

## NEUROSYSTEMS

# Daily rhythms and sex differences in vasoactive intestinal polypeptide, VIPR2 receptor and arginine vasopressin mRNA in the suprachiasmatic nucleus of a diurnal rodent, *Arvicanthis niloticus*

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## Abstract

Diurnal and nocturnal animals differ with respect to the time of day at which the ovulatory surge in luteinizing hormone occurs. In some species this is regulated by the suprachiasmatic nucleus (SCN), the primary circadian clock, via cells that contain vasoactive intestinal polypeptide (VIP) and vasopressin (AVP). Here, we evaluated the hypothesis that chronotype differences in the timing of the luteinizing hormone surge are associated with rhythms in expression of the genes that encode these neuropeptides. Diurnal grass rats (*Arvicanthis niloticus*) were housed in a 12/12-h light–dark cycle and killed at one of six times of day (Zeitgeber time 1, 5, 9, 13, 17, 21; ZT 0 = lights-on). *In-situ* hybridization was used to compare levels of *vip*, *avp* and VIP receptor mRNA (*vipr2*) in the SCN of intact females, ovariectomized females, ovariectomized females given estradiol and intact males. We found a sex difference in *vip* rhythms with a peak occurring at ZT 13 in males and ZT 5 in intact females. In all groups *avp* mRNA rhythms peaked during the day, from ZT 5 to ZT 9, and had a trough in the dark at ZT 21. There was a modest rhythm and sex difference in the pattern of *vipr2*. Most importantly, the patterns of each of these SCN rhythms relative to the light–dark cycle resembled those seen in nocturnal rodents. Chronotype differences in timing of neuroendocrine events associated with ovulation are thus likely to be generated downstream of the SCN.

## Introduction

In female rodents, mating and the preovulatory surge in luteinizing hormone (LH) occur several hours before the active period (Sodersten *et al.*, 1981; de la Iglesia & Schwartz, 2006; Kriegsfeld & Silver, 2006). In nocturnal rats and hamsters this is around the time of lights-off whereas in the diurnal rodent *Arvicanthis niloticus* (grass rat) this occurs around lights-on (McElhinny *et al.*, 1999; Mahoney & Smale, 2005). In some nocturnal rodents the circadian time-keeping system regulates these events (Alleva *et al.*, 1971) through the suprachiasmatic nucleus (SCN) (Weigand & Terasawa, 1982), the primary circadian pacemaker in mammals (Klein *et al.*, 1991).

The SCN appears to coordinate the timing of the LH surge at least in part via rhythmic release of vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP) (Perreau-Lenz *et al.*, 2004) at synapses with gonadotropin-releasing hormone (GnRH) neurons, and perhaps indirectly via projections to neurons containing kisspeptin

or gonadotropin-inhibitory peptide (Kriegsfeld & Silver, 2006; Gibson *et al.*, 2008). In rats and hamsters, GnRH neurons, which stimulate the release of LH, are contacted by VIP fibers (van der Beek *et al.*, 1997), and SCN lesions eliminate approximately 80% of these connections (van der Beek *et al.*, 1993). Disrupting VIP production delays and diminishes the amplitude of the LH surge (Harney *et al.*, 1996; van der Beek *et al.*, 1999), and rhythms in *vip* mRNA are quite different in females and males, as is the ability to produce a surge (Becu-Villalobos *et al.*, 1997; Krajnak *et al.*, 1998b).

AVP also appears to mediate the circadian influence on LH secretion (Perreau-Lenz *et al.*, 2004). AVP fibers contact GnRH neurons in the medial preoptic area of female rats and hamsters and in the supraoptic nucleus of female cynomolgus monkeys (Thind *et al.*, 1991; van der Beek *et al.*, 1998; Huhman & van der Beek, 1996). SCN lesions in laboratory rats reduce the number of these contacts (van der Beek *et al.*, 1998) and the proestrous LH surge can be blocked by an intracerebroventricular injection of an AVP receptor antagonist (Funabashi *et al.*, 1999).

The role of these neuropeptides in the circadian regulation of reproductive events has not been characterized in diurnal species.

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Here, we used grass rats as a model to evaluate the hypothesis that chronotype differences in the timing of the LH surge arise from differences in temporal patterns of production of *vip* and/or *avp* in the SCN by determining whether these patterns: (i) differ in female grass rats from those of female laboratory rats, (ii) differ in males, which are unable to produce a surge, and females which do, and (iii) are influenced by ovarian secretions essential for the surge to occur. In addition, we evaluated the possibility that *vip* might act differently within the SCN of these groups of animals by examining mRNA for one of its receptors.

## Materials and methods

### Animals

All experiments were approved by the Michigan State University Committee on Animal Use and Care in accordance with standards in the National Research Council *Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize the number of animals used in these experiments. Adult male and female *A. niloticus* (> 60 days) bred from a laboratory colony maintained at Michigan State University were singly housed, kept in a 12/12-h light–dark cycle and provided food (Teklad rodent chow 8640, Harlan Industries, Madison WI, USA) and water *ad libitum*. A red light (< 5 lux) was left on continuously in each animal room. We used four groups of animals: adult males, adult intact females, ovariectomized (OVX) females and ovariectomized females treated with estradiol benzoate capsules. Adult females were reproductively mature. No attempts were made to assess their reproductive state further as these animals, like many rodents, do not exhibit spontaneous estrous cycles. Vaginal smears and ovarian histology suggest that almost all females housed alone are in a continuously diestrous state (McElhinny, 1996). Brains from animals were collected at six time points: Zeitgeber time (ZT) 1, 5, 9, 13, 17 and 21. Sample sizes are given in Table 1.

Females were bilaterally ovariectomized while under isoflurane anesthesia. Incisions were closed with subcutaneous sutures and treated with Nolvasan (Fort Dodge Animal Health, Fort Dodge, IA, USA).

TABLE 1. Sample sizes analysed for each time point, group and mRNA transcript

	ZT					
	1	5	9	13	17	21
<b>VIP</b>						
Intact female	6	6	6	5	5	5
OVX	3	4	4	5	4	5
OVX+E	3	4	4	4	3	3
Male	5	4	4	5	5	4
<b>VIPR2</b>						
Intact female	6	6	6	5	5	5
OVX	3	4	4	5	4	5
OVX+E	3	5	4	4	3	3
Male	5	4	3	4	5	4
<b>AVP</b>						
Intact female	6	6	6	5	5	5
OVX	4	4	3	5	4	5
OVX+E	4	5	4	4	3	4
Male	5	4	4	2	5	4

ZT, Zeitgeber time. VIP, vasoactive intestinal polypeptide; VIPR2, VIP receptor 2; AVP, arginine vasopressin. OVX, ovariectomized; E, estradiol treatment.

Following ovariectomy animals were given saline (1 mL 0.9%, s.c.) and Buprenex (0.03 mg/kg i.m.; Reckitt & Coleman, Richmond, VA, USA) to minimize and control pain. Females recovered from surgery for a minimum of 10 days and were given Buprenex (0.03 mg/kg i.m.) to control pain during their recovery. For animals given estradiol capsules, the capsules were soaked in sterile saline overnight, and then soaked in sterile 100% ethanol for 2 h just prior to implantation. Females were given two s.c. silastic capsules containing estradiol benzoate (OVX+E; 180 µg/mL oil; 0.5-mm inner diameter, 2.15-mm outer diameter, 20 mm long, Sigma-Aldrich, St. Louis, MO, USA). Capsules were implanted at ZT 11 for OVX+E females that were killed at ZT 1, 5 and 9 (after exposure to estradiol for 38, 42 and 46 h, respectively) and at ZT 0 for estradiol-treated females killed at ZT 13, 17 and 21 (after exposure to estradiol for 37, 41 and 45 h, respectively).

Finally, the animals were injected with an overdose of pentobarbital (0.7 mL), decapitated, and their brains removed and frozen in an isopentane bath cooled with dry ice. Animals killed during the dark portion of the light–dark cycle were decapitated under red light. Brains were cut into four series (12 µm) on a freezing cryostat. Coronal sections were collected from the medial septum to just past the caudal SCN. One tissue series was processed for *in-situ* hybridization for the detection of *vip*, *vipr2* or *avp* mRNA, respectively.

### RNA probes

The probes for *vip* and *vipr2* were prepared by RT-PCR from RNA extracted from whole *A. niloticus* hypothalamus. For *vip* we used a forward primer (5'-ACCCGCCTTAGAAAGCAAAT-3') and reverse primer (5'-TCCTCAATTGCTACCCTTGC-3') to amplify a 303-bp fragment of *vip* products (Genbank accession no. FJ750350). For *vipr2* we used forward and reverse primers based on the *vipr2* gene sequence for the mouse (Genbank accession no. BC138572.1) to amplify a 536-bp fragment (Genbank accession no. FJ750351). Fragments were cloned into pCR II-TOPO vector (Invitrogen, Carlsbad, CA, USA) using the TOPO TA cloning system and following the manufacturer's protocol. The amplified fragment was then sequenced to verify its identity and orientation. For *vip*, the cDNA fragments corresponded to rat (96%), mouse (96%) and *Arvicantis ansorgei* (98%) published sequences. For *vipr2* the cDNA fragments corresponded to 95% in rat and to 96% in mouse. The 232-bp rat vasopressin probe was a kind gift from Drs S. Watson, A. Seasholz (University of Michigan, Ann Arbor, MI, USA) and S. Bhatnagar (University of Pennsylvania, Philadelphia PA, USA). This probe corresponds to Exon C of the rat vasopressin gene (Genbank accession no. X01637.1). S<sup>35</sup>-labeled antisense probes were prepared from these templates. Purified linearized plasmid was incubated at 37°C for 1.5 h in a transcription buffer that included either T7 or SP6 viral RNA polymerase, depending on orientation of inserts, and <sup>35</sup>S-labeled UTP and CTP (MP Biomedicals, Solon, OH, USA). The resulting probe was treated with DNase (Invitrogen) to prevent further polymerization. Free nucleotides were removed using Micro Bio-Spin Chromatography Columns (Bio-Rad Laboratories, Hercules, CA, USA). For controls, the same procedures were performed using a probe corresponding to the sense sequence of the respective genes.

### In-situ hybridization

At the time of the *in-situ* hybridization procedure, tissue from all animals (i.e. all time points, sexes and hormonal conditions) was processed identically and in parallel. Slides were thawed and immediately fixed in 4% paraformaldehyde (freshly prepared, in 0.1 M phosphate buffer pH 7.3, Sigma-Aldrich) for 1 h, washed extensively

in 2× saline-sodium citrate buffer, acetylated in 0.1 M triethanolamine/0.25% acetic anhydride (Invitrogen) for 10 min, and dehydrated through increasing concentrations of ethanol (50–100%). The  $^{35}$ S-labeled probe was diluted in hybridization buffer (50% formamide, 10% dextran sulfate, 3× saline-sodium citrate buffer, 50 mM sodium phosphate buffer, pH 7.4, 1× Denhardt's solution, 0.1 mg/mL yeast tRNA and 10 mM dithiothreitol; Invitrogen) to yield an approximate concentration of  $2.5 \times 10^6$  counts/80  $\mu$ L. Each slide was covered with 80  $\mu$ L diluted probe and then coverslipped. The slides were then placed in plastic trays lined with filter paper soaked with 50% formamide. The trays were sealed and incubated at 55°C overnight. Following hybridization, coverslips were floated off in 2× saline-sodium citrate buffer, and slides were rinsed an additional three times in 2× saline-sodium citrate buffer at room temperature. Slides were then incubated in RNase A (200  $\mu$ g/mL; Invitrogen) at 37°C (1 h). Slides were rinsed in decreasing concentrations of saline-sodium citrate buffer (2×, 1× and 0.5×) at room temperature, and washed in a final stringency of 0.1× saline-sodium citrate buffer at 65°C for 1 h, cooled to room temperature in dH<sub>2</sub>O and dehydrated through increasing concentrations of alcohols (50–100%). Slides were exposed to X-ray film to obtain autoradiographs (Kodak Biomax-MR, Kodak, Rochester, NY, USA).

#### Quantification

Autoradiographs of brain sections were captured digitally and used to analyse mRNA levels. For each animal, the hybridization signal was measured bilaterally from two sections representing the mid SCN, using a circular template. The mid SCN was defined using anatomical landmarks as previously described (Smale & Boverhof, 1999). The magnitude of the signal from the radioactive probes in single-labeled sections was determined using Scion Image (NIH, Bethesda, MD, USA), which automatically determined signal above background in each brain region. A background value was obtained from the corpus callosum in each section, and only pixels with a mean gray value exceeding the background value by 2.5 standard deviations were included in the analysis. Results are expressed as the mean integrated density of signal pixels divided by the total number of pixels in the selected area that surpassed threshold, which we defined as optical density. All photography and analysis was performed by an experimenter blind to the identity of each slide.

#### Statistical analysis

We used an ANOVA with time as the independent variable and signal as the dependent variable. A separate analysis was done for each group of animals (male, intact female, OVX female, OVX+E female). We used a 2 × 6 ANOVA to examine the effects of sex (male to intact female) and time on mRNA signal. This analysis enabled us to compare the results from grass rats with those previously reported in laboratory rats (Krajnak *et al.*, 1998b). We also used a 2 × 6 ANOVA to determine the effects of estrogen treatment and time on hybridization signal by comparing OVX and OVX+E females. When significant main effects were found ( $P = 0.05$ ), a Tukey *post-hoc* analysis was performed. When outliers were identified by the statistical program they were removed from analyses and graphs (SYSTAT v10, Systat Software Inc., Chicago, IL, USA).

#### Results

Representative images of radioactive *in-situ* hybridization signals for each mRNA are shown in Fig. 1. Hybridization for *vip* was most visible in the ventral portion of the nucleus while those for *vipr2* and

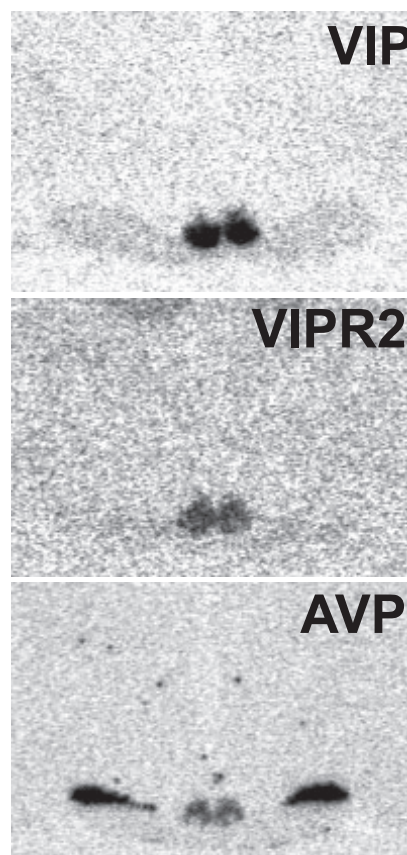


FIG. 1. Representative autoradiographs of the radioactive *in-situ* hybridization for *vip*, *vipr2* and *avp* in the SCN of the diurnal grass rat. Scale bar = 10 mm. VIP, vasoactive intestinal polypeptide; VIPR2, VIP receptor 2; AVP, arginine vasopressin; SCN, suprachiasmatic nucleus.

*avp* were concentrated in the medial and dorsal portion of the nucleus. We also saw relatively dark *avp* mRNA signal within the supraoptic nucleus. No labeling of the SCN was seen in tissue treated with sense control probes.

#### *vip*

Males and intact females had a daily rhythm in the expression of *vip* mRNA signal. There was a significant effect of time on the signal of *vip* mRNA in both males ( $F_{5,21} = 9.4$ ,  $P = 0.001$ ) and intact females ( $F_{5,26} = 4.9$ ,  $P = 0.003$ ) (Fig. 2). In males, the *vip* signal peaked just after lights-off (ZT 13) at which point it was significantly higher than at any of the other time points ( $P = 0.003$  or less for all *post-hoc* comparisons). In contrast, in intact females, the peak in signal occurred 8 h earlier, at ZT 5. Optical density was significantly higher at this point than at ZT 1 ( $P = 0.02$ ) or ZT 21 ( $P = 0.001$ ). When we compared intact females with males we found a significant interaction between time and sex ( $F_{5,47} = 5.39$ ,  $P = 0.001$ ).

OVX+E females did not have a significant rhythm in *vip* mRNA signal. The effect of time approached significance for OVX females ( $F_{5,18} = 2.7$ ,  $P = 0.054$ ). However, the 2 × 6 ANOVA revealed a main effect of hormone treatment such that OVX females had more signal than did OVX+E females ( $F_{5,36} = 8.8$ ,  $P = 0.005$ ).

#### *vipr2*

There was a significant effect of time on *vipr2* mRNA in males ( $F_{5,19} = 3.25$ ,  $P = 0.028$ ) and intact females ( $F_{5,27} = 8.56$ ,



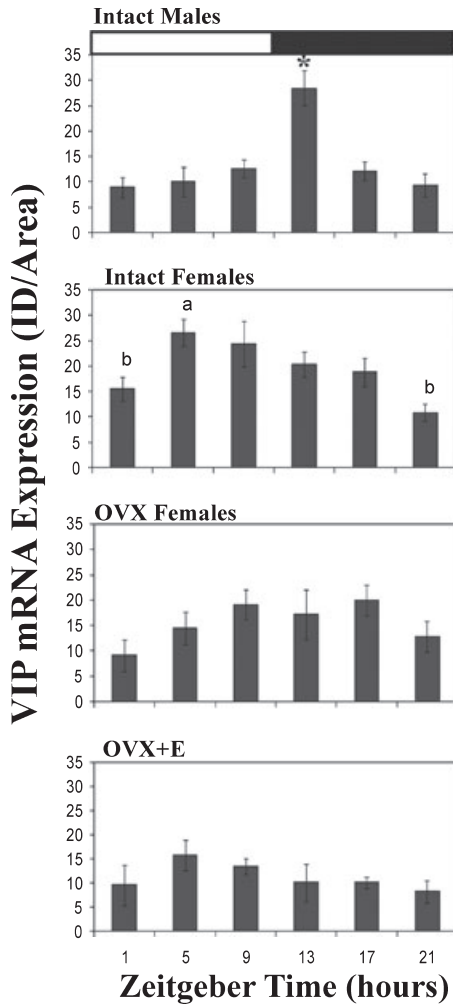


FIG. 2. Daily patterns in the *vip* mRNA signal in the SCN of males, intact females, ovariectomized (OVX) females and OVX females given two estradiol capsules for 2 days. Values are expressed as mean  $\pm$  standard error. White and black bars indicate the time of lights-on and lights-off, respectively. \*Significantly greater than all other time points ( $P = 0.003$ ). Within each graph, bars with different letters above them are significantly different from one another ( $P = 0.02$ ). See text for specific  $P$  values for each comparison. VIP, vasoactive intestinal polypeptide; SCN, suprachiasmatic nucleus.

$P = 0.001$ ). In males, the peak of signal at ZT 13 was significantly greater than at ZT 17 ( $P = 0.018$ ). In contrast, in intact females, the signal of *vipr2* mRNA at ZT 9 was significantly less than at every other time point ( $P = 0.03$  or less for all comparisons). We found a significant interaction between time of day and sex (male vs. intact female) with respect to the pattern of *vipr2* signal across the day ( $F_{5,46} = 2.92, P = 0.023$ ).

There was a significant effect of time on *vipr2* mRNA in OVX females ( $F_{5,18} = 3.97, P = 0.013$ , Fig. 3). OVX females were similar to intact females in that the *vipr2* signal trough measured at ZT 9 was less than that at ZT 21 ( $P = 0.03$ ). There was no main effect of time on the signal of *vipr2* in OVX+E females. We found a significant interaction between steroid treatment and time of day when OVX and OVX+E females were compared ( $F_{5,36} = 3.523, P = 0.01$ ). Specifically, OVX+E females had less signal overall when compared with OVX females, and the pattern of *vipr2* expression across the day differed between OVX and OVX+E females.

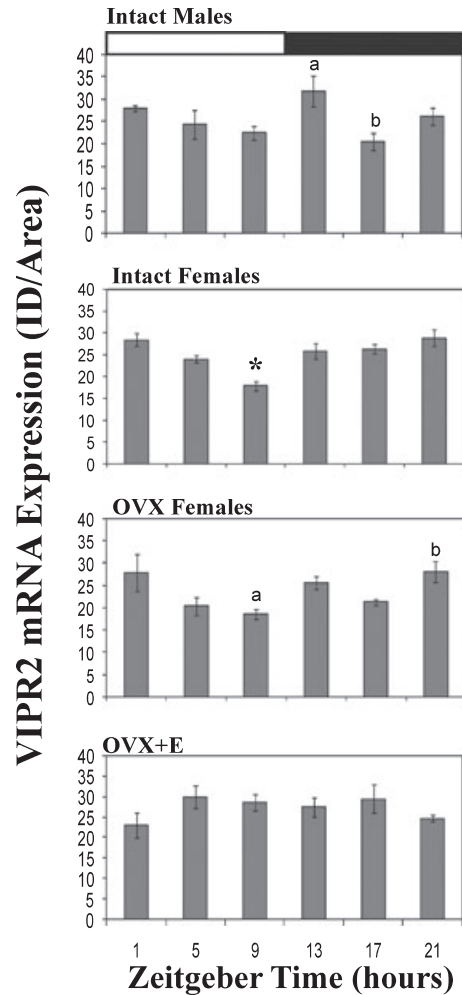


FIG. 3. Daily patterns in the *vipr2* mRNA signal in the SCN of males, intact females, ovariectomized (OVX) females and OVX females given two estradiol capsules for 2 days. Values are expressed as mean  $\pm$  standard error. Within each graph bars with different letters above them are significantly different from one another ( $P = 0.03$ ). \*Significantly less than all other time points ( $P = 0.03$ ). See text for specific  $P$  values for each comparison. VIPR2, VIP receptor 2; SCN, suprachiasmatic nucleus.

avp

There was a daily rhythm in the expression of *avp* mRNA in all groups, and the patterns were the same across groups. There was a significant effect of time on *avp* signal in all groups (males:  $F_{5,18} = 11.36, P = 0.001$ ; intact females:  $F_{5,26} = 11.23, P = 0.001$ ; OVX females:  $F_{5,20} = 17.54, P = 0.001$ ; OVX+E females:  $F_{5,18} = 13.29, P = 0.001$ ). *Post-hoc* analyses revealed a general pattern with peak signal occurring between ZT 5 and 9, and trough levels occurring at ZT 21 (Fig. 4). In males, the levels of *avp* at ZT 5 and 9 were higher than at ZT 17 and 21 ( $P = 0.001$  for each comparison). Additionally, levels at ZT 1 were higher than at ZT 21 ( $P = 0.05$ ). For intact females the values at ZT 1, 5, 9 and 13 were significantly higher than at ZT 21 ( $P = 0.008$  or less for each comparison). The signal measured at ZT 17 was also significantly lower than that at ZT 5 or 9 ( $P = 0.003$  or less for each). In OVX females the signals of *avp* mRNA at ZT 1, 5, 9 and 13 were all significantly higher than that measured at either ZT 17 or 21 ( $P = 0.01$

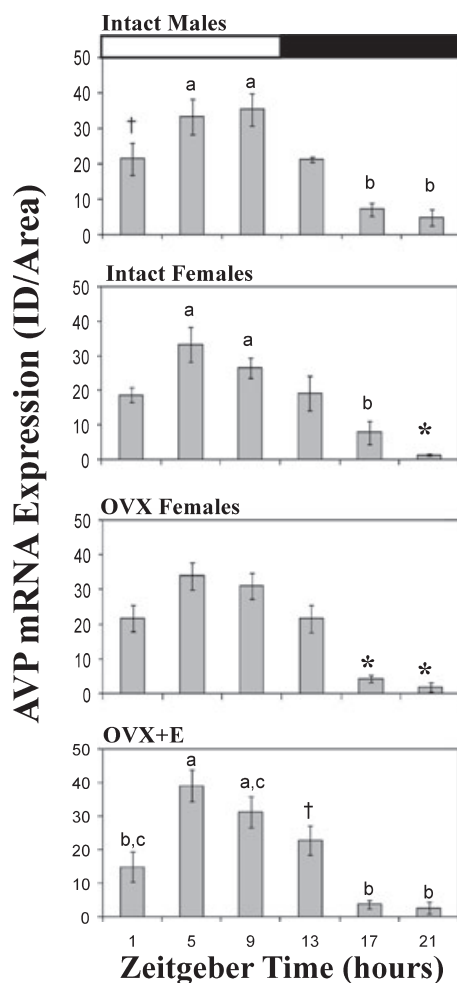


FIG. 4. Daily patterns in the *avp* mRNA signal in the SCN of males, intact females, ovariectomized (OVX) females and OVX females given two estradiol capsules for 2 days. Values are expressed as mean  $\pm$  standard error. Within each graph bars with different letters above them are significantly different from one another ( $P = 0.01$ ). †Significantly greater than ZT 21 ( $P = 0.05$ ). \*Significantly less than ZT 1, 5, 13 and 19 ( $P = 0.01$ ). See text for specific  $P$  values for each comparison. AVP, arginine vasopressin; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time.

or less for all comparisons). Finally, in OVX+E females *post-hoc* analyses revealed that the signal of *avp* mRNA at ZT 5 was significantly greater than that measured at ZT 1, 17 or 21 ( $P = 0.004$  or less for all comparisons). The mRNA levels at ZT 9 were also greater than at ZT 17 or 21 ( $P = 0.004$  for both comparisons). Lastly, signal at ZT 21 was significantly lower than that at ZT 13 ( $P = 0.021$ ) in these OVX+E females. No significant effect of sex was found between males and intact females nor was there an interaction between sex and time. Similarly, no effect of steroid hormone exposure (E caps) or interaction between treatment and time were found when OVX and OVX+E females were compared.

## Discussion

In nocturnal rodents, AVP and VIP appear to influence the timing of physiological rhythms through their oscillatory production and release from cells within the SCN. These effects are likely to be mediated by projections from the SCN to other brain regions, as well as through

interactions among SCN cells. Here, we evaluated the hypothesis that differences between day- and night-active species with respect to the timing of the LH surge could be produced by differences in patterns of *avp*, *vip* or *vipr2* production in the SCN. Specifically, we obtained data on these patterns in diurnal grass rats that could be compared directly with published data on male and female laboratory rats. Additionally, we compared females that cannot produce an LH surge (OVX) with females that can (OVX+E). Our major findings were that the SCN of *A. niloticus* has (i) daily rhythms in the expression of *vip* mRNA that are influenced by hormones and are sexually dimorphic, (ii) a robust daily rhythm of *avp* mRNA that is similar in males and females, and (iii) the phases of all of these rhythms are very similar to those seen in nocturnal species (Krajnak *et al.*, 1998a; Krajnak *et al.*, 1998b; Kallo *et al.*, 2004).

### Daily rhythms in *vip* mRNA in the SCN

Male and intact female grass rats had daily rhythms in *vip* gene expression but the patterns of these rhythms were quite different (Fig. 2). In males, the peak occurred just after lights-off (ZT 13), whereas in intact females it occurred during the light portion of the light–dark cycle (ZT 5). It is likely that these patterns are regulated, at least in part, by estradiol secreted by the ovaries. In OVX females given estradiol the pattern of *vip* expression resembled that of intact females, with the highest levels occurring at ZT 5 (Fig. 2), although in this case the effect of time of day was not statistically significant. In comparison, OVX females had a rhythm with relatively high levels of *vip* mRNA during the dark portion of the light–dark cycle. Although this rhythm only approached significance ( $P = 0.054$ ), it resembled that of males more than those of females in the other two conditions.

The influence of ovarian hormones observed here provides further support for the hypothesis that VIP signals originating in the SCN are involved in regulation of ovulation in some species. A variety of more general features of SCN function can be modified by the ovarian hormones that are essential for the production of the LH surge in nocturnal species. For example, in ovariectomized female rats, treatment with an estrogen advances the rhythm of the clock gene *Per2* mRNA in the SCN (Nakamura *et al.*, 2005) and increases connexin32 mRNA transcripts (Shinohara *et al.*, 2001). Injection of OVX female rats with an estrogen at midday or at midnight leads to significant decreases in *Cry2* mRNA within the SCN 24 h later (Nakamura *et al.*, 2001). Ovariectomy of female rats reduces expression of the immediate early gene *Fos* in the SCN, an effect that is restored by treatment with estradiol (Peterfi *et al.*, 2004). These effects of estrogens on gene expression patterns may be mediated through steroid receptor-containing cells that project to the SCN (de la Iglesia *et al.*, 1999). These effects may also be mediated by receptors within the SCN itself. Estrogen receptor alpha and beta, and/or their mRNAs, have been detected in the SCN of mice (Vida *et al.*, 2008), rats (Cintra *et al.*, 1986; Su *et al.*, 2001), humans (Kruijver *et al.*, 2003) and *Octodon degus* (a diurnal rodent; D. Hummer, M.M. Mahoney and T. Lee, unpublished observations). Rising levels of estrogen on the afternoon of proestrus may influence the expression of mRNA and peptide rhythms within the SCN, perhaps by directly acting on steroid receptors located in this nucleus. If ovarian steroid hormones alter the expression of SCN genes in a sex-dependent manner, as is the case for *vip*, this may be one mechanism by which SCN output signals and high estrogen levels coordinate to trigger the preovulatory LH surge.

Despite the fact that *A. niloticus* is a diurnal species, both the daily pattern and the sex difference in *vip* mRNA expression were similar to those reported for nocturnal laboratory rats and mice. Male rats have a peak at ZT 14 (Kallo *et al.*, 2004) and in male mice (*Mus musculus*)

the peak occurs at ZT 14.5 (Dardente *et al.*, 2004) whereas in the current study the peak was at ZT 13 in male grass rats. By contrast, intact female *A. niloticus* had a peak in *vip* at ZT 5 and significantly lower levels of this transcript at the end of the evening and beginning of the light portion of the light–dark cycle (ZT 21 and 1, Fig. 2), a pattern similar to that of female laboratory rats (Krajnak *et al.*, 1998b). Interestingly, the rhythms in *vip* mRNA expression seen here differ somewhat from that reported for a related diurnal species, *Arvicanthis ansorgei*, in which the peak occurred at ZT 17 (Dardente *et al.*, 2004). This would suggest that *vip* mRNA rhythms are quite different in these two closely related diurnal species. However, there are other possible explanations. The two studies differ with respect to the statistical analyses that were used to assess the rhythm patterns (cosinor analysis vs. ANOVA) and the time points sampled. In addition, the report on *vip* rhythms in *A. ansorgei* did not indicate the sex of the animals used (Dardente *et al.*, 2004). The *vip* patterns of males and females are quite different in *A. niloticus* (current study), as well as in laboratory rats (Krajnak *et al.*, 1998b) such that combining the data from the two sexes could obscure the patterns.

The similarity between the patterns of *vip* mRNA rhythms seen here and those reported in rats and mice suggest that this feature of SCN function is unrelated to chronotype. The timing of the LH surge is 12 h out of phase in *A. niloticus* and laboratory rats (McElhinny *et al.*, 1999), yet the time of the daily peak in *vip* mRNA is similar in females of these two species. These data thus suggest that differences in how targets downstream from the SCN respond to VIP are likely to contribute to differences in neuroendocrine rhythms of nocturnal rodents and grass rats.

#### Daily rhythms in *vipr2* mRNA in the SCN

There was a modest rhythmic pattern of *vipr2* mRNA in the SCN of both male and female grass rats (Fig. 3). In males the peak occurred immediately after lights-off (ZT 13). This pattern resembles that seen in male laboratory rats in which *vipr2* levels decrease across the day, then peak at the time of lights-off (Shinohara *et al.*, 1999; Kallo *et al.*, 2004). In laboratory rats, however, *vipr2* mRNA remains elevated throughout the night (ZT 14–24) whereas in grass rats, it declined to a trough at ZT 17 before returning to high levels again at ZT 21. This finding was unanticipated as species differences in rhythm patterns within the SCN are not common (reviewed in Smale *et al.*, 2003), and it raises the question of whether *vipr2* is functionally related to, or may even contribute to, the differences in behavioral or physiological rhythms expressed in males of these two species. In the SCN of intact female *A. niloticus*, *vipr2* expression was lower at ZT 9 than at any other time point and otherwise did not change across the day (Fig. 3). We do not know if this is unusual as patterns of this mRNA transcript have not been characterized in females of any other species. Thus, there may be a modest rhythm and sex difference in *vipr2* expression, but these daily expression patterns need to be more fully characterized in addition to determining rhythmic expression of this receptor is functionally related to the ability to mount an LH surge.

#### Daily rhythms in *avp* mRNA in the SCN

Male and female *A. niloticus* had robust rhythms in *avp* mRNA expression across the light:dark cycle. In all four treatment groups, levels of transcript were high during the day (between ZT 5 and 9) and were relatively low in the late evening between ZT 17 and 21. This pattern is fundamentally similar to those of nocturnal laboratory rats (Krajnak *et al.*, 1998b; Kallo *et al.*, 2004), Siberian hamsters (Duncan *et al.*, 1995), Syrian hamster (Duncan *et al.*, 2001), mice and another

diurnal grass rat, *A. ansorgei* (Dardente *et al.*, 2004). We did not detect any differences in the pattern of *avp* expression between groups of animals. However, the *avp* mRNA rhythm produced within the SCN could still influence the timing of the LH surge in female grass rats, as it appears to do in laboratory rats (Palm *et al.*, 1999, 2001).

#### Conclusions

The current data reveal that rhythms in *vip* and *avp* mRNAs within the SCN are fundamentally similar in diurnal grass rats and nocturnal murid rodents, reinforcing the view that the SCN has more similarities than differences across species, even those whose temporal niche is very different. Indeed, the SCN of *A. niloticus* also resembles those of nocturnal rodents with respect to rhythms in expression of *Fos* (Schwartz *et al.*, 2004) and clock genes (Ramanathan *et al.*, 2006) as well as the SCN output signal prokineticin 2 (Lambert *et al.*, 2005). Here, we have taken this a step further by showing that two additional output pathways, those involving *vip* and *avp*, are the same in *A. niloticus* as in nocturnal rodents. In the case of *vip*, the phase of the mRNA rhythm is similar in diurnal and nocturnal species even though the rhythms are different in males and females. Taken together, these data support the view that differences between diurnal and nocturnal species arise from differences in responsiveness to SCN signals. A recent elegant study of *A. ansorgei* has provided direct evidence that this is the case for rhythms in secretion of glucocorticoids (Kalsbeek *et al.*, 2008). In the future, it will be important to determine whether these conclusions apply more generally to nocturnal and diurnal species in other taxonomic groups, as well as how differences in responsiveness to SCN signals might come about.

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#### Abbreviations

AVP, arginine vasopressin; E, estradiol; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; OVX, ovariectomized; SCN, suprachiasmatic nucleus; VIP, vasoactive intestinal polypeptide; VIPR2, VIP receptor 2; ZT, Zeitgeber time.

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