**The contribution of the pineal gland on daily rhythms and masking in diurnal grass rats,
Arvicanthis niloticus.**

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ABSTRACT

Melatonin is a hormone rhythmically secreted at night by the pineal gland in vertebrates. In diurnal mammals, melatonin is present during the inactive phase of the rest/activity cycle, and in primates it directly facilitates sleep and decreases body temperature. However, the role of the pineal gland for the promotion of sleep at night has not yet been studied in non-primate diurnal mammalian species. Here, the authors directly examined the hypothesis that the pineal gland contributes to diurnality in Nile grass rats by decreasing activity and increasing sleep at night, and that this could occur via effects on circadian mechanisms or masking, or both. Removing the pineal gland had no effect on the hourly distribution of activity across a 12:12 light-dark(LD) cycle or on the patterns of sleep-like behavior at night. Masking effects of light at night on activity were also not significantly different in pinealectomized and control grass rats, as 1 hr pulses of light stimulated increases in activity of sham and pinealectomized animals to a similar extent. In addition, the circadian regulation of activity was unaffected by the surgical condition of the animals. Our results suggest that the pineal gland does not contribute to diurnality in the grass rat, thus highlighting the complexity of temporal niche transitions. The current data raise interesting questions about how and why genetic and neural mechanisms linking melatonin to sleep regulatory systems might vary among mammals that reached a diurnal niche via parallel and independent pathways.

Keywords: Circadian Rhythm; Masking; Melatonin; Pineal; Sleep

1. Introduction

Adaptations, such as the ability to occupy temporal niches, allow a species to better survive within an environment. For example, animals that are active during the day (i.e., diurnal),

have developed more sophisticated visual systems than those that operate at night (i.e., nocturnal) (Hall et al., 2012; Tan et al., 2005). Diurnal and nocturnal animals represent the two extremes of the temporal niche spectrum. Studying these two niches provides the opportunity to understand how species alter their activity patterns when they must switch from one temporal niche to another, and what impact these changes have on deeply engrained physiological processes such as circadian rhythms. The changes in the circadian system needed to facilitate a temporal switch from nocturnal to diurnal patterns are believed to occur downstream of the central pacemaker, called the suprachiasmatic nucleus (SCN) (Smale et al., 2003). However, the neural circuitry responsible for this switch has not yet been concretely established. One possibility for the downstream action of the clock is via a variation in hormone sensitivity. In particular, melatonin is a hormone that appears to play a distinctly different role between nocturnal and diurnal species.

Melatonin is rhythmically produced by the pineal gland and is elevated in both nocturnal and diurnal species during the dark phase of the daily light:dark (LD) cycle and during the subjective night when animals are maintained in constant darkness (DD; Reiter, 1993). Melatonin is believed to play a very different role with respect to activity in diurnal and nocturnal species. In nocturnal owls and tench (*Tinca tinca*), activity is not altered by exposure to melatonin, whereas in diurnal sparrows, quail and goldfish, activity is decreased following melatonin exposure (Lopez-Olmeda et al., 2006; Murakami et al., 2001). It is important to note here that these nocturnal birds and fish evolved from a diurnal lineage; therefore, animals that adopted a nocturnal behavioral pattern exhibited a loss of function with respect to melatonin on activity (Gerkema et al., 2013; Hunt et al., 2009). Mammals, however, have had a very different evolutionary past. Mammals experienced a “nocturnal bottle neck” during their evolutionary

history, in which they went through major adaptive changes to avoid competition with other diurnal reptiles (Gerkema et al., 2013). However, this view has been recently challenged with the recent finding that some dinosaurs were nocturnal as well (Schmitz & Motani, 2011).

Nonetheless, diurnal mammalian species have switched from nocturnal to diurnal behavioral patterns under many different parallel pathways.

The sleep-promoting effects of exogenously administered melatonin have been extensively studied in humans (e.g. Dollins et al., 1993, 1994; Nave et al., 1996; Zhdanova et al., 1995, 1996). When melatonin is administered at times of day during which it is not already elevated via endogenous mechanisms, melatonin has a sleep-promoting effect, suggesting that its endogenous role is to inhibit wakefulness-generating mechanisms during the night (Lavie, 1997). These findings were also replicated in several species of non-human primates (Hao and Rivkees, 2000; Hughes and Badia, 1997; Inui and Hazeki, 2010; Zhdanova, 2005; Zhdanova et al., 2002). Further data on the role of the pineal gland in the regulation of daily sleep patterns in humans has come from studies of individuals who experience injuries that interfere with signals to the pineal gland (Biering-Sorensen and Biering-Sorensen, 2001; Scheer et al., 2006) or have the organ surgically removed (Kocher et al., 2006; Petterborg et al., 1991). These studies revealed that such individuals have an increase in sleep dysfunction, though it is impossible to rule out the possibility that other aspects of their conditions are the source of the problem. However, in some such cases, administration of melatonin improved sleep-related symptoms (Etzioni et al., 1996; Jan et al., 2001; Lehmann et al., 1996). Therefore, one could hypothesize that melatonin is integral in promoting diurnal behavior in animals. This, however, would be a gross generalization, as little is known about direct effects that the pineal gland may have on sleep and activity in diurnal mammals other than primates.

Another way in which the pineal gland could promote sleep in diurnal species is via an influence on mechanisms mediating the masking response of animals to light. Masking refers to a process whereby light directly affects behavior, such as the induction of sleep in nocturnal species and enhanced wakefulness in diurnal ones. Two studies have revealed heightened masking responses to light in pinealectomized vs. control nocturnal rats (Quay, 1970; Vilaplana et al., 1994). Comparable studies with diurnal species have not been reported. However, treatment with melatonin has been found to alter the masking of body temperature and sleep by light at night in humans. Young men given an infusion of melatonin and then exposed to bright lights showed a reduction in the light-induced increase of body temperature (BT; Strassman et al., 1991) and had shortened latencies to sleep onset (Burgess et al., 2001) when compared to controls. Melatonin may, therefore, work through multiple avenues to promote diurnal behavior.

There are many examples of laboratory rodent models of diurnality (Challet et al., 2002; Garcia-Alleque et al., 1999; Hut et al., 1999; Katona et al., 1998; Schumann et al., 2005; Weinert et al., 2007). However, the influence of melatonin on the expression of sleep or masking to light in these models has not yet been examined. Additionally, only one group has examined the influence of pinealectomy on the circadian system of a diurnal rodent (Martinet and Zucker, 1985). They found no difference between pinealectomized and control groups, but only a few circadian characteristics were measured: phase angle of entrainment in 10:14LD, rate of reentrainment for a 6h phase advance, and tau in constant light. In the present study, we examined the effects of pinealectomy on several factors that impact the expression of diurnal activity patterns in Nile grass rats (*Arvicanthis niloticus*), including circadian rhythms of activity, masking to light, and sleep. The present work tests the hypothesis that the pineal gland

contributes to diurnality in Nile grass rats by decreasing activity and increasing sleep at night, and that this could occur via effects on circadian mechanisms or masking, or both.

2. Methods

2.1 General Procedures

Eighteen adult female *Arvicanthis niloticus* (grass rats) from the breeding colony at Michigan State University were singly housed in Plexiglas cages (34x28x17 cm) for the duration of these experiments. The facilities were maintained at a temperature ranging between 20-25°C with a 12:12 light:dark (LD) schedule. Animals were given food (PMI Nutrition Prolab RMH 2000, Brentwood, MO) and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University and were treated in accordance with the guidelines of the National Institutes of Health on the care and use of animals.

Animals were randomly assigned to either the pinealectomy (n=10, Pinx) or control (n=8, Sham) group. All animals were anesthetized using isoflurane (2-5%). The surface of the head was then shaved and sanitized with betadine. Animals were secured in a stereotaxic apparatus (Stoelting Co.; Wood Dale, IL) and then injected subcutaneously (s.c.) with Buprenex (0.05mg/kg) and lidocaine (6mg/kg) prior to the surgery. A 2 cm incision was made on the scalp to expose the surface of the skull. Above Lambda, a circular hole was made with a 5 mm trephine and, using fine forceps, the pineal gland was quickly extracted. Two researchers examined the extracted gland to confirm that it had the distinct texture and shape of the pineal; samples were also stored in PBS and later examined by D.D.S. under a dissecting scope to further confirm that the pineal gland had been removed. The hole in the skull was packed with

gel foam and the incision was closed with autoclips. At the conclusion of the surgery, animals were injected with sterile saline (2 cc, 0.9% NaCl) and Ketoprofen (5mg/kg of body weight). Animals then received the analgesic Meloxicam (0.1 mg/kg of body weight) orally in apple every 24 hrs for the next 2 days. Sham animals followed the same surgical protocol without the removal of the pineal gland. After a recovery period of at least two weeks, animals were exposed to a series of different lighting conditions during which activity was recorded via infrared motion detectors (IRs, Visonic Tel Aviv, Israel) that sent signals to a computer in an adjacent room equipped with the VitalView Program (Minimitter, Bend, OR, USA).

2.2 Rhythms

Grass rats were monitored in a 12:12 LD cycle for 1 week, then placed into constant darkness (DD) for 2 weeks and then transferred into constant light (LL) for 2 weeks. Animals were then placed in a 12:12 LD cycle until all animals were entrained, at which point a masking protocol was applied (see below). Finally, the LD cycle was phase shifted to determine the rate of reentrainment after a 6 hr phase delay followed by a 6 hr phase advance.

Actograms were produced via ClockLab (Actimetrics, Wilmette, IL, USA) which also automatically determined onset and offset of activity and calculated the period of the rhythm, tau (τ). The experimenter visually inspected these points prior to analysis, as the program occasionally improperly designated onset/offset; in such cases errors were corrected manually (as in Schrader et al, 2009). Based upon the onsets and offsets, we also calculated the duration of the active period of the daily/circadian rhythms, alpha (α). Using Microsoft Excel we examined the raw activity data and determined the total activity per cycle, based on the circadian period in DD, LL, and LD. Additionally, for 5 days in LD, we calculated the activity profile in 1 hr-bins

across 24 hrs by totaling the activity during the one hour period and dividing by the total activity during the day. Finally, two individuals (DDS & AAA) independently examined the actograms to determine when rhythms had reentrained after phase shifts of the LD cycle; this point was defined as the first day of a period of at least three days during which the time of activity onsets were consistent (e.g., Jechura et al., 2006). During these analyses and corrections, experimenters were blind to the experimental groups of the individual animals.

Effects of lighting and surgical condition on alpha and total activity were assessed with a mixed 2-way ANOVA in which lighting condition (DD, LL, and LD) was treated as a within-subjects variable and surgical condition (Pinx and Sham) as a between-subjects variable. To assess effects of lighting and surgical condition on tau we also performed a mixed 2-way ANOVA; in this case there were two lighting conditions (DD and LL, as the within subjects variable), and surgical condition (Pinx and Sham) was again the between subjects variable. For the ANOVAs, Eta-squared values (η^2) were calculated to determine effect size. Finally, we used independent-sample t-tests to determine the differences between Pinx and Sham animals in their days to reentrain and Cohen's d to determine effect size, following both phase delays and phase advances of the LD cycle.

2.3 Masking

To determine if the pineal gland plays a role in masking, animals maintained on a 12:12 LD cycle were cycled through a three-day protocol, so that every third day the animals were exposed to a 1 hr light pulse (LP) during the dark phase or a one hour dark pulse (DP) during the light phase (Shuboni et al., 2012). DPs were administered first in a random order at ZT2, 10, and 6, and LPs were administered second also in a random order at ZT14, 22, and 18. Microsoft Excel

was used to sum the total amount of activity during the 1 hr of each of the 6 pulses, and the same 1 h on the preceding day. For dark and light pulses we used a three-way ANOVA to assess effects of the time of the pulse (3 times), lighting (dark/light pulse and control) and the surgical conditions (Pinx and Sham). Effect size was reported as Eta-squared values (η^2).

2.4 Sleep

Here, we examined the patterning of sleep across the dark phase of a 12:12 LD cycle to test the hypothesis that pineal melatonin contributes to the consolidation of sleep at night in diurnal grass rats. Two low-light lens CCTV cameras connected to a time-lapse video recorder that condensed a 12 hr period onto a 2 hr tape were used to record behavior across the dark phase of the LD cycle. The tapes were transferred to DVD media that could be seen on a computer screen and the behavior was scored with a custom-made data acquisition program (S & K Computer Products, Toronto, Ontario, Canada; Lonstein and Stern, 1997). “Sleep-like behavior” (referred to here as “sleep”) was scored when the animal adopted a distinct posture, lying down in a slumped position with the head on the floor of the cage (Figure 5A). An observer (T.K.H.G.) blind to the condition of the animal recorded onsets and offsets of each sleep bout across the night. From these data we calculated the total amount of sleep, the number of sleep bouts, and the duration of those bouts during the 12 hr recording period. For analysis of the total sleep bout number and average bout duration, we used independent sample t-tests to compare Pinx and Sham groups and Cohen’s d values to report effect size. To analyze the effect of surgery on the amount of sleep per hour across the 12 hr period of darkness, we used a mixed two-way ANOVA with time as the within-subjects variable and surgical condition (Pinx and Sham) as the between subjects variable and reported the Eta-squared (η^2) to demonstrate effect size.

3. Results

3.1 Rhythms

In LD conditions (Figure 1), there was a main effect of time on hourly rates of activity ($F[23, 345] = 17.95, p < 0.001, \eta^2 = 0.54$), but there was no significant effect of surgical condition ($F[1, 15] = 0.19, p = 0.666, \eta^2 = 0.01$), and there was no interaction between these two variables ($F[23, 345] = 2.07, p = 0.085, \eta^2 = 0.12$). In both groups of animals, the highest levels of activity occurred during the 12 hr light period and at the transitions between light and dark phases of the LD cycle (Figure 1).

Animals were placed in constant conditions (i.e., DD and LL; see Figure 2A for representative actograms); when comparing the period of activity in the three lighting conditions, alpha was affected by lighting condition ($F[2, 26] = 30.32, p < 0.001, \eta^2 = 0.70$) but not by surgery ($F[1, 13] = 0.00, p = 0.993, \eta^2 < 0.01$) or by an interaction between lighting and surgery ($F[2, 26] = 0.38, p = 0.636, \eta^2 = 0.03$). Alpha was significantly longer in LL than both LD and DD (Figure 2B). Tau did not differ in Pinx and Sham groups ($F[1, 14] = 1.81, p = 0.200, \eta^2 = 0.01$), and there was no interaction between lighting condition and surgery ($F[1, 14] = 0.11, p = 0.740, \eta^2 = 0.01$). There was a main effect of lighting condition ($F[1, 14] = 90.79, p < 0.001, \eta^2 = 0.87$) such that the period of the rhythm was significantly longer in LL than in DD (Figure 2C).

Animals were then phase shifted, both via a 6hr phase advance and a 6hr phase delay; see Figure 3A and 3B for representative actograms. The rate of reentrainment from the phase delay was significantly faster than phase advances (Figure 3C; $F[1, 11] = 5.54, p = 0.038, \eta^2 = 0.34$), but, again there was no effect of surgery ($F[1, 11] = 0.19, p = 0.673, \eta^2 = 0.02$) nor was there an interaction ($F[1, 11] = 0.01, p = 0.927, \eta^2 = 0.01$).

3.2 Masking

The masking responses to light did not differ significantly as a function of surgical condition (i.e., there was no main effect of surgery); this was the case for both dark pulses ($F[1, 15] = 0.66, p = 0.43, \eta^2 = 0.04$) and light pulses ($F[1, 15] = 0.71, p = 0.41, \eta^2 = 0.05$). Activity significantly increased in response to light (i.e., there was a significant main effect of light) at all three times sampled during the dark period (Figure 4) among both Pinx and Sham grass rats ($F[1, 15] = 61.25, p < 0.00, \eta^2 = 0.80$); there was no significant effect of dark pulses during the day ($F[1, 15] = 0.12, p = 0.73, \eta^2 < 0.01$). There was a significant main effect of time at night ($F[2, 30] = 14.99, p < 0.01, \eta^2 = 0.50$) but not during the day ($F[2, 30] = 0.61, p = 0.55, \eta^2 = 0.04$). There were no significant interactions for any pair of conditions for dark pulses (data not shown) and only a significant interaction between time and lighting for the light pulses ($F[2, 30] = 5.05, p = 0.01, \eta^2 = 0.25$). The duration of the active phase in different lighting periods can also be an indicator of masking, and this, too, was unaffected by removal of the pineal gland (Figure 2B).

3.3 Sleep

Representative photographs of an awake animal and an asleep animal are provided in Figure 5A. Sleep bout numbers (Figure 5B) and durations (Figure 5C) did not differ in Pinx and Sham groups ($t[12] = 1.42, p = 0.182, d = 0.75$ and $t[12] = 3.5, p = 0.006, d = 0.15$ respectively). Hourly rates of sleep across the dark phase of the LD cycle (Figure 5D) were affected by time ($F[11, 132] = 16.36, p < 0.001, \eta^2 = 0.58$), but not by surgical condition ($F[1, 12] = 0.04, p = 0.845, \eta^2 < 0.01$), nor were they affected by an interaction between these two variables ($F[11,$

132] = 0.59, $p = 0.713$, $\eta^2 = 0.05$).

4. Discussion

Our results suggest that melatonin does not contribute to diurnality in grass rats, as it does in some primates and non-mammalian vertebrates. This conclusion stems from data on activity rhythms, masking, and analysis of the distribution of sleep across the night in animals in which the pineal gland has been removed.

4.1 Rhythms

Temporal patterns of general activity in a 12:12 LD cycle did not differ between pinealectomized and control groups. This was the case for the overall amount of activity during the light vs. dark phases of the cycle, as well as the pattern of changes in hourly activity across the 24 hr LD cycle (Figure 1). We also found no evidence that the pineal gland plays a role in modulation of the endogenous circadian timekeeping system, as revealed by placing animals in constant conditions (Figure 2A) and during phase shifts of the LD cycle (Figure 3A). In some other mammals, melatonin influences tau and how tau responds to changes in lighting intensity, but in others it does not (Aguilar-Roblero and Vega-Gonzalez, 1993; Aschoff et al., 1982; Cassone, 1992; Cheung and McCormack, 1982; Morin, 1993; Morin and Cummings, 1981; Yanovski et al., 1990). In addition, administration of exogenous melatonin in LD conditions does not significantly impact the expression of circadian rhythms in *Octodon degus* (Vivanco et al., 2007). Based upon the removal of the pineal gland, our results suggest that melatonin does not influence circadian rhythms in grass rats (Figure 2C). In another diurnal rodent, the golden mantled ground squirrel, the responses of tau to changes in light intensity were similarly

unaffected by removal of the pineal gland (Martinet and Zucker, 1985). Rates of reentrainment in grass rats were also unaffected by Pinx (Figure 3C), which was also the case in ground squirrels (Martinet and Zucker, 1985), but rates of reentrainment are affected in some nocturnal rodents under certain conditions (Finkelstein et al., 1978; Quay, 1970, 1971). Taken together, data from ground squirrels, degus, and grass rats raise the possibility that the influence of light on the circadian clock of diurnal rodents is not modulated by melatonin, as it appears to be in some other rodents.

4.2 Masking

In intact diurnal mammals, light pulses at night directly suppress melatonin and stimulate an increase in activity (Redlin, 2001), raising the possibility that the light-induced decrease in melatonin could contribute to positive masking. If that were the case, then pinealectomy should decrease the masking responses to photic stimuli. However, this did not appear to be the case in grass rats, as 1 hr light pulses during the dark phase of a 12:12 LD cycle increased activity in both groups and the magnitude of the response did not differ between them (Figure 4). The stimulatory effect of light at night on activity is thus unlikely to be facilitated by a decrease in melatonin, providing further evidence that melatonin does not influence activity/rest state in these animals.

Data from grass rats maintained in constant conditions also suggest that masking was unaffected by pinealectomy. Specifically, the duration of the active period increased when conditions changed from DD to LL (Figure 2B), and the increase was identical in intact and Pinx animals. The effect of light intensity on alpha is likely to reflect masking, with light expanding

the active period and darkness reducing it in a diurnal mammal. These results are similar to what we have seen previously in intact grass rats (Gall et al., 2013).

4.3 Sleep

Finally, there was no indication that removal of the pineal gland affected sleep. Video analysis revealed no difference between pinealectomized animals and control animals in the overall temporal distribution of sleep across the dark phase or the duration or number of sleep bouts during that time (Figure 5). It should be noted that studies of sleep in pinealectomized nocturnal rodents have yielded contradictory results (Fisher and Sugden, 2010; Mendelson and Bergmann, 2001; Mouret et al., 1974; Wang et al., 2003). However, there is some evidence that humans may be different in this regard. There are data suggesting that humans without a functional pineal gland have sleep that is less consolidated and that extends into the day considerably more than it does in humans with normal patterns of melatonin secretion (Macchi and Bruce, 2004; Slawik et al., 2012). Though most of this data comes from case studies (Macchi and Bruce, 2004), it does suggest that the pineal contributes to the consolidation of human sleep at night, presumably through secretion of melatonin, which has clear soporific effects in other diurnal primates, birds and fish (Hao and Rivkees, 2000; Hughes and Badia, 1997; Inui and Hazeki, 2010; Lopez-Omeda et al., 2006; Murakami et al., 2001; Zhdanova et al., 2001, 2002, 2005). Although the pineal gland may contribute to diurnality in those animals, the current data suggest that this is not the case in grass rats.

4.4 Conclusions and Implications

As noted above, there are numerous reports of soporific effects of melatonin in diurnal vertebrates (for review, Tzischinsky et al., 2001) including human and non-human primates (Hao and Rivkees, 2000; Hughes and Badia, 1997; Inui and Hazeki, 2010; Matsumoto, 1999; Zhdanova et al., 2002, 2005; Zhdanova and Wurtman, 1997). There are no data that we are aware of addressing the question of whether the pineal gland might play a direct role in the regulation of activity or sleep in non-primate mammals that have independently evolved a diurnal pattern of adaptation to the day-night cycle. Katz (2011) and others have argued at a general level that when common behaviors evolve independently, evolution is likely to occur via similar changes in neural organization, because the potential mechanisms that could produce a given behavioral pattern are limited. In the current study, we aimed to examine whether this might be the case when it comes to potential changes in the role played by the pineal gland at two independent evolutionary transitions from nocturnality to diurnality by comparing previous reports of the role of the pineal gland in primates to our data within a murid clade. The current data suggest that the pineal gland does not play a role in rhythms, masking, or sleep in diurnal grass rats. However, one limitation of our study includes the lack of radioimmunoassay of melatonin for our pinealectomized animals. In addition, we did not administer supra-physiological levels of melatonin in this study. Further investigation into the distribution of melatonin receptors within diurnal grass rats and primates may provide insight into why melatonin has such a different role in the two species. Testing other diurnal species would also, allow for more complex comparisons to be made about the role of melatonin. Other basic mechanisms that promote sleep at night and activity during the day may be the same, but the role that the pineal gland plays in primates appears to be absent in diurnal grass rats.

355 **Conflict of Interest**

356 The authors declare that they have no conflict of interest.

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Figure 1

Figure 2

Figure 3

Figure 4

Light Pulses

Figure 5

A

B

Bout Number

C

Bout Duration

D

Results:

1)

2)

3)