

THE THYMUS AS A PERIPHERAL NEURAL-IMMUNE MODULATOR THROUGH MELATONIN

Project Summary: The hormone melatonin is essential in driving seasonal variations in immunity as a result of a changing photoperiod (day length). The principal center of melatonin production is the pineal gland (PG) in the brain and a critical circadian oscillator directing seasonality in the immune system is the suprachiasmatic nuclei (SCN). Regrettably, the control enacted by peripheral centers of melatonin production, such as the thymus, remains unexamined. This study aims to investigate the role of the thymus in neural-immune modulation in a mammalian model exhibiting seasonal changes in immunity and behavior. Under winter-like photoperiods, the experiments proposed here will evaluate thymus production of melatonin in pinealectomized (PG removed) animals. In addition, bi-directional communication via melatonin will be assessed between the thymus and SCN in pinealectomized animals under both normal conditions and during a pathogenic challenge. I hypothesize that the thymus is an important organ in melatonin-based neural-immune interactions in the context of photoperiodism.

I. Background and Introduction

Recent studies have led to the discovery of complex neural-endocrine-immune interactions that lead to seasonal changes in the physiology and behavior of vertebrates (Martin et al. 2008). Neuroendocrine and circadian modulation of immunity is of great interest to ecologists because they represent a distinctive set of mechanisms that modulate immunocompetence and influence the overall fitness of an organism. A study of the role of key components in these processes will unravel just how intimately the nervous and immune systems intertwine and thus expand knowledge on this chapter of vertebrate evolutionary history. Although much progress has been made in the field of neural-immune interactions (neuroimmunomodulation) not all the intricacies

and processes are completely elucidated. A major component that has been the subject of intense research is the hormone melatonin. Melatonin transmits day-length (photoperiod) information to regulate sleep-wake cycles and influence major physiological systems in vertebrates (Hardeland, 2008). The entrainment of endogenous rhythms to a changing photoperiod throughout the year conveys information regarding seasonality (i.e. shorter photoperiod = winter) and serves to direct the necessary physiological changes that promote survival of the organism (Martin et al. 2008). The ecological challenges for many organisms are dynamic and this is reflected in the variability of important defenses such as the immune response. The reasons for these variations may involve cues anticipating periods of increased pathogenic threats and the energetic benefit of oscillating the robustness of the immune response over the seasons (Martin et al. 2008). For instance, energetically expensive processes such as reproduction occur when resources are plentiful but immune defenses take priority during challenging periods to ensure survival and secure future reproductive success. Hence, physiological trade-offs in vertebrates procure the successful transmission of an individual's genes (fitness) in the face of a hostile environment (Martin et al. 2008).

Underlying much of the complexity of neuroimmunomodulation is melatonin, a hormone with a multifaceted array of functions. Melatonin is synthesized in the pineal gland (PG) where it is directly delivered to other areas of the brain and can easily cross the blood-brain barrier and enter circulation (Freeman et al. 2007). Circadian oscillations lead to peak melatonin release during the night and eventual cessation throughout the day. The pleiotropic effects of melatonin on the immune system have been well characterized *in vivo* and *in vitro* (Guerrero and Reiter, 2002; Wronka et al. 2008). Besides controlling sleep-wake cycles, melatonin's effects range from suppressing the inflammatory response to inducing thymus growth and increasing

concentrations of circulating lymphocytes (Freeman et al. 2007; Guerrero et al. 2002). Melatonin receptors have also been reported in the immune system and extra-pineal biosynthesis of this hormone occurs in the thymus organs of humans and mice (Naranjo et al. 2007). Melatonin production in the thymus is intriguing as it suggests a more complex role for this organ beyond T-cell maturation.

The role of peripheral centers of melatonin-based regulation of immunity merits investigation. One reason for this is that the effects of pineal-derived melatonin on immunity have already been intensely examined and yet extra-pineal sources (e.g. thymus) outside the central nervous system (CNS) have not received the same degree of attention. Areas of the brain such as the suprachiasmatic nucleus (SCN) have been found to explicitly mediate induction of phenotypes typical of a reduced photoperiod and thereby indicating seasonality. These phenotypes include attenuating behavioral responses to infection such as anorexia (loss of appetite) and cachexia (muscle atrophy, fatigue); presumably to bolster energy supplies to the immune system in the face of pathogenic threats (Freeman et al. 2007). The immune-regulative functions of peripheral sites of melatonin biosynthesis are elusive because they remain largely unexplored!

CNS oversight¹ of the multiple functions of melatonin is fundamental (Figure 1A) but the ubiquity of peripheral melatonin production can no longer be considered merely ancillary given that bi-directional communication between the nervous and immune systems has been established (Skwarlo-Sonta et al. 2003). In addition, the existence of a fixed, extra-pineal site of melatonin production in the thymus points to an unexplored avenue of paracrine control in seasonal immunity. I do not aim to make a complete distinction of CNS versus peripheral control of melatonin's photoperiodic effects. The intimate connections established between the neural,

¹ CNS= pineal + SCN control in the brain.

endocrine, and immune systems would be ignored by pursuing such a strategy. Also, the principal transmission of circadian rhythms, such as photoperiodism, by points found outside the CNS is unlikely. Instead, melatonin derived from peripheral lymphoid organs could add to the robustness of an organism's defenses during winter seasons. Therefore, I am interested in finding if the thymus will enact immune system readiness based on available photoperiodic information (Figure 1B).

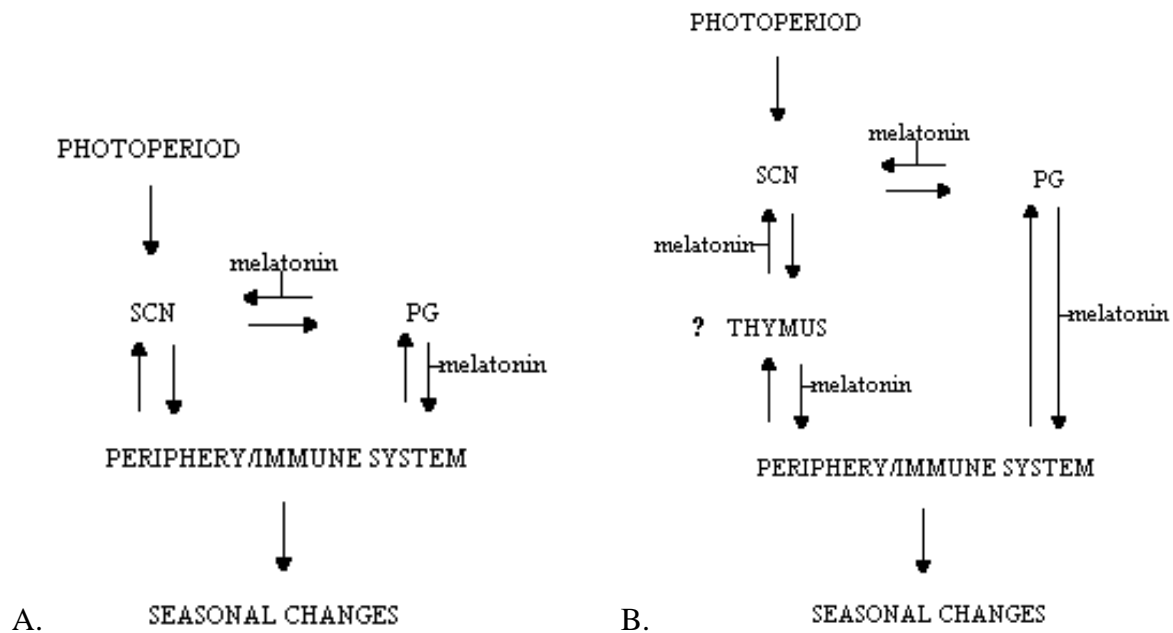


Figure 1. Melatonin-associated neural-immune interactions. **A. CNS control of seasonal immunity.** Photoperiod information is transmitted to the suprachiasmatic nucleus (SCN) which induces melatonin production in the pineal gland (PG). Pineal-derived melatonin acts on the SCN and both centers drive the full suite of seasonal changes in immunity and behavior. **B. Proposed role of the thymus.** The thymus may also partly drive seasonal changes in immunity under winter-like photoperiodic conditions and convey neuroimmunomodulatory information to the SCN to possibly drive complementary changes in behavior. Also, perceived infection (not shown) may prompt neural-immune interactions mediated by the thymus. Direction of melatonin signaling is indicated by melatonin-connected arrows and unlabeled arrows indicate bi-directional

communication via signals other than melatonin (e.g. cytokines, other hormones, neuronal control).

II. Hypothesis

Based on extensive background, I hypothesize that the thymus is an important organ in melatonin-based neural-immune interactions in the context of photoperiodism. Although the PG is mostly responsible for melatonin biosynthesis, the thymus's ability to synthesize this hormone suggests a key placement of this organ in the pathways that lead to seasonal changes in immunity based on a changing photoperiod. Otherwise, the thymus merely responds to melatonin signals originating in the CNS and seasonal neural-immune interactions are arranged so that the immune system, including the thymus, mainly conforms to control from higher areas in the brain (null hypothesis). Instead, the thymus can play a dynamic role in bi-directionality and neural-immune modulation.

III. Proposed Study

Photoperiodic organisms are needed to study peripheral control of melatonin-induced seasonal immunity. Small avian or mammalian subjects are commonly employed because they exhibit evident and measurable changes in behavior and physiology in response to short, winter-like photoperiods (Martin et al. 2008). I have opted to use Siberian hamsters (*Phodopus sungorus*) because they meet this criteria, are easily maintained, and surgical removal of the PG, SCN, and the thymus is relatively simple and effective for experimentation. (Freeman et al. 2007).

Phase 1. Assay of melatonin biosynthesis in the thymus of pinealectomized hamsters.

The first phase of experimentation will assay melatonin production in the thymus under the influence of a short photoperiod (SP) in an animal whose pineal gland has been removed (pinealectomized). Since the thymus is perfectly capable of its own melatonin production (Liu et

al. 2007) circadian biosynthesis of this hormone will occur even in the absence of the PG since an intact SCN will continue to perceive photoperiodic information. Studies suggest that pineal-derived melatonin may principally target components of the immune system (e.g. lymphocytes) and also trigger further melatonin biosynthesis at the thymus (Naranjo et al. 2007). The prediction linked to the present experiments is that the conversion of photoperiodic information to a biochemical signal will still be accomplished in pinealectomized hamsters by the thymus and lead to immune modulation. A marked increase in non-thymus, tissue-derived melatonin is concerning as this may lead to an overestimation of the contribution from the thymus. A suitable control for this response is to measure the levels of circulating melatonin in pinealectomized and thymectomized hamsters under identical photoperiodic conditions as their pinealectomized-only counterparts. Circulating melatonin levels in control animals should remain negligible. Also, tissue-derived melatonin does not readily enter circulation (Guerrero and Reiter, 2002; Mjewski et al. 2005), so any circulating melatonin would originate from the thymus.

The overall scheme involves pinealectomizing the test subjects and subsequently exposing them to SP conditions. After a suitable period of SP exposure, presumably enough for detectable melatonin biosynthesis, the test subjects are sacrificed and the thymus is harvested. After the surgical removal of the thymus, the organ is processed so that we are able to extract any medium containing melatonin. This may involve physical disintegration (homogenization) of the organ and multiple rounds of centrifugation to remove any cellular debris and other, heavier, material. The remaining supernatant will be a mixture of many metabolites and we can use an enzyme linked immune-sorbent assay (ELISA) to detect the presence of melatonin. To establish melatonin production in the thymus under SP conditions in a group of normal test subjects, we would forgo pinealectomy and detect melatonin levels from the thymus extractions of these

pineal-intact animals. A comparison of melatonin levels in the thymus of pinealectomized, pineal-intact, and pinealectomized/thymecotized hamsters under SP conditions will allow us to ascertain if melatonin production occurs in the thymus under these conditions. Circulating (blood) melatonin levels will also be measured in all three groups to verify these predictions.

Innervation of the thymus is modest (Litvinenko et al. 2005) and since pineal-derived melatonin primarily targets circulating components of immunity, neuronal stimulation, probably from the SCN, may be involved in melatonin production at the thymus. However, this notion may be invalidated if pineal-derived melatonin is primarily responsible for driving melatonin biosynthesis at the thymus. Consequently, if melatonin production occurs in the thymus of pinealectomized hamsters under SP conditions, then circadian-driven peripheral melatonin production at the thymus will be supported although the actual transmission of such information will remain a matter of conjecture.

Phase 2. Assay of neural-immune bi-directional interactions via melatonin.

Since the receptors for melatonin are abundantly expressed in many tissues of a vertebrate organism, melatonin has the potential to be a very effective and central hormonal signal of seasonal changes. The ability of melatonin to easily cross the blood-brain barrier suggests that this hormone can effectively reach centers of control in the brain, such as the SCN, and sustain a dialogue between the brain and the peripheral tissues (Litvinenko et al. 2005). The SCN is a principal biological clock that controls circadian rhythms and attenuates detrimental behaviors in the face of infection (Freeman et al. 2007). The ability of thymocytes to carry out melatonin biosynthesis (Liu et al. 2007) presents the possibility of communication with brain centers such as the SCN in the absence of pineal-derived melatonin. Although it is the pineal gland that mainly delivers melatonin to the SCN (Freeman et al. 2007) the documented presence of a high

density of melatonin receptors makes this area of the brain attractive for detecting any melatonin produced in the periphery for neural-immune discourse. The pinealectomized, pineal-intact, and pinealectomized/thymectomized cohorts from the previous phase would still be of use since the SCN would have remained intact, thereby limiting the number of animals that must be sacrificed. To recapitulate, all three cohorts will be exposed to identical SP conditions and after a suitable period the animals would be sacrificed, their brains dissected, and the SCN areas harvested so that melatonin can also be detected by ELISA (Freeman et al. 2007). The same control also applies as the removal of both the pineal and thymus glands will allow us to discern the influence of tissue-derived melatonin (not from PG or thymus) on the SCN, which is assumed to be negligible.

If endogenous production of melatonin from the thymus is insufficient for detection at the SCN then an alternative/supplementary experiment is to challenge the animals with a viral respiratory infection using the Sendai virus. A new set of pinealectomized and pinealectomized/thymectomized (control) animals are used. The purpose of a viral infection is to elicit the inflammatory response and subsequent adaptive immune response. Subjects that are noticeably affected by the viral challenge will be selected for the study. In pinealectomized animals, the thymus must once again compensate for melatonin production as circulating lymphocytes detect the infection and mount an appropriate response. Central to this immune response is the secretion of the various cytokines that not only promote inflammation but also induce thymocytes to produce melatonin (Naranjo et al. 2007). In the face of infection, it is quite possible that the immune system is able to communicate with the brain not only through cytokines but also by inducing the production of melatonin at the thymus. Examination of the SCN will confirm if melatonin production incited by the infection is sufficient for detection. If the presence of

melatonin is clearly established in the SCN of these animals, then we can presume that melatonin-producing organs like the thymus are involved not only in receiving immunomodulatory signals from the brain but can, in turn, convey neuromodulatory signals from the immune system. Thus, an avenue of interactions that has been poorly explored will now be better illuminated for future research.

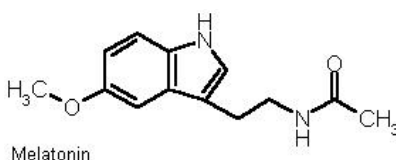
The remarkable suite of interactions that this study proposes to explore will reveal that the thymus is at the very interface of the central and peripheral oscillators that prepare an organism for survival during harsh conditions. This is quite impressive considering that the thymus has been previously deemed rather obsolete after T-cell maturation. Enhancing the immune system is clearly vital in the face of prolonged pathogenic threats during certain seasons. The newly gained understanding of thymus action through melatonin signaling will provide a wider-ranging view of neuroimmunomodulation and further reveal the incredible survival strategies of vertebrate life.

Your friend and mine, the Siberian Hamster:



References

1. Freeman DA, Kampf-Lassin A, Galang J, Wen JC & Prendergast BJ (2007). Melatonin acts at the suprachiasmatic nucleus to attenuate behavioral symptoms of infection. *Behav Neurosci* 121 (4), 689-697.
2. Guerrero JM & Reiter RJ. Melatonin-Immune System Relationships (2002). *Curr Top Med Chem* 2 (2), 167-79.
3. Hardeland R (2008). Melatonin, hormone of darkness and more- occurrence, control mechanisms, actions and bioactive metabolites. *Cell Mol Life Sci Online First Publication* 17 March.
4. Litvinenko GI, Shurlygina AV, Verbitskaya LV, Panteleeva NG, Melnikova EV & Trufakin VA (2005). Circadian dynamics of cell composition of the thymus and lymph nodes in mice normally, under conditions of permanent illumination, and after melatonin injection. *Bull Exp Biol Med* 140 (2), 213-6.
5. Liu S, Cai Y, Sother RB, Guan Y & Chan P (2007). Chronobiological analysis of circadian patterns in transcription of seven key clock genes in six peripheral tissues in mice. *Chronobiol Int* 24(5), 793-820.
6. Martin LB, Zachary WM & Nelson RJ (2008). Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. *Philos Trans R Soc Lond B Biol Sci* 363 (1490), 321-39.
7. Mjewski P, Adamska I, Pawlak J, Baranska A & Skwarlo-Sonta K (2005). Seasonality of pineal gland activity and immune functions in chickens. *J Pineal Res* 39 (1), 66-72.
8. Naranjo MC, Guerrero JM, Rubio A, Lardone PJ, Carrillo-Vico A, Carrascosa-Salmoral MP, Jiménez-Jorge S, Arellano MV, Leal-Noval SR, Leaf M, Lissen E & Molinero P (2007). Melatonin biosynthesis in the thymus of humans and rats. *Cell Mol Life Sci* 64 (6), 781-90.
9. Skwarlo-Sonta K, Majewski P, Markowska M, Oblap R & Olszanska B (2003). Bidirectional communication between the pineal gland and the immune system. *Can J Physiol Pharmacol* 81 (4), 342-9.
10. Wronka M, Maleszewska M, Stepinska U & Markowska M (2008). Diurnal differences in melatonin effect on intracellular Ca(2+) concentration in chicken spleen leukocytes in vitro. *J Pineal Res* 44 (2), 134-40.



Budget Justification

The finances requested to fund this study will cover accommodations and equipment related to animal subject housing and testing conditions, surgical procedures, immunodetection procedures, and the training and employment of at least two undergraduate assistants. Cost estimates are provided for each component. All resources and research space to be utilized will be located in the Medical Research Building of the Arizona Health Sciences Center at the University of Arizona, Tucson, Arizona.

Animal Subject Housing and Testing Conditions:

Siberian hamsters (*Phodopus sungorus*)

Approximately 50 subjects of both sexes of healthy appearance and 1-3 months of age are needed to conduct the proposed experiments. Sex of the animals is inconsequential as the reproductive effects of a changing photoperiod are not of interest. Sex differences are expected to be negligible and were not of concern in the literature supporting this study. At least 50 test subjects are required for all phases of experimentation and the collection of sufficient data for a valid statistical analysis of the results. Approximate cost- \$ 500

Test subject housing and testing accommodations

Test subjects are kept in individual cages to prevent excessive breeding and fed a balanced diet. Cages are designed with proper ventilation and space for a stress-free hamster environment. Testing conditions will include lighting conditions similar to the hamster's natural environment and with a timer device that allows for adjustment of "day length." Approximate cost- \$ 3,000

Viral challenge

Sufficient aliquots of Sendai virus (murine parainfluenza virus type 1) for respiratory pathogenic challenge; will be applied using an intranasal spray. \$ 5,000

Surgical Procedures:

Surgical equipment and space

Provision of appropriate surgical tools for organ removal, sufficient space for test subject preparation for surgery, and sufficient space for surgical preparation for the investigator is necessary. Appropriate anesthetics to subdue test subject for surgery and drugs for euthanizing animals upon completion of procedure are also needed. Blood collection equipment is included.

Approximate cost- \$ 31,500

Immunodetection Procedures:

ELISA reagents and equipment

A commercial ELISA kit suitable for detecting melatonin must be purchased along with all necessary plastic-ware needed to conduct the assays. Approximate cost- \$ 2,000

Undergraduate Assistants:

Training and employment

In order to conduct the experiments in an expedient and coordinated manner I will need at least two undergrad assistants to help care for the animals, assist in surgery, and conduct the ELISA assays. These assistants will be paid a stipend upon committing for at least 10 months or two semesters for this project. Institutional Animal Care and Use Committee (IACUC) training will be provided by the University of Arizona. This experience will help train prospective scientists or physician-scientists. Approximate cost- \$ 8,000

Total cost- \$ 50,000

Estimated project duration (including training and research space setup) – 18-20 months

