Summary of the proposed project

Mistiming critical seasonal behaviors carries serious consequences for individual survival. Animals minimize the risk of mistiming by integrating extrinsic environmental and social cues with intrinsic physiology and behavior. However, the mechanisms by which they do so are poorly understood. Environmental cues have been shown to modulate the hormone melatonin in vertebrates[1, 2]. Similarly, the arginine vasotocin (AVT)/arginine vasopressin (AVP) neuropeptide system regulates a variety of reproductive and social behaviors [3-5]. Importantly, recent evidence suggests that melatonin modulates the AVT/AVP neuropeptide system [3, 6, 7]. However, no studies have examined whether the effects of melatonin on AVT are mediated directly via a receptor-mediated mechanism within AVT neurons, or indirectly via an as yet unidentified intermediate factor. Moreover, whether or not melatonin has a functional role in regulating male sociosexual behavior is poorly understood. Using the green treefrog (Hyla cinerea) as a model, I will investigate whether melatonin and AVT mediate the integration of environmental and social signals with seasonal reproductive physiology and behavior. Specifically, I propose to use double-label immunohistochemistry to determine whether melatonin acts directly on AVT cells, and a field study to observe the effects of exogenous melatonin and AVT on male calling behavior. My research will test the novel hypothesis that AVT neurons integrate melatonin signals to regulate seasonal sexual behavior. This project will provide insight into how animals synchronize physiology and behavior with environmental and social information. These data will significantly advance our understanding of the neuroendocrine pathways that mediate complex seasonal behaviors, and the results will be applicable to all vertebrates.

Introduction

Animals must be able to accurately tell time in order to synchronize seasonal reproductive behavior with the correct social and environmental context. An inability to do so could mean missed mating opportunities, parturition during a resource-poor season, or other deleterious consequences. Animals minimize these risks by integrating extrinsic environmental and social cues with intrinsic physiology and behavior to time critical seasonal activities. However, the mechanisms by which they do so are poorly understood. While we have a fair understanding of how seasonal environmental cues are transduced into endocrine signals [2], as well as which neurohormones are important in regulating sociosexual behavior [4, 5], we know very little about how these neuroendocrine mechanisms interact to orchestrate seasonal reproduction.

In vertebrates, the production of melatonin, an indoleamine hormone, is controlled by environmental cues such as temperature and photoperiod. Because melatonin levels vary with environmental cues, melatonin provides an "endocrine clock" to all cells equipped with melatonin receptors [1, 2]. Thus, melatonin has been implicated in regulating many physiological and behavioral processes, particularly seasonal reproduction [1, 2]. The neurohormone AVT (and its mammalian homologue AVP) is a potent regulator of social and sexual behavior [4]. Seasonal and sex differences in the AVT/AVP neuropeptide system have been well documented in many vertebrate groups [4]. The neuroendocrine mechanisms regulating these seasonal differences, however, remain enigmatic. Recent preliminary evidence indicates that melatonin modulates the production and/or release of AVT in the brain [7]. I hypothesize that melatonin and AVT mediate the integration of environmental and social signals with seasonal reproductive physiology and behavior. I will test this hypothesis using green treefrogs (Hyla cinerea), a well-studied and robust model of seasonal sociosexual behavior.

Green treefrogs (Hyla cinerea) are an excellent model organism for investigating the neuroendocrine control of seasonal sexual behavior due to their robust seasonal breeding behavior [8]. Like most anurans, male reproductive success depends upon producing advertisement calls in the appropriate social and seasonal contexts [9]. During the breeding
season (approximately April to August), males gather in lek-type aggregations and call to attract conspecific females. AVT regulates the production of these advertisement calls, and recent evidence suggests that melatonin modulates AVT in _H. cinerea_. Interestingly, these data also indicate that melatonin’s effect on the number of AVT immunoreactive neurons in the brain is sexually dimorphic: only males exhibit a change in AVT cell number in response to melatonin treatment.

Lutterschmidt and Wilczynski hypothesized that melatonin modulates the number of AVT neurons in the brain via an inhibition of AVT release. Several lines of evidence support this hypothesis. For example, in male _H. cinerea_, AVT cell number increases as day length decreases toward the end of the breeding season, presumably due to diminished AVT release. Additionally, melatonin has been shown to decrease AVP release from cultured slices of suprachiasmatic nucleus in rats (_Rattus rattus_). Although this evidence is compelling, no studies have examined whether the effects of melatonin on AVT are mediated directly via a receptor-mediated mechanism within AVT neurons, or indirectly through an as yet unidentified intermediate factor. Moreover, whether or not melatonin plays a functional role in regulating male sociosexual behavior is poorly understood. Any funding awarded would be applied to field research and collection expenses associated with the questions posed below. This project will address both the neuroendocrine mechanisms by which seasonal and social cues are integrated in the brain, and their impacts on seasonal reproductive behavior. This will advance our understanding of the link between neuroendocrine mechanisms and complex seasonal behaviors in vertebrates.

**Question to be addressed**

_Do melatonin and AVT mediate the integration of environmental and social signals with reproductive physiology and behavior?_ I will test for co-localization of melatonin receptors within AVT neurons using double-label immunohistochemistry. In the field, I will manipulate melatonin and AVT levels during the mating season and assess their effects on male calling. These experiments will allow me to test the hypothesis that melatonin and AVT interact to integrate environmental and social cues, and that this integration has direct consequences on seasonal breeding behavior.

**Proposed Research**

For each of the aims outlined below, adult green treefrogs will be collected from natural breeding populations at established field sites within the Sam Houston State University Biological Field Station in Huntsville, Texas. For question 1, 8 males and 8 females will be euthanized and their brains rapidly dissected and fixed for immunohistochemistry. All tissues will be sectioned on a cryostat into 4 alternate series, mounted onto subbed slides, and stored at -20°C until assay. For field behavior studies (question 2), all animals will be released at the site of capture at the conclusion of the experiment.

1. **Does melatonin act directly on the AVT neuropeptide system?**

   I will use double-label immunohistochemistry to determine if melatonin receptors are co-localized within AVT neurons. Immunohistochemistry procedures for AVT will be similar to Lutterschmidt and Wilczynski [7]and O’Bryant and Wilczynski [3]. As preliminary data for these experiments, we have developed an assay for melatonin receptor subtype 1a using a commercially available antibody (Fig. 1; goat anti-melatonin receptor from Santa Cruz, sc-13179). Similar assays will be developed for melatonin receptor subtypes 1b and 1c [10]. The proportion of AVT-immunoreactive neurons co-expressing melatonin receptor will be quantified using fluorescence microscopy and compared via multivariate ANOVA with brain region and sex as factors.

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**Fig. 1.** Photomicrograph showing cell bodies (arrowheads) immunoreactive for melatonin receptor type 1a in green treefrogs.
2. Do melatonin and AVT interact to regulate male calling behavior?

To determine if melatonin interacts with AVT to regulate reproductive behavior, I will record calling behavior of males after treatment with exogenous melatonin and/or AVT (Fig. 2). Methods and hormone doses used will be similar to [11] and [12]). Briefly, I will locate and record the calling behavior of each male frog for a period of 15 minutes using a Sennheiser ME67 long shotgun microphone and a Marantz PMD-661 recorder. Care will be taken to not disturb the frogs while this baseline level of calling is recorded. I will then capture and inject each frog according to one of the pharmacological manipulations outlined in Figure 2. Note that we selected a single dose of 20 μg AVT because previous studies in this species have demonstrated that this dose produces a measurable change in calling behavior[11]. After allowing the frog to re-acclimate for 15 minutes, I will record its post-treatment calling behavior for 30 minutes. In order to account for potential effects of melatonin on locomotor behavior, movements of each frog will be monitored and recorded during the 15-minute re-acclimation period and the 30-minute behavior period. Previous studies in this species have demonstrated that male frogs resume calling soon after capture and injection and tend to move only centimeters away from the point of capture before resuming calling behavior[11]. Data will be quantified using Adobe Audition software and analyzed by ANOVA. The proposed timeline for these experiments appears below:

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**Predicted Outcomes:**

Because the effects of melatonin on AVT cell number were sexually dimorphic[7], I predict that melatonin receptors will co-localize with AVT in males, but not females. If co-localization is not observed, then it would suggest that melatonin indirectly modulates AVT cell populations in the brain. Importantly, this would not preclude the possibility that melatonin regulates male reproductive behavior, as will be tested in Question 2. I predict that treatment of male frogs with exogenous melatonin will decrease calling in a dose-dependent manner. If melatonin acts by decreasing AVT release, as previously hypothesized[7], then simultaneous treatment with melatonin and AVT should rescue calling behavior.

**Importance of Proposed Research**

My research will test the novel hypothesis that AVT neurons integrate environmental cues with seasonal sociosexual behavior via melatonin signaling. Using *H. cinerea* as a model, this project will provide valuable insight into how animals synchronize physiology and behavior with environmental and social information. These data will significantly advance our understanding of the neuroendocrine pathways that mediate complex seasonal behaviors, and the results will be applicable to all vertebrates.
References Cited:


