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Functional connections between the pineal gland and immune system

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Abstract. Experimental findings which support a functional connection between the pineal gland and immune system in mammals and birds are summarized. Developmental and age-related changes in pineal gland function appear, at least partially, to be related with immune system efficiency. Mechanisms whereby melatonin influences immune system function are complex, but include participation of mediators (endogenous opioids, cytokines, hormones, zinc pool) as well as specific binding sites on the immune cells. Melatonin, as a highly lypophilic compound, may also penetrate immune cells without mediation of specific receptors and act within as a potent free radical scavenger and as an anti-aging and oncostatic factor. The immune system may, in turn, *via* synthesis and secretion of soluble factors, cytokines, influence pineal gland function, thereby closing the information loop to maintain homeostasis in order to face the harmful environment.

Review

Key words: pineal gland, melatonin, immune system, circadian rhythm, melatonin receptors, anti-aging

INTRODUCTION

The pineal gland is known as a regulator of seasonal changes in physiological functions of several species, particularly in seasonal breeders (Pévet 1987, Reiter 1989). It is a neuroendocrine gland responsible for the transformation of external signals, mainly photoperiodic information, into a hormonal output, interpreted thereafter by internal target structures. Photoperiodic information is utilized by the pineal gland in that it synthesizes and releases its principal hormone, melatonin, in a rhythmical fahsion, characterized by a low level during the day and an increase during the night. This circadian rhythm of melatonin production is observed in all vertebrate species examined to date, regardless of their diurnal or nocturnal pattern of locomotor activity, indicating that melatonin's role in an organism is to provide information on darkness and not about nocturnal rest. Another essential feature of the diurnal rhythm of melatonin synthesis is its dependence on the length of the night period, thus the pineal gland may act as both "a clock and a calendar" (Reiter 1993b). Interpretation of the melatonin message within the body is essential to adapt the physiological functions of an animal to environmental conditions and needs, and this adaptation would increase the probability of its survival. Immune system activity is one of the physiological capabilities most responsible for the survival of an individual, whereas the survival of the species is guaranteed by reproductory system function.

CIRCADIAN RHYTHM OF IMMUNE SYSTEM FUNCTION

The immune system has long been considered as functioning autonomously, but now good experimental evidence exists for bidirectional interactions with the nervous and endocrine systems (for reviews see Goetzl and Sreedharan 1992, Fabris 1993, Homo-Delarche and Dardenne 1993). The role of the immune system consisting of the defence against harmful microorganisms, foreign molecules or malignant transformed cells has to be coordinated over a 24 h period in order to achieve optimal efficiency. Therefore, it is not surprising that, as with other body functions, immune system activity undergoes circadian changes, and the reciprocal synchrony is of importance with regard to the maintenance of homeostasis. On the other hand, disease may alter such a rhythm and modify its temporal coordination (Lévi et al. 1992). Adrenal corticosteroids were the first hormonal factors considered to be regulators of the diurnal rhythm of the immune system.

There is substantial evidence that particular subtypes of immune cells, as well as other immune parameters, fluctuate differentially over a 24 h period and exhibit different phase relationships with circulating corticosteroid levels (e.g. Eskola et al. 1976, Fernandes et al. 1976, Abo et al. 1981, Skwarło-Sońta et al. 1983, 1987, Indiveri et al. 1985, Angeli et al. 1990, McNulty et al. 1990, Levi et al. 1992). This implies the existence of some other factor(s) responsible for the regulation of the circadian rhythm of immune system function and the pineal gland, in particular its principal hormone melatonin, may be a prime candidate. There are several reasons, but the most important are the following: (1) pineal gland function shows a circadian and seasonal periodicity in most species and declines with age; (2) circadian rhythm of melatonin synthesis strongly depends on lighting conditions; (3) melatonin participates in the control of several biological rhythms, including those associated with aging and affective and psychosomatic diseases, which, in turn, are related to an increased incidence of infections, autoimmune disorders and cancer (for review see Pierpaoli and Maestroni 1987). However, to date, there is scanty experimental evidence demonstrating the role of melatonin as a synchronizer of circadian rhythmicity of various immune parameters in humans (Angeli et al. 1990), rats (McNulty et al. 1990, Ding et al. 1995), and chickens (Rosołowska-Huszcz et al. 1991, Skwarło-Sońta et al. 1991, 1992).

EFFECT OF EARLY PINEALECTOMY ON THE DEVELOPMENT OF IMMUNE SYSTEM

The first experimental approach to demonstrate reciprocal interdependence between the pineal gland and immune system consisted of the ablation of the former in order to examine the development and function of the latter. Reports have suggested that surgical pinealectomy may exert a stimulatory, inhibitory or no effect on immune function in the mammalian species examined to date. These controversial results seem to be related to the stage of the animals development when pinealectomy was perfomed, especially in the context of extrapineal melatonin production (retina, Harderian gland, etc.) (Nowak et al. 1989). Nevertheless, during *in utero* phase of altricial mammal development, the pineal gland appears sometime (in human - 3 weeks) prior to the appearance of the thymus rudiment, and displays transitory neuronal connections with higher centres. The signals coming from hypothalamus *via nervus pinealis* which disappears before birth may be conveyed to the thymus to accomplish its normal development (Carderelli 1990).

Surgical pinealectomy during early embryonic development of mammals is methodologicaly difficult, if not impossible, whilst the avian embryo offers an excellent model for the study of this kind. There are several reasons for this, and the most important are that: (1) the avian embryo develops without direct influence from the mother organism; (2) its nervous, endocrine and immune systems develop almost simultaneously, and (3) their immune system contains two separated primary lymphoid glands: thymus, comparable with that in mammals, controlling cell-mediated immune response, and, existing only in birds, bursa of Fabricius, a lymphoid gland furnishing cells which participate in humoral immune response.

Using chicken embryos pinealectomized at 96 h of incubation, therefore developing without any influence of the pineal gland, Janković et al. (1994a) clearly demonstrated functional interrelationships between the pineal gland and development of the immune system. This early embryonic pinealectomy caused a retarded development of both the thymus and bursa of Fabricius, decreased cellularity of both primary lymphoid glands, a decrease in the humoral immune response (number of bursal and splenic hemolysin producing cells) as well as a diminishing of several activities of cell-mediated immunity. Moreover, they also observed a significant change in the concentration of serotonin, dopamine and noradrenaline in spleen, brain and hypothalamus; in particular pinealectomy caused a decrease in the content of all biogenic amines examined in the hypothalamus and an increase in serotonin content in brain and spleen. It seems also to prove an involvement of the chicken pineal gland in the development of the neuroendocrine network, which in turn may influence immune system development.

However, it has to be stressed that in this experimental approach the effect of early embryonic pinealectomy was measured in 17-day-embryos, and it may be compensated later by melatonin coming from extrapineal sources, and therefore, different effects may be observed in the later stages of postnatal development.

IMMUNOMODULATORY ACTION OF MELATONIN MEDIATED BY OPIOID PEPTIDES

Since the early seventies, evidence accumulated for the influence of the pineal gland on the immnue system in laboratory rodents but the results were controversial (for review see Meastroni 1993). However, most papers suggested the necessity of the intact pineal gland for the normal function of the thymus, and, in addition, the reported oncostatic effect of melatonin also implied an immunoregulatory activity (reviewed by Blask 1984).

Functional pinealectomy, achieved by exposure of mice for some generations to constant illumination to cause a suppression of melatonin synthesis, produced all the symptoms of runting syndrome

with severe impairment of body growth and antibody production, and an atrophy of lymphoid tissues (Maestroni and Pierpaoli 1981). Similarly, a pharmacological inhibition of pineal gland function in mammals may be made by either evening application of the β-adrenergic antagonist propranolol (which reduces the night peak of melatonin synthesis mediated by noradrenaline released from post-ganglionic sympathetic neurones in darkness) or by daily injections of p-chlorophenyl-alanine, an inhibitor of the synthesis of serotonin, which is an essential melatonin precursor (Maestroni et al. 1986). Both treatments significantly reduced antibody production (measured by the hemolytic plaque-forming cell number, PFC, in the spleen of mice immunized with sheep red blood cells, SRBC, a commonly used non-pathogenic, thymo-dependent antigen), and evening administration of melatonin restored the normal immune response. An immuno-enhancing effect of melatonin was strictly dependent on the time of administration (Maestroni et al. 1987): the daily evening injections increased PFC number whereas the same morning treatment was without effect. Stimulation of antibody production by melatonin was also dose-dependent: between 10 and 10^4 μg/kg b.w. it induced a significant increase in PFC number, whereas a high pharmacological dose of 200 mg/kg b.w. significantly reduced the response to SRBC. The effect of melatonin was not observed in non-immunized mice nor *in vitro*, indicating that it influences the antigen-activated immunocompetent cells *via* some mediator(s). Exogenous melatonin completely counteracted the immunosuppressive effect of corticosterone given in drinking water or acute anxiety-restraint stress on thymus weight and PFC number as well as it prevented paralysis and death of mice infected with sublethal doses of *encephalomyocarditis* virus after actue stress. All these immunostimulatory and anti-stress effects of melatonin were abolished by concomittant administration of naltrexone, a specific antagonist of the opiod μ-receptors. The antagonizing influence of naltrexone was also dose-dependent, indicating that the presumed mediator of melatonin action on immune cells may be a member(s) of the family of endogenous opioids (EOS), produced under melatonin influence by antigen-activated immunocompetent cells (Maestroni et al. 1987, 1988, 1989). This suggestion was based on results which indicated that not only EOS exert numerous and controversial effects on the immune system and lymphocytes have surface receptors for endorphins and enkephalins (for review see Wybran 1985), but also immune cells are able to synthesize and secrete the opioid-like factor(s) (Smith and Blalock 1981). Participation of melatonin-induced immune opioids (MIIO) in an immunomodulatory action in mice was proved experimentally. First, it was found that exogenous β-endorphin and dynorphin 1-13 mimicked the immuno-enhancing and anti-stress effect of melatonin in its circadian-dependent and naltrexone-sensitive influence (Maestroni and Conti 1989). Subsequently, splenocytes taken from antigen-activated mice, incubated with melatonin were shown to synthesize and release the agent(s) restoring the thymus weight of prednisolone-treated, unprimed mice (Maestroni et al. 1989), and acting as an immunoenhancing and anti-stress factor on thymus cellularity and antibody production in SRBC-immunized mice (Maestroni and Conti 1990). Again, the effect exerted by these active supernatants was circadian-dependent and naltrexone-sensitive (Maestroni et al. 1989). Similar but less consistent results were obtained when mitogen-activated human immunocompetent cells were incubated with melatonin, as only four out of ten blood donors provided cells responsive to melatonin (Maestroni and Conti 1990).

Selective depletion of T cell subpopulations from activated spleen cell suspensions by monoclonal antibodies have demonstrated that the target cells for melatonin-stimulated release of opioid agonist(s) were $L3T4^+$ ($CD4^+$), and the active factor could be the compound(s) combining the properties of β-endorphin and dynorphin 1-13 (Maestroni and Conti 1990). Subsequently, the specific binding of a T-helper-derived agonist to thymic opioid receptors and its cross-reactivity with anti-β-endorphin and anti-met-enkephalin antisera finally demon-

strated an opioid character of the factor which counteracted the depression induced by stress-associated corticosteroids. As the presence of endogenous melatonin and antigen activation were necessary to express this activity, Maestroni and Conti (1991) proposed the existence of a melatonin--immuno-opioids network with a physiological function consisting of providing a correct immune recovery after the depression caused by elevated corticosteroids associated with stressful situations.

Recently, Maestroni (1993) suggested the use of melatonin as an immunotherapeutic agent for immunodeficiency, i.e. in conditions where melatonin was shown to be most active, and indicated acquired-immunodeficiency-syndrome (AIDS) as a most dramatic example of this situation. Rationale for supposed beneficial potential of melatonin in asymptomatic HIV-positive patients is that it is the same target, i.e. lymphocytes $CD4^+$, for both HIV and melatonin, which could be helpful before the development of AIDS, when the T-helper lymphocytes still function normally.

OTHER MECHANISMS INVOLVED IN THE IMMUNOMODULATORY ACTIVITY OF MELATONIN IN MAMMALS

During the last twenty years the number of published papers describing bidirectional interrelationships between the pineal gland, melatonin and the immune system have increased several fold (Conti and Maestroni 1994), most of them have been aimed to elucidate the mechanism(s) involved. General remarks arising from those numerous experiments is that the effects exerted by melatonin on particular aspects of immune system function vary depending on species, age and sex of animals used as well as on experimental protocol, including season, melatonin dose and route of administration. We will try to enumerate some of them, but it implies that melatonin exerts its multidirectional influence using several different mechanisms, at least within the immune system.

An anti-glucocorticoid effect of daily melatonin injections on several metabolic parameters, including prevention of thymus atrophy was found in young female rats unprimed with any antigen (Aoyama et al. 1986). This result was obtained with a pharmacological melatonin dose and indicated the possibility of an anti-glucocorticoid activity even in non-immunized animals which is in contrast with the results of Maestroni et al. (1986, 1987, 1988), but both experimental protocols differ, in particular in species, melatonin dose and route of application.

In 1989 del Gobbo and co-workers demonstrated that pinealectomy significantly reduced interleukin-2 (IL-2) production and natural killer (NK) cytotoxic cell activity, whereas acute treatment with a pharmacological dose of melatonin was able to reverse the effect of pinealectomy on both parameters (Del Gobbo et al. 1989). On the other hand, there are other results which indicate a melatonin--induced suppression of human NK activity *in vitro* (Lewiński et al. 1989).

IL-2 is a lymphokine released by activated T--helper lymphocytes and plays a fundamental role in antitumor defense which is effected primarily by NK cells. Therefore, melatonin acting as an immunostimulatory factor and controlling IL-2 production, together with its well established oncostatic role, seems to be an excellent candidate for use in clinical oncology. There are several papers indicating that a concomitant administration of melatonin with IL-2 during immunotherapy of cancer could be in some tumor histotypes a well-tolerated, effective treatment possibly allowing a diminished IL-2 dose and therefore reducing its side effects (e.g. Lissoni et al. 1993a, b, 1994). As the research on the oncostatic activity of melatonin, including its clinical applications and therapeutic approaches, become more and more frequent, it will not be discussed in this review.

Melatonin seems to also modulate the secretion of another cytokine playing a key role in the activity of the immune system, γ-interferon (INF-γ). Here again, the effect of melatonin diametrically differs

in studies in rodents with those in humans. Melatonin stimulated INF-γ secretion from mouse splenocytes and this effect was several times higher in cells isolated at night than in the morning (Colombo et al. 1992). In cultured human peripheral blood lymphocytes (PBL) melatonin did not induce production of INF-γ and TNF, but it inhibited the production of both cytokines when the cell culture was stimulated with PHA. This effect was not opioid-mediated but seemed to be dependent on the seasonal time of blood collection (DiStefano and Paulesu 1994). In another experimental approach, low melatonin concentrations stimulated INF-γ release by human PBL activated with T-cell mitogen, whereas in higher concentrations it was inhibitory (Muscettola et al. 1994). In humans a feed-back circuit between the pineal gland and γ-interferon was described: melatonin inhibited γ-interferon production, which, in turn, stimulated peripheral blood mononuclear leukocytes to synthesize melatonin (Arzt et al. 1988, and Finocchiaro et al. 1988, 1991).

Melatonin also exerts a very strong anti-viral activity in mice, both in normal animals, dexamethasone injected, or stressed, and this effect was or was not counteracted by naltrexone, depending upon age of animals used (Ben-Nathan et al. 1994).

Another mechanism of melatonin action, antagonizing the corticosteroid-induced involution of the thymus in mice was proposed by Pierpaoli and co-workers (Pierpaoli and Yi 1990, Lesnikov et al. 1992). These authors obtained some indications that melatonin may exert its circadian immuno--modulatory, thymotropic and anti-stress activity by the regulation of the synthesis and/or secretion of hypothalamic or peripheral thyrotropin-releasing hormone (TRH) and this effect was not thyroid-dependent. An electrolytic lesion of the anterior hypothalamic area resulted in an involution of the thymus and a reduction in peripheral blood lymphocyte number, reversed significantly by daily evening treatment with TRH or melatonin. Again, these restoring effects were not dependent on the stimulation of the thyroid gland, but were antagonized by naltrexone. On the basis of the above results, Pierpaoli's research group indicated TRH as a suitable candiadte for a transducer of pineal-recorded external stimuli, leading to the adaptation of an organism to environmental variables, including regulation of immunity as a reaction to the antigens, stress and other noxious agents.

On the other hand, TRH is a potent prolactin (PRL) releasing factor (reviewed by Martinez de la Escalera and Weiner 1992), and recently the participation of PRL in the anti-stress effect of melatonin on the immune system of rats was hypothesized (Liebman et al. 1993). Exogenous melatonin decreased basal PRL release and potentiated the PRL--increasing effect of stress and this action was EOS-mediated. PRL is a well known stress-induced factor (for reviews see Van de Kar et al. 1991 and Kelley et al. 1992) and its immunostimulatory activity, antagonizing the effect of glucocorticoids (Bernton et al. 1992) in both mammals and birds is well established (for review see Berczi 1992, and Skwarło-Sońta 1992), with similar immune system target, as for melatonin (T-cell immunity).

Finally, it was found that pinealectomy impairs and melatonin enhances the antibody-dependent cellular cytotoxicity in mice, and this effect was sex- and seasonally-dependent, suggesting that sex hormones may be involved in the pineal effect on some parameters of immune system function (Giordano and Palermo 1991, Vermeulen et al. 1993, Palermo et al. 1994). As melatonin is a principal environmental signal transducer controlling gonadal development and function, at least in seasonally breeding species (Morgan 1990), and on the other hand, sex steroids are implicated in the well documented sexual dimorphism in the immune response (Grossman et al. 1991), the above mentioned mechanism of melatonin action upon the immune system seems to be important.

Taking the experimental results cited above together, it is admissible the existence of a muli-step transmission of information coming from external environment *via* pineal gland-melatonin-TRH-PRL and/or GnRH-gonadotrophins-sex steroids to the immune system. This mechanism(s) may operate parallelly to the melatonin-induced immuno-opioids (MIIO, proposed by Maestroni and co-workers,

1994), and/or direct action, using specific melatonin receptors on immune cells (see below). This last possibility was recently supported by the demonstration that melatonin *in vitro* significantly inhibited the incorporation of [^{3}H]thymidine into both normal mouse and human lymphocytes and T-lymphoblastoid cell lines whereas it stimulated the myeloma cell proliferation (Persengiev and Kyurkchiev 1993). This first report dealing with a direct melatonin influence on lymphoid cell proliferation opens, we believe, a new direction of research on the mechanism(s) of melatonin action within the immune system.

ELECTROMAGNETIC ENVIRONMENT AND PINEAL GLAND FUNCTION

As was already discussed, light inhibits melatonin synthesis within the pineal gland. However, visible light constitutes only a very narrow portion of the total electromagnetic spectrum, which varies from a very short, thus high energy ionizing radiation (X-rays, UV), to an extremely low frequency (ELF), long waves, with energy so low that it is incapable of extracting electrons from atoms (non-ionizing electromagnetic waves). ELF waves derive from electrical power lines and electrical appliances and produce charges or currents on the surface of bodies or objects exposed. Widespread use of electric power markedly changes the electromagnetic environment, especially in countries with well-developed industry. On the other hand, magnetic field readily pass through all materials including the body, and can induce eddy currents in it. There are several studies suggesting that the exposure of humans to high or unusual electric and magnetic fields induces a higher than normal incidence of cancer (Reiter and Richardson 1992, Reiter 1993, 1994).

Experiments on laboratory rodents have demonstrated that unusual electric, magnetic or electromagnetic fields induce a reduction of melatonin synthesis and secretion, *via* an inhibition of N-acetyltransferase activity, a key enzyme responsible for the circadian rhythm of melatonin. Since both visible light and ELF electromagnetic fields evoke the same effect in the melatonin synthesis pathway, a similar mechanism of action may be suggested. A diminished melatonin message or perturbed circadian rhythm may, in turn, have a significant physiological consequences, including diminished antioxidant activity and anti-cancer protection (see below), (Reiter 1994).

On the other hand, it has been demonstrated that the exposure of the rat brain occipital region to static magnetic fields results in a significant immunopotentiation (Jankovi et al. 1994). In aging rats, implantation of micromagnets to the brain occipital region not only enhanced both the cellular and humoral immune response to a T-dependent antigen, but also abrogated immunodeficiency induced by pinealectomy. This indicates the role of the pineal gland, and presumably melatonin, in magnetic field-induced immunopotentiation, and on the other hand, demonstrates a functional relationship between the central nervous system, pineal gland, immune system and static magnetic fields. The presence of melatonin binding sites within the central nervous system implies the involvement of the brain in the immunomodulatory activity of the pineal gland.

ANTI-AGING MELATONIN ACTIVITY

One of the most impressive effects evoked by the pineal gland is its anti-aging activity demonstrated in laboratory rodents in which grafting of the entire pineal from young donors (Pierpaoli et al. 1991, Lesnikov and Pierpaoli 1994) or melatonin treatment (Maestroni et al. 1989, Caroleo et al. 1994,) caused a significant rejuvenation in old animals. Aging or senesence is a multifactorial postmaturational process that results in the heterogenous patterns of a progressive morbidity and disability (Seeman and Robins 1994), manifested also by a measurable decline in immune function (Ershler 1993). There are several theories of aging, among them the free radical theory hypothesizes that the

aging and associated degenerative processes result from the accumulation of free radicals, continuously produced as a normal product of aerobic cellular metabolism (Meydani 1992). It is also well documented that active oxygen species are produced upon exposure of organisms to ionizing radiation, ultraviolet light, and some chemical carcinogens. Free radicals are atoms or molecules having an unpaired electron, i.e. O^{-}_2, HO*, H_2O_2, and usually they are short-lived because of their highly reactive nature. They can damage molecules important to cellular function, including DNA, and this type of damage can be both mutagenic and carcinogenic. There are multiple enzymatic and non-enzymatic antioxidant defense systems in cells to protect against the damaging effects of free radicals. These defense systems consist of the removal of free radicals or in slowing their production, and in addition, there are also molecules which scavenge or quench free radicals once they are produced. These molecules are more useful in preventing or delaying aging and age-related diseases. Among the best known free radical scavengers are tocopherol (vit. E), ascorbate (vit. C), glutathione, and recently, an antioxidative role of melatonin was proposed. It was demonstrated that melatonin was several times more potent as a hydroxyl radical scavenger than glutathione and mannitol. Moreover, in rats treated with safrole, a highly toxic carcinogen which causes hepatic DNA damage *via* production of toxic free radicals, melatonin administered in relatively low doses (750- to 1,500-times lower than that of safrole) was extremely efficient in DNA protection (Reiter et al. 1994).

Free radicals are molecules participating, together with cytokines, in a developing immune response, especially in the destruction of toxic bacteria or pathologically changed cells. Recently it was demonstrated that activated macrophages synthesize another type of free radical, nitric oxide, which, as well as ending bacterial destruction, activates the generation of a new very active hydroxyl radical. Immune cell-derived free radicals are extremely useful to effect an immunosurveillance against abnormal cells, but, unfortunately, they can also act against their own lymphocytes leading to some tissue dammage. The safe level of immune-derived free radicals strongly depends on the presence of antioxidant agents, including the intracellular level of zinc (Favier 1993).

Recently, it was demonstrated that melatonin can modulate zinc turnover (Mocchegiani et al. 1994a, b). With advancing age not only plasma melatonin declines progressively, but this decrease is also accompanied by thymic degeneration, reduced plasma level of the zinc-bound biologically active form of thymic hormone, thymulin (Dardenne et al. 1982), and by low zinc plasma levels. In old mice, both melatonin treatment or pineal grafting into the thymus, corrected all immune parameters, including thymus cellularity, peripheral lymphocyte number and responsiveness as well as restored the zinc pool to similar to that observed in young mice (Mocchegiani et al. 1994b).

It is important to notice that the activity of melatonin as a very potent endogenous antioxidant may be exerted everywhere within the body, because, as a highly lipophilic compound, it can easily pass through all morpho-physiological barriers, and protect every portion of the cell from radical-induced damage. Moreover, there is substantial evidence that melatonin is produced in many organs, in addition to the pineal gland, including peripheral blood leukocytes (Finochiaro et al. 1991), and therefore may represent a very important component in the modulation of immune function. This point of view seems to be strongly supported by the well known fact that advancing age, in both humans and laboratory animals, is associated with a progressive reduction in the concentration and circadian rhythm of melatonin (Reiter 1992). On the other hand - aging is characterized by a deficiency in the cellular immune response as a result of relatively inert memory T cells and reduced T cell activity, influencing also humoral immnuity. The senescence of the immune system has been proven to be causative in the development of age-associated autoimmune phenomena, infectious diseases and neoplasia (Ershler 1993). It is admissible, that age-related accumulation of free radicals, in parallel

with a decline in endogenous melatonin rhythm, may be, at least partly, responsible for a senescence of immune system efficiency.

INFLUENCE OF IMMUNE SYSTEM ON PINEAL GLAND FUNCTION

If the pineal gland really participates in the transmission of information between the external environment and internal *milieu,* and plays a role in the modulation of immune system function, it is essential that the immune system in turn sends its own information to the pineal gland, and these signals have to be adequately transformed to maintain homeostasis. Experiments which indicate that the pineal gland is able to receive this kind of information have just started to appear, and it seems that cytokines, a soluble factors produced by immune cells, are the best candidates to effect this very important function. First, it has been demonstrated that INF-γ enhanced melatonin production by the rat pineal gland in organ cultures (Withyachumnarnkul et al. 1990a), whilst, on the other hand, it suppressed NAT activity stimulated with isoproterenol, a β-adrenergic receptor agonist (Withyachumnarnkul et al. 1991). Two possible mechanisms of INF-γ action upon the pineal gland in rats have been proposed: a faciliation of tryptophan entry into the sympathetic nerve terminal and pinealocyte, and down-regulation of the β-adrenergic receptors on the pinealocyte membrane. The end result, either an increase or a decrease of melatonin production, depends on the doses of INF-γ and the level of noradrenergic stimulation of the pinealocyte (Withyachumnarnkul et al. 1990b).

The existence of bilateral interplay between the pineal gland and immune system has recently been supported by another experiment in rats, indicating a significant dose-dependent decrease in serum melatonin level under the influence of exogenous recombinant human interleukin 1β (IL-1β), abolished by anti-human IL-1 receptor antibody (Mucha et al. 1994). IL-1 is a cytokine with pleiotropic activity within the neuroendocrine and immune systems, and the above results seem to indicate an important role in transmitting information between the immune system and pinealocytes, by direct action *via* specific receptors. Recently, the same research group have demonstrated that colony-stimulating factors (G-CSF and GM-CSF), glycoproteins secreted by activated immune cells, mainly macrophages, stimulated both *in vivo* and *in vitro* rat pineal melatonin synthesis, in a dose-dependent way (Żylińska et al. 1995).

EFFECT OF MELATONIN ON IMMUNITY IN BIRDS

All the previously mentioned experimental results and hypotheses described an immuno-regulatory function of the pineal gland and melatonin in mammals and this field of research is well understood because of possible application of exogenous melatonin in human immunotherapy. Nevertheless, the pineal gland was demonstrated to play an essential role in the transduction of external information into the organisms of all vertebrates, with numerous differences between mammals and lower vertebrates in terms of several pineal gland functions, especially with respect to the direct photosensitivity and the mechanism of melatonin synthesis regulation.To our knowledge, there is no published information concerning immunoregulation by the pineal gland in ectotherms, whereas that in birds is very scanty, but, as it was already mentioned, avian species offers a very useful experimental model to examine pineal - immune interrelationships.

It was demonstrated in several experiments that in the chicken, the circadian rhythm of different immune parameters was strongly dependent upon the presence of an intact pineal gland, e.g. pinealectomy at the first week of age influenced the diurnal rhythm of both granulocyte number and serum lysozyme concentration in 5-week-old chickens, and both rhythms were restored by daily melatonin but not vehicle injections (Rosołowska-Huszcz et al. 1991). Also, in chickens with an intact pineal gland,

exogenous melatonin modified the circadian rhythm of immune system function (measured by the number of white blood cells, WBC, and their fractions, as well as by the level of circulating agglutinins anti-SRBC and PFC number), and the effect was more dependent on the time of hormone administration than on the dose used (Skwarło-Sońta et al. 1991, 1992). However, we were not able to demonstrate any immunostimulatory effect of melatonin in the chicken, even in experimental conditions in which it stimulated PFC number in mice (Skwarło-Sońta et al. 1992).

Recently, the effect of pinealectomy on nonspecific immune parameters as well as serum hormones level were examined in adult male and female ring doves (Rodriguez and Lea 1994). Immune parameters were examined 6 month after surgery (pinealectomy or sham operation) once a day, thus no information was obtained about the effect of pinealectomy on the circadian rhythm of immune parameters, but here again, there is no clear cut indication about an immunostimulatory effect of the pineal gland. On the contrary, some parameters were increased in pinealectomized birds, some not influenced, and an increased production of free radicals in heterophils, indicating a reduced melatonin concentration in pinealectomized birds. Immunization with a soluble antigen, normal sheep serum, similarly influenced phagocytic parameters in both pinealectomized and sham operated birds.

The above mentioned results are in apparent contradiction with those obtained recently by Janković et al. (1994a) who demonstrated a diminished immune response in pinealectomised chicken embryos. The necessity of the pineal gland in developing chicken embryos to effect a normal immune response, found by Janković research group (1994a) does not exclude the possibility of compensation of melatonin synthesis in extrapineal sites in the later stages of postnatal life.

In the chicken with an intact pineal gland melatonin in a dose effective in mice did not antagonize the immunosuppressive effect of corticosterone given in drinking water, and its effect was not naltrexone-dependent. However, a very high melatonin dose (2,000 μg/kg BW), and naloxone counteracted the effect of corticosterone on some parameters, suggesting a different sensitivity to melatonin and opioid antagonists in mammals and birds (Skwarło-Sońta et al. 1994a).

Recently it was demonstrated that in the chicken between 2 and 8 weeks of age the pineal gland is infiltrated by lymphocytes which form an accumulation well organized, like in peripheral lymphoid organs (Olah 1995). Intra-pineal lymphoid cells were characterized using the monoclonal antibodies, and they appeared to be T cells as well as Ig-producing plasma cells, forming a real lymphopineal tissue. The physiological significance of the transitory lymphoid infiltration of the pineal gland is unknown, but it may be a very special manifestation of the neuroendocrine-immune interactions.

The results obtained to date seem to indicate that the difference between mammals and birds in terms of pineal gland function may be extended to its immunomodulatory potential, but needs further experimentation. It is especially necessary to elucidate the effect of sex, age and season upon the influence of melatonin on particular immune parameters in birds, as these factors were demonstrated to modify the immunomodulatory activity of melatonin in mice (Giordano and Palermo 1991, Vermeulen et al. 1993, Palermo et al. 1994, Maestroni et al. 1994).

MELATONIN RECEPTORS WITHIN THE IMMUNE SYSTEM

For several years the central nervous system was considered as a site by which melatonin may modulate the rhythmicites, observed in mammals. The coincidence of the peaks of melatonin concentration and its receptors in the brain in terms of diurnal rhythms may determine the responsiveness to melatonin of the neuroendocrine system (Anis et al. 1989, Stankov and Reiter 1990, Stankov et al. 1991a, b). Using 2-[^{125}I]iodomelatonin, an agonist with a high specific activity, binding of melatonin in peripheral organs [e.g. gastrointestinal tract (Lee

and Pang 1991), reproductive organs (Ayre et al. 1992, Wang et al. 1992), kidney (Song et al. 1992)], including the immune system, have been demonstrated, implying a direct action of melatonin on target tissues outside the central nervous system.

In 1989 Niels in the hamster, and thereafter Yu and co-workers (1991) in other mammals and birds demonstrated the presence of iodomelatonin binding sites in spleen, with higher density in birds (duck, chicken) than in mouse, whilst no binding sites were detected in the spleen ofthe rat. Binding of 2-[^{125}I]iodomelatonin to the spleen membrane preparations was specific, reversible, and time-dependent, and binding parameters (Kd and Bmax) suggested that these binding sites belonged to the M1 class of melatonin receptors proposed by Dubocovich in 1988 (Pang et al. 1991a). Subsequently, high affinity specific binding of melatonin was described in membrane preparations form spleens of guinea pig (Poon and Pang 1992, 1994), chicken (Pang and Pang 1992) and pigeon (1993), duck thymus (Liu et al. 1992, cited after Liu and Pang 1992) and bursa of Fabricius (Liu and Pang 1992), rat thymus (Martin-Cacao et al. 1993) and in human blood leukocytes (Lopez-Gonzalez et al. 1992, Guerrero et al. 1994). Our own data (Skwarło-Sońta et al. 1994b) has demonstrated in 4 week-old cockerels the presence of very abundant 2-[^{125}I]iodomelatonin binding within the brain and a much lower binding sites density in lymphoid tissues, as well as in the gonads. Of the lymphoid tissues examined, the highest iodomelatonin binding was found in membrane preparations isolated form the bursa of Fabricius (comparable with those of Liu and Pang 1992), much lower in the spleen, and only traces in the thymus.

In human peripheral blood leukocytes Scatchard analysis revealed two classes (high- and low-affinity) of melatonin binding sites on lymphocytes, whilst granulocytes possessed only one classe, a low-affinity binding site (for review see Guerrero et al. 1994). The high-affinity melatonin binding site on peripheral lymphocytes may be responsible for recognition of the circulating hormone by the lymphocytes, the low-affinity binding sites are unlikely to be related to a physiological role of melatonin within immune system. Moreover, the authors suggest that these lymphocyte low-affinity binding sites may be due to the presence of granulocytes in the blood lymphocyte preparations.

Similarly, in partially purified membrane preparations from the rat thymus, two classses of melatonin binding sites have been identified: high- and low-affinity sites, which, additionally, exhibit a decrease in binding capacity during the two first weeks of age: melatonin binding was highest in newborn rats but in the second week of age it was as low as in adult rats (Martin-Cacao et al. 1993). Our recent studies on early postnatal development of melatonin binding sites within the chicken immune system have also revealed a decrease in hormone binding during the first weeak of age (Dziwiński et al., unpublished data). In membranes prepared from whole thymi, specific melatonin binding increased from 0.25 fmol/mg protein at 2nd day after hatching to 0.60 fmol/mg protein at 7th day, and thereafter it decreased to the very low value (approx. 0.10 fmol/mg protein) remaining constant until the 4th week of age. The pattern of postnatal change in melatonin binding sites in the thymus of rats and chickens are different and their physiological relevance remains to be establish. However, it may be considered as one of the reasons for the different effects of melatonin on immune parameters observed in the *in vivo* experiments.

It is also of interest that when membranes were obtained from isolated chicken thymocytes and remained tissue debris, both thymic subfractions exhibited melatonin binding ability, which was maximal at the 4th day after hatching, and decreased thereafter to the value observed in the whole gland after the 1st week of age. This result implies the possible involvement of melatonin in the intrathymic education of developing T cells in chickens, as well as some role of the thymic microenvironment in melatonin message transmission between blood and immune cells.

Developmental changes in melatonin binding sites number were also measured in membrane preparations from the bursa of Fabricius and spleen,

obtained from both whole glands and their subfractions (Dziwiński et al., unpublished data). Melatonin binding site density varied with gland and age: highest and variable was in the spleen, suggesting that this secondary lymphoid gland contains a more heterogenous cellular composition in relation to sensitivity to melatonin. In membranes from whole bursa of Fabricius, melatonin binding was relatively stable until the 21st day of life, but, surprisingly, it increased continuously in isolated bursal lymphoid cells. On the other hand, in the lymphoid glands examined, melatonin binding by tissue debris was highest in the bursa of Fabricius. The physiological significance of these results and precise localisation of melatonin binding sites within the chicken immune system deserve further studies, but results presented to date, together with those obtained in developing rats seem to indicate a developmental interdependence between the immune system and pineal gland. They are in line with previously demonstrated impairment of immune potential by neonatal pinealectomy (Devecerski 1963) as well as with the recent results published by Janković et al. (1994) on the effect of embryonal pinealectomy on the development of the chicken immune system.

Our data (Skwarło-Sońta et al. 1994b) obtained in 4 week-old cockerels also demonstrated that neither Kd nor density of melatonin binding sites were modified by immunization with SRBC, which caused a significant stimulation of serum anti-SRBC agglutinins. This result suggests that in immunocompetent birds, melatonin binding by both primary (thymus and bursa of Fabricius) and secondary (spleen) lymphoid organs is unrelated to immune system activation by a T-dependent antigen. It remians in sharp disagreement with results obtained by Poon et al. (1994) in the duck treated for 7 days with pharmacological doses of cortisol (1 mg per 2-week-old bird daily). These authors obtained a significant reduction of Bmax in cortisol injected birds, concomitant with a decrease in body and primary lymphoid organ weights (thereby relative organ weights were not influenced) and no significant increase in serum melatonin content. Reduction of melatonin binding site number in cortisol-injected, very young birds was attributed by the authors to a change in their immune status, though *not* clearly documented. Our own results (Skwarło-Sońta et al. 1994b, and unpublished data) demontstrated in the 5-week-old chicken, treated for 5 days with corticosterone (a major adrenal corticoid in avian species, De Roos 1961) in drinking water, at a dose approx. of 1,2 mg/bird daily, a reduction of relative thymus and bursa of Fabricius weight by 40-65 and 52-60%, respectively, simultaneous with a 60% decrease in serum antibody level. A reduction of melatonin binding density under the influence of cortisol injections, observed by Poon et al. (1994) is probably rather related to binding site down regulation by the increased serum melatonin concentration, than with any depressed activity of the immune system. On the other hand, Wang et al. (1993) have reported a significant increase in the number of melatonin binding sites on spleen membrane preparations, concomitant with unchanged binding by the brain in hydrocortisone-treated pigeons.

Melatonin binding sites within the immune system undergo diurnal changes, with the higher density during the day (Liu and Pang 1992) and an increase under constant illumination (Poon and Pang 1992).

To date, information on melatonin signal transduction and the post-receptor mechanism of its action within the immune system is very scarce (Lopez-Gonzalez et al. 1992, Poon et al. 1993, Poon and Pang 1994, 1994a) but seems to indicate that the melatonin binding site is a single type of receptor coupled to a G-protein. However, it has recently been demonstrated that melatonin binding sites in chicken primary (bursa of Fabricius) and secondary (spleen) lymphoid organs may contain subtypes of receptor which form different receptor-G protein-effector complexes (Poon and Pang 1994b). In human lymphocytes, melatonin had no influence on the basal cAMP level however it stimulated cGMP and increased the effect of VIP on cAMP production (Lopez-Gonzalez et al. 1992). It was suggested that in human blood lymphocytes, high-affinity binding sites are coupled with the potentiation of the VIP effect on cAMP, and low-affinity binding

sites are coupled with activation of cGMP production. On the other hand, physiological concentrations of Na^+ and Ca^{2+} seem to play a modulatory role in melatonin binding to its receptor in the guinea pig spleen (Poon and Pang 1994). Very recently Guerrero's research group have published results indicating that high-affinity melatonin binding sites in rat splenocytes fulfilled all the necessary criteria for a single class of receptor. Moreover, melatonin was demonstrated to inhibit cAMP production stimulated by forskolin, similar way to that observed using pituitary cells (Morgan et al. 1989). The affinity of these binding receptors suggest that they may recognize physiological melatonin concentrations in the serum (Rafii-ElIdrissi et al. 1995).

It remains to be established whether or not melatonin binding sites within the immune system may participate directly in the immunomodulatory activity of this hormone, proven to date at least in mammals. In fact, when the effect of different doses of exogenous melatonin on its binding sites was examined in guinea pig spleens, a dose-dependent decrease of binding density was observed (Poon and Pang 1994a). It is the first indication that melatonin, by a modulatory influence on its own binding site, may act directly upon the immune system function. However, it is impossible to answer, whether and in what extent described melatonin treatment modified that function, as immune parameters were not measured in this experiment.

CONCLUSIONS

The results reviewed here indicate that the pineal gland, a neuroendocrine organ, is involved in a bidirectional interrelationship with the immune system (Fig. 1). A special position of the pineal gland in this scheme is due to its particular ability to transduce external information on light and the electro-magnetic field, not perceived by both neuro- and endocrine systems, into a biochemical message understood by the whole body. This message, consisting of daily rhythms of melatonin synthesis and release, is thereafter transmitted to the immune system using several intermediate mechanisms, including the endogenous opioid system, various hormones, cytokines as well as the specific melatonin binding sites within immnue system itself, which, in turn, is able to produce some factors (cytokines) which control pineal gland function. The highly lypophilic melatonin molecule may also penetrate every cell of the body, including immune cells, scavenge produced free radicals and act as a very potent anti-aging and anti-neoplastic factor. Reciprocal interrelationships between the pineal gland, nervous, endocrine and immune systems during development are essential for the normal acquiring of immune competence. The pineal gland is also an important component of the neuroendocrine system responsible for the resetting of circadian rhythmicity of several physiological processes, therefore it also seems to participate in the control of temporal organization of immune system function.

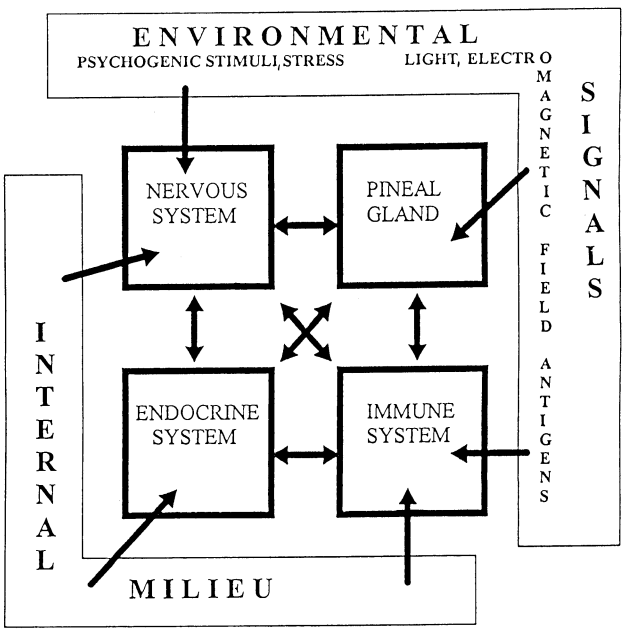

Fig. 1. Pineal gland involvement in the signal transduction between environmental information and immune, nervous and endocrine systems.

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