords: Melatonin, pineal gland, immune-pineal axis, cytokines, LPS, corticosteroid, nuclear factor kappa B

INTRODUCTION

Organisms are continuously in contact with aggressors, which need to be sensed and destroyed. The innate immune system recruits leukocytes to infected sites in order to identify and remove bacteria, cell debris and macromolecules. The inflammatory response also comprises a recovery phase responsible for the healing of the tissue. The four cardinal signals of the inflammatory response described by Celsus (30 BCE – 38 AD) include redness, heat, swelling and pain. Later, Galen (Claudius Galenus, AD 129 – 199/217) proposed that if there is not a proper resolution of the acute inflammation, a fifth signal appears: the lost of function.

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The initial steps of the inflammatory response are the release of mediators by resident cells and the migration and activation of neutrophils, which release factors that summon other leukocytes and generate reactive oxygen and nitrogen species. These reactive species kill bacteria and any other nearby cells. The efficiency of the inflammatory response relies on a proper migration of cells to the site of the lesion. Spurious migration and activation of cells leads to a disruption of the healthy tissue.

Many authors have proposed an anti-inflammatory role for exogenous melatonin [for review see 1, 2]; however, these effects were obtained with very high concentrations, some 100 to 1000 times higher than those found in nocturnal plasma. Therefore, they were not attributed to a nocturnal melatonin surge.

Studies evaluating the adaption of the immune response elegantly suggested that melatonin secreted by the pineal gland plays a role in regulating the innate and acquired immune responses [3–5]. The redirection of energy consumption from reproduction to survival in

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the winter was due to the increase in the duration of the nocturnal melatonin peak [6]. In summary, two lines of research attributed a role in the immune response to melatonin: one focusing on the so-called pharmacological effect, and the other on the increase in the duration of nocturnal pineal synthesis.

In this review, we will describe experimental data, which suggest that the hormone of darkness, melatonin, impairs neutrophil migration in healthy subjects, providing conditions for maintaining circulating leukocytes in the blood stream. In addition, pineal melatonin synthesis is suppressed by pathogen-associated molecular patterns (PAMPs) and pro-inflammatory cytokines, creating the conditions for a proper mounting of an inflammatory response, independently of the hour of the day. As the inflammatory response progresses to the healing phase, melatonin produced by activated immune-competent cells plays the role of an antioxidant and anti-inflammatory molecule. Melatonin synthesized at the site of the lesion has no relationship to photoperiodic information, and it is worthwhile to remember that the primitive biological role played by this indolamine was that of an antioxidant molecule [1]. In order to close the cycle that defines an immune-pineal axis, inflammatory mediators contribute to restoring pineal gland activity. We summarize this cycle in Fig. 1, which shows our working hypothesis during the last ten years. In this review, we will revisit the experiments that allow for the proof of this working hypothesis.

VASCULAR PERMEABILITY – A PROCESS REGULATED BY THE PINEAL GLAND HORMONE

At the end of 1990, an undergraduate student, looking to model biological data, measured the difference in the size of the paws of mice injected or not with BCG (bacillus Calmette-Guerin) for 90 days at the light and the dark phase of the day. The modeling of the chronic phase ("plateau") of the inflammatory response was adjusted to a senoidal curve with a period of 24 h [7]. The paw was thicker during the day and thinner at night (Fig. 2).

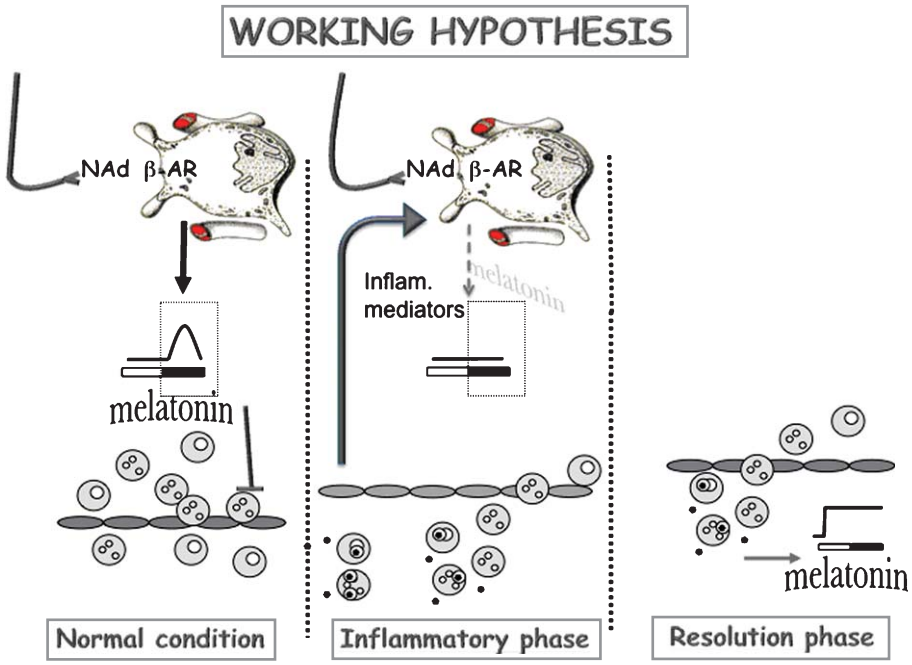

Fig. 1. The Immune-Pineal Axis working hypothesis – In *normal conditions* (left panel) the activation of β-adrenoceptors by noradrenaline released from sympathetic neurons induces the synthesis of melatonin, which is promptly released in the blood and liquor. Melatonin impairs the migration of leukocytes from the blood stream, avoiding unwanted triggering of inflammatory responses. At the *inflammatory phase* (central panel) the early mediators of inflammation, such as the cytokine TNF, blocks pineal melatonin synthesis induced by noradrenaline, impairing the nocturnal melatonin surge and favoring the migration of cells to the site of lesion. When the inflammatory response enters the *recovery phase* (right panel) the activated leukocytes that migrate to the site of lesion produces melatonin, which attains very high concentrations and together with other anti-inflammatory mediators contributes for the ending of the inflammatory response.

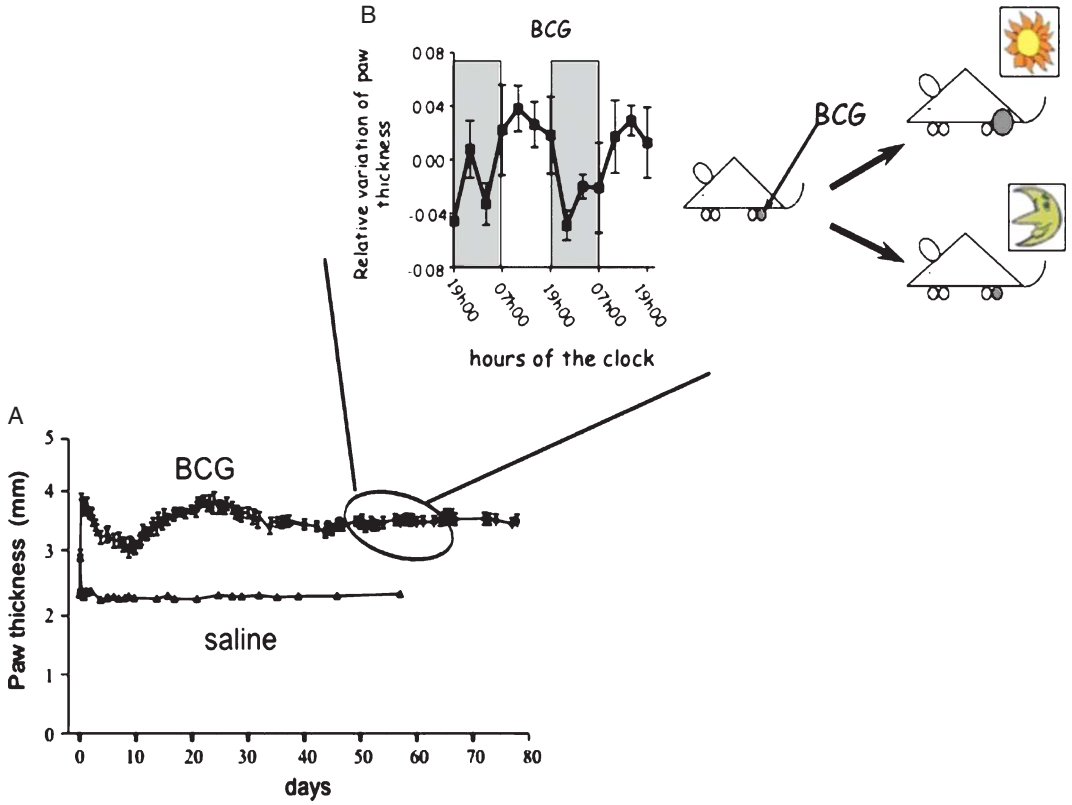

Fig. 2. Daily rhythm of inflamed mice paw. A - Daily size (mm) of the paw of mice injected with BCG ($n = 19$) or saline ($n = 10$) at day zero. The measures obtained at daytime and nightime for 80 days shows the time-course of a chronic inflammatory response. An immediate increase in paw-size at day zero and a second slow increase at day 30, followed by an stable plateau. The mathematical modeling of the plateau disclosed a senoidal rhythm with a period of 24 h. B – The 24 h variation in paw size was confirmed by measurements obtained at regular 4 h intervals 30 days after BCG inoculation. The acrophase was between 09h00 and 13h00 and the nadir between 21h00 and 01h00. As shown in the graphics, lights were turned on and off at 07h00 and 19h00, respectively. Data are expressed as mean ± standard deviation of the relative variation of mean paw thickness along the record period for each mouse. Reprinted from [7] with permission of "John Wiley and Sons".

Dark-mediated activation of glutamatergic pathways from the suprachiasmatic (SCN) to the paraventricular nucleus (PVN) of the hypothalamus activates the sympathetic input of the pineal gland, which is innervated by the conarii nerves that originate in the superior cervical ganglia (for a review, see [8]). The sympathetic terminals release noradrenaline and ATP, which act on α1 and β1 adrenoceptors and P2Y1 purine receptors [9, 10]. The activation of β-adrenoceptors is essential for the translation of the gene and/or activation of the key enzyme in melatonin synthesis (alkyl-arylamine-N-acetyltransferase, AA-NAT), which converts serotonin to N-acetylserotonin (NAS), the precursor of melatonin. The enzyme hidroxy-indole-O-methyltransferase (HIOMT) converts NAS to melatonin.

The suppression of the nocturnal melatonin surge by removing the pineal gland or the superior cervical ganglia for more than 30 days abolished the daily variation in paw size [7]. The nocturnal administration of melatonin restored the rhythm of the lesion. The same variation profile was observed when we measured the changes in vascular permeability; the mechanism underlying changes in paw size (Fig. 3).

Serum levels of glucocorticoids are consistently and markedly increased during chronic inflammation [11], and adrenalectomy increases vascular permeability in inflamed areas [12]. Therefore, both hormones, melatonin and corticosterone, could be responsible for the daily variation in vascular permeability. As a matter of fact, pinealealectomy and adrenalectomy abolished the nocturnal reduction in vascular permeability induced by BCG; however, melatonin was able to restore the rhythm in both experimental conditions (Fig. 3). In addition, the nocturnal rise in the excretion of the main melatonin metabolite

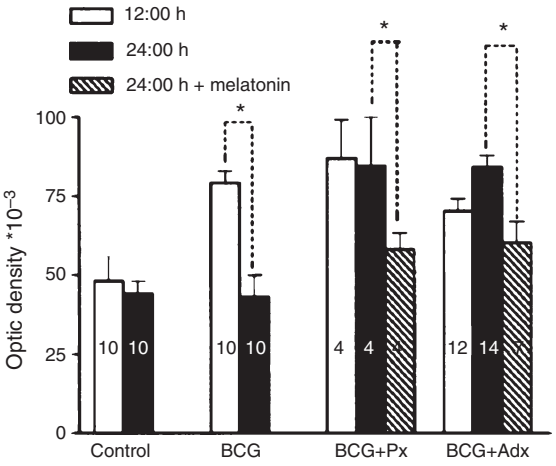

Fig. 3. The variation of the vascular permeability in mice paw at daytime and nighttime 45 days after inoculation of BCG. The vascular permeability was evaluated at midday and midnight by the method of Evans-blue-albumin outflow. No difference between day and nighttime was observed in the paws injected with saline. The higher vascular permeability observed at daytime in BCG inoculated mice was abolished by pinealectomy (Px) or adrenalectomy (Adx). Nocturnal administration of melatonin reversed the effect of both pinealectomy and adrenalectomy. Numbers inside the bars show the number of mice in each experiment. Dotted lines indicate the two compared groups. * - groups are significantly different at $p < 0.05$. Results are expressed as mean ± standard deviation. Reprinted from [39] with permission of "John Wiley and Sons".

in the urine, 6-sulfatoximelatonin, was abolished in adrenalectomized chronic inflamed mice. This surprising result strongly suggested that the pineal gland is a target for corticosterone.

ENDOTHELIAL LAYER REACTION TO ENDOCRINE AND PARACRINE MELATONIN

Cell migration from blood to tissue implies the crossing of the endothelial barrier. Endothelial cells form a unicellular layer that is in direct contact with the blood stream. These cells play a role in controlling either the vascular tone or the migration of cells from blood to tissue.

Melatonin concentration at the pM-nM range, which is found in nocturnal plasma, reduces the rolling and adhesion of leukocytes to endothelial cells [13]. The pharmacological profile of melatonin analogs suggests that the control of rolling and adhesion is mediated by MT2 and MT3 melatonin receptors, respectively. Both responses are blocked by the competitive antagonist luzindole. The partial agonist of the MT2 melatonin receptor, 4-PPDOT, mimics the inhibition of rolling, while the agonist for the MT3 receptor, MCA-NAT, inhibits leukocyte adhesion to endothelial cells.

The adhesion of neutrophils to endothelial cells is mediated by the interaction of neutrophils with β2 integrins (CD18), with adhesion molecules of the immunoglobulin superfamily expressed in endothelial cells [14]. Melatonin did not reduce the expression of CD18 induced by leukotriene B4 (LTB4) or formyl-methionyl-leucyl-phenylalanine (fMLP) [15], or the neutrophil-endothelial interaction due to fMLP [13] (Lotufo et al., 2001). Melatonin reduces the interaction induced by LTB4. Since the effect of fMLP is mediated by neutrophil activation, while LTB4 activates both endothelial cells and neutrophils, it was concluded that melatonin at low concentrations acts on endothelial cells.

Melatonin regulates endothelial cell line proliferation and protects both endothelial cells lineages and primary cultures against oxidative stress, DNA damage and death [16, 17]. Studying the effect of melatonin in primary cultures in rat endothelial cells, we observed that melatonin also inhibits the neutrophil-leukocyte interaction induced by lipopolysaccharide (LPS), a PAMP found in the membrane of gram-negative bacteria [18]. In addition, the nitric oxide (NO)-mediated relaxation of the rat aorta, induced by LPS, was also inhibited by melatonin. In this context, the melatonin effect was due to the inhibition of the nuclear factor kappa B (NFKB) pathway and was not blocked by luzindole. The concentration of melatonin required for the inhibition of the LPS effect is in the nM-mM range and was initially thought to be a pharmacological effect. However, it is now well known that activated neutrophils and macrophages produce melatonin, at the site of the lesion [19, 20].

An interesting output of this line of research was that melatonin primes endothelial cells for reducing the expression of the inducible nitric oxide synthase (iNOS) and adhesion molecules, such as PECAM-1 and ICAM-1 [21]. In other words, the expression of these molecules in primary cultured endothelial cells was inversely correlated with the level of melatonin in the blood of the donor animals [21]. In addition, a significant increase in the adhesiveness of endothelial cells obtained from donors with lower melatonin plasma concentration is observed. Considering the increasing interest in applying cell therapy, obtaining grafts with endothelial cells in the quiescent state will certainly favor more homogenous responses.

In summary, both endocrine and paracrine melatonin reduces the reactivity of endothelial cells, impairing their adhesiveness to leukocytes. In the first

case, the effect is mediated by the activation of melatonin membrane receptors, while in the second case, melatonin inhibits the PAMPs-activated NFKB pathway. The endocrine origin of melatonin is the pineal gland, while in inflamed tissues, activated immune-competent cells are the paracrine source of melatonin.

THE PINEAL GLAND AS A TARGET OF INFLAMMATORY MEDIATORS

The pineal gland has evolved from the roof of the forebrain (for a review, see 22), in the main part of the epithalamus, and is formed by three types of cells: pinealocytes, glia cells and neurons. In non-mammal vertebrates, the pinealocytes are photoreceptors, and react directly to light, while in mammals, they are not directly sensitive to light. The pinealocytes are the cells responsible for synthesizing the main pineal hormone, melatonin, also known as the hormone of darkness.

Astrocytes and microglia present in the pineal gland have a specific distribution. Astrocytes are located at the proximal end of the gland and along the entire stalk [23, 24]. On the other hand, microglia are part of the parenchyma [25]. Both astrocytes and microglia react to PAMPs derived from gram-positive (LPS) and gram-negative (LTA – lipotheic acid) bacteria [26]. Microglia was suggested to mediate neuroendocrine-immune interactions of the pineal gland [27]. Both microglia and astrocytes expresses IL-1β, however, there is an intriguing difference between in vivo and ex vivo cellular expression of this cytokine. In vivo the expression is higher in astrocytes, while in vitro (organ or cell culture) the microglia expression increases. In addition, the metabolism of serotonin in cultured pineal glands is directly affected by intereferon-gamma (IFN-γ), IL-1β, TNF and transforming growth factor-β1 [29]. All these cytokines interfere on the metabolism of serotonin [29], however, its translation to melatonin output requires a direct evidence. Cytokines signalize through and modulate the level of activation of several transcription factors, therefore, their final effect on melatonin output cannot be extrapolated from the understanding of serotonin profile. An early paper that deals with the effect of INF-γ on melatonin synthesis showed a very complex effect [30]. IFN-γ potentiates cultured rat pineal gland production of melatonin induced by 10 nM or 1 mM isoproterenol, while blocks the effect of 100 nM of the β-adrenergic agonist [30]. Therefore, there are many evidences showing that not only melatonin interact with immune competent cells, but also the pineal gland is able to react to molecules that signalize innate and acquired immune reactions.

The concept of a bidirectional communication between pineal gland and the endocrine system was initially proposed in birds [31]. More recently, we extended the idea and disclosed the mechanisms underlying the effect of PAMPs and cytokines on the mammal pineal gland [32].

The main transduction pathway that signals PAMPs is the NFKB transduction pathway, which occurs in phylogenetically distinct species from insects to mammals [33–35]. This transduction pathway is triggered by membrane receptors of the toll-like receptor (TLR) family that are able to distinguish between different kinds of PAMPs and by activated receptors for pro-inflammatory cytokines. This pathway is turned off by corticosterone, a well-known anti-inflammatory mediator [36]. Despite its well-characterized function in the immune system, NFKB also plays a role in the normal central nervous system [37], including the suprachiasmatic nuclei [38], and in pineal physiology [39].

The NFKB family is highly conserved across species. In mammals, it is composed of five proteins characterized by the REL homology domain (RHD), which contains the crucial functional regions for DNA binding, dimerization, nuclear localization, and interaction with the inhibitory kappa B proteins. The RelA (also known as p65), RelB and c-REL isoforms, but not the p50 and p52 isoforms, have a transactivating domain (TAD) in the C-terminal portion, which is responsible for inducing gene transcription. Upon stimulation, the inhibitory kappa B protein is phosphorylated, ubiquitinated, and degraded by proteasome, releasing the NFKB dimer. The presentation of the nuclear signal localization favors the translocation of the dimer, which binds to specific DNA elements (kappa B motifs) in the promoter region of target genes. Dimers that lack TAD, such as p50/p50, are predicted to block gene transcription (see the reviews [40, 41]).

The pineal gland rhythmically expresses p50/p50 homodimers during the day [39]. A continuous increase in nuclear NFKB from dawn to dusk is abruptly reduced just after lights are turned off (Fig. 4). Melatonin blocks the nuclear translocation of NFKB in cultured pineal glands and pinealocytes. Although we do not know the role of this pathway in pineal physiology, this is a pivotal pathway for the immune-pineal axis.

Pro-inflammatory mediators

The mammalian pineal gland acts as a sensor for injury, as TNF blocks noradrenaline-induced melatonin synthesis in cultured rat pineal glands [42]. Activation of the NFKB pathway transiently blocks *Aa-nat* translation and the melatonin precursor N-acetylserotonin synthesis. The transiency of the inhibition of *Aa-nat* translation was blocked by cycloheximide, strongly suggesting that TNF signalizes the synthesis of some protein(s) that reverse the blockage of melatonin synthesis [42].

The pineal gland also reacts to bacterial PAMPs, such as LPS and LTA [26], and we recently showed that it expresses membrane receptors for LPS, such as TLR4 and CD14, which are known to signal thorough the NFKB pathway [43, 44]. These receptors are expressed in the three types of cells, astrocytes, microglia and pinealocytes (data not published). The systemic injection of LPS induces the expression of the interleukine-1β (IL-1β) gene [45] and the incubation of rat pineal glands with LPS leads to the expression of the TNF and TNF receptor subtype I [43]. This effect is mediated by the nuclear translocation of p50/p50 and p50/RelA. The activation of the NFKB pathway by LPS also blocks melatonin synthesis and the later synthesis of TNF and TNFR1, which may amplify the signal [43]. A similar process could occur in humans, as a negative correlation between the increase in TNF after an acute inflammation and the level of nocturnal melatonin surge was observed [47, 48].

In summary, the pineal gland is instrumented to answer to PAMPs and pro-inflammatory cytokines through a classical pathway for signaling injury. The most evident output is the suppression of the nocturnal melatonin surge, which allows a full transmigration of immune-competent cells, independent of the hour of the day. Inasmuch, this reduction in the nocturnal melatonin surge probably plays a role in the development of sickness behavior, which in the case of infections implies in reduction of ingestive, social and sexual behaviors, the induction of lethargy and thermoregulatory changes and an alteration in the sleep/wake cycle (for a review see [49]). As a matter of fact, it is well recognized that the behavioral tolerance to endotoxin is enhanced by adaptation to winter photoperiods [3, 49].

Anti-inflammatory mediators

The inflammatory response comprises a process of activation followed by a process of deactivation in order to promote a well-tuned response that rescues the organism from aggression and restores the healthy state. In the recovery phase, anti-inflammatory mediators should play a role in restoring the nocturnal melatonin surge. Here we will describe the effect of corticosterone, which inhibits the NFKB pathway.

Corticosterone has a dual effect on noradrenaline-induced melatonin synthesis in cultured rat pineal glands [50]. Low concentration potentiates noradrenaline-induced transcription and activity of the enzyme AA-NAT and the synthesis of melatonin, while high concentration has no effect on the noradrenaline-induced NAS and melatonin synthesis [42, 50, 51]. The corticosterone effect is mediated by the activation of glucocorticoid receptors and the inhibition of the NFKB pathway [50]. The effect of low doses of noradrenaline observed in cultured pineal glands can be translated to nocturnal sympathetic stimulations, as administration of corticosterone by trans-pineal infusion has no effect on daytime melatonin output, but increases the nocturnal melatonin peak [51]. Therefore, corticosterone potentiates noradrenaline-induced melatonin synthesis either in vitro or in vivo.

Taking into account that chronic inflamed animals have a higher level of circulating corticosterone than normal animals [52], these recent results probably explain why in our initial studies, the nocturnal melatonin surge is suppressed after the adrenalectomy of mice [53]. In other words, a balance between pro-inflammatory mediators and corticosterone on NFKB nuclear translocation should be important for determining the nocturnal melatonin surge in chronic inflamed animals.

SYNTHESIS OF MELATONIN BY IMMUNE-COMPETENT CELLS

The shuttle between pineal and extra-pineal production of melatonin implies that activated immune-competent cells are able to produce melatonin that is released in a paracrine manner. In 2004, two independent groups working with rat macrophages [20] and human lymphocytes [19] showed that these cells produce high concentrations of melatonin. More recently, this information was extended to other defense cells, such as human colostrum macrophages [47, 48].

The human colostrum confers protection against gastrointestinal and respiratory infection in infants [54]. The main cell lineages in this secretion (>80%) consist of macrophages and neutrophils specialized in phagocytosis [55]. They are able to kill

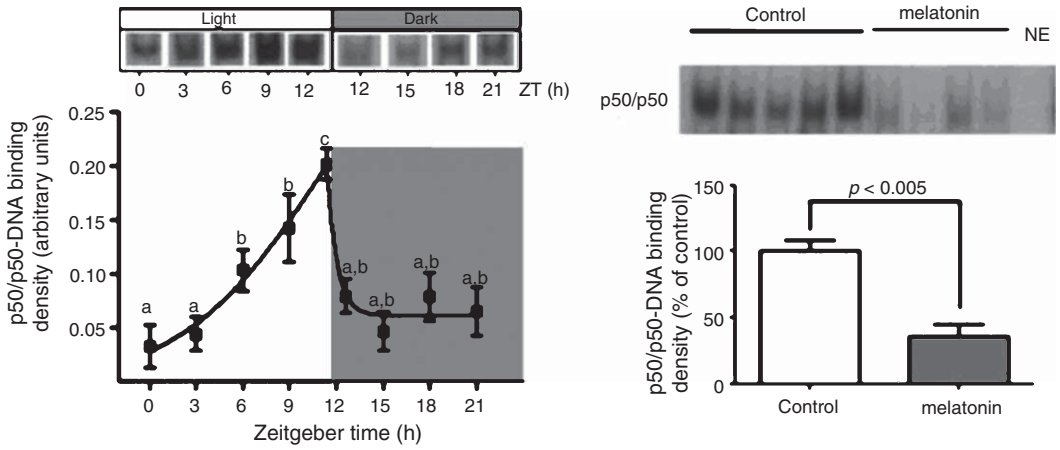

Fig. 4. NFKB transcription factor activity in pineal glands from healthy rats and non-stimulated cultures. LEFT PANEL - Daily variation of the content of NFKB in the nuclear fraction of pineal glands obtained from rats maintained under a 12 h:12 h LD cycle, killed at 3 h intervals. The gel in the superior panel is a typical eletromobility gel-shift assay (EMSA) showing the content of *p*50/p50 at each zeitgeber time. The lower panel shows the mean values and the standard error of the mean for 4–12 glands per timepoint. Different letters represent significant difference between the groups ($a \neq b \neq c$; $p < 0.05$ one-way ANOVA). RIGHT PANEL - Nuclear NFKB content in cultured rat pineal glands incubated or not with melatonin (1 mM, 3 h). EMSA shows the NFKB translocated to the nuclear extract cultured pineal glands. The densitometry confirms that melatonin inhibits NFKB nuclear translocation in pineal glands. Data are expressed as mean values and the standard errors of the mean for 4–5 glands per group. Differences among groups were tested by student t test. Abbreviation: NE = no extract. Reprinted from [53] with permission of " Informa UK Ltd."

enteropathogenic Escherichia coli (EPEC) [56] without activating oxidative pathways, as observed with the great majority of phagocytes [57]. It is interesting to note that EPECs and zymosan induce the synthesis of high concentrations of melatonin by human macrophages [47] and enhance the ability of these cells to phagocyte EPEC and zymosan [58].

In the majority of immune-competent cells, either melatonin or one of its metabolites plays the role of an anti-inflammatory and anti-oxidant substance. This is one of the most studied effects of melatonin, and many recent excellent reviews are available [2, 59–61]. These effects have the function of finishing the defense response, avoiding the generation of chronic inflammatory responses and unnecessary tissue damage.

In summary, melatonin secreted by immune-competent cells acts as a paracrine, or even autocrine hormone, regulating the function of these cells and the defense response.

THE IMMUNE-PINEAL AXIS HYPOTHESIS

The pineal gland, classically considered the photoneuroendocrine transducer organ of mammals, is now being discovered as target for molecules that signal immune responses. In pathophsysiological conditions, mediators of inflammation modulate the synthesis of melatonin, in such a manner that pro-inflammatory mediators inhibit while anti-inflammatory mediators potentiate melatonin production.

In addition, during the course of an immune response, activated leukocytes produce melatonin, which reaches a concentration of a 100 to 1000 times higher than that found in the circulation, contributing to the improvement of bacterial phagocytosis and the recovery of the lesioned area.

Restoration of nocturnal melatonin surge is obtained not only by the reduction of pro-inflammatory signaling in the pineal gland, but also by the action of anti-inflammatory mediators. It is important to mention that the classical pathway for signaling danger (the NFKB pathway) is involved in the suppression and restoration of the nocturnal melatonin surge.

Therefore, during the mounting of an inflammatory response, the organism is disconnected from environmental cues, and the darkness hormone, melatonin, gains another function. This is a primitive function linked to organism self defense, and not self-adaptation (Fig. 1).

This new integrative view of the relationship between the pineal gland and the immune response certainly will help to explain several findings about melatonin effects regarding to pain [62, 63], diseases

that involve inflammatory responses, such as stroke [64–66] or chronic inflammatory diseases [for review see 67, 68].

In conclusion, the pineal gland is not only a transducer of photoperiodic information, but is also a constitutive player in the innate immune response. In other words, melatonin is not only the darkness-hormone, but also one of the many anti-inflammatory molecules that are produced at the sites of lesions during the recovery phase of an inflammatory response. Therefore, there is a constant crosstalk between the periphery and the pineal gland [31], which we defined as the Immune-Pineal Axis [32].

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