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Sex-specific differences in the circadian pattern of action potential firing by rat suprachiasmatic nucleus vasopressin neurons

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Abstract

The suprachiasmatic nucleus (SCN) of the hypothalamus serves as the master circadian clock in mammals. Most SCN neurons express the inhibitory neurotransmitter GABA (gamma amino butyric acid) along with a peptide cotransmitter. Notably, the neuropeptides vasopressin (VP) and vasoactive intestinal peptide (VIP) define two prominent clusters within the SCN: those located in the ventral core (VIP) and those forming the dorsomedial "shell" of the nucleus (VP). Axons emerging from VP neurons in the shell are thought to mediate much of the SCN's output to other brain regions as well as VP release into the cerebrospinal fluid (CSF). Previous work has shown that VP release by SCN neurons is activity dependent and SCN VP neurons fire action potentials at a higher rate during the light phase. Accordingly, CSF VP levels are higher during daytime. Interestingly, the amplitude of the CSF VP rhythm is greater in males than females, suggesting the existence of sex differences in the electrical activity of SCN VP neurons. Here we investigated this hypothesis by performing cell-attached recordings from 1070 SCN VP neurons across the entire circadian cycle in both sexes of transgenic rats that express green fluorescent protein (GFP) driven by the VP gene promoter. Using an immunocytochemical approach we confirmed that >60% of SCN VP neurons display visible GFP. Recordings in acute coronal slices revealed that VP neurons display a striking circadian pattern of action potential firing, but the characteristics of this activity cycle differ in males and females. Specifically, neurons in males reached a significantly higher peak firing frequency during subjective daytime compared to females and the acrophase occurred ~1 h earlier in females. Peak firing rates in females were not significantly different at various phases of the estrous cycle.

KEYWORDS
circadian, females, males, suprachiasmatic nucleus, vasopressin

1 | INTRODUCTION

The suprachiasmatic nucleus (SCN) consists of two clusters of neurons located on each side of the third ventricle directly above the optic chiasma.[1] Electrophysiological studies have shown that the average rate of action potential firing by SCN neurons is high during the light phase and low during the dark phase,[2–4] reflecting a circadian (~24 h) rhythm of electrical activity that is maintained even when the SCN is physically isolated,[5] or when SCN neurons are removed from the brain and placed in dissociated culture.[6] Bilateral lesions of the SCN in rodents abolish most circadian rhythms[7–9] and many of these can be restored by transplantation of donor SCNs in the third ventricle of SCN-lesioned animals.[10–12] The SCN is therefore widely recognized as the master circadian clock in mammals.[13,14]

Most SCN neurons produce the inhibitory neurotransmitter GABA (gamma amino butyric acid) and also coexpress a neuropeptide. Two major subsets of SCN neurons are defined by the peptides they coexpress: vasopressin (VP; ~37% of the neurons) and vasoactive intestinal peptide (VIP; ~24% of the neurons).[15] VIP neurons form the ventral core of the SCN and these neurons receive axonal inputs from the retino-hypothalamic tract that entrain the clock's timing to the local light cycle.[16] By contrast, VP neurons form a dorsomedial "shell" that sits atop the VIP core. VP neurons in this area send axonal projections to the rest of the brain[17] and also through the ependymal lining of the third ventricle for secretion into the cerebrospinal fluid (CSF).[18] We have recently shown that VP release from the axon terminals of SCN neurons is activity-dependent,[19,20] indicating that circadian changes in the secretion of this peptide are regulated by the rate of action potential firing by SCN VP neurons.

One of the overt rhythms generated by the SCN is the daily increase in VP concentration within CSF that occurs during the light phase.[21–23] This pattern is consistent with the higher firing rate of identified SCN VP neurons observed in rats at circadian time (CT) 5–8 (CT 5–8) compared with CT 14–17.[24] Interestingly, the values of CSF VP concentration are higher in males than females at all circadian times, and the amplitude of the VP rhythm is also greater in males.[22] These observations suggest that the circadian pattern of action potential firing by SCN VP neurons may be different in the two sexes. Indeed, it was recently reported that the average firing rate of mouse SCN shell neurons (i.e., putative VP neurons) is higher at CT4–6 in males than in females.[25]

To establish if sex differences exist in the circadian activity profile of SCN VP neurons we obtained cell-attached recordings from large numbers of single, genetically identified SCN VP neurons in hypothalamic slices prepared from male and female transgenic rats that express enhanced green fluorescent protein (GFP) driven by the VP gene promoter.[26] Our results show sex differences in aspects of the circadian activity of rat SCN VP neurons.

2 | MATERIALS AND METHODS

2.1 | Animal housing and brain collection

All procedures used in this study followed the Canadian Council guidelines on Animal care (https://www.ccac.ca/) and experimental protocols were approved by the Facility Animal Care Committee of McGill University (protocol AUP1190). Male and female transgenic Wistar rats (2 to 4 months old) expressing enhanced GFP driven by the VP gene promoter[26] were housed in a temperature-controlled room (21 ± 2°C) and maintained under 12:12 h light–dark cycle with lights on at 7:00 AM. Because individual brain slices were only kept for ~6 h, animals were sacrificed at different zeitgeber times (ZT) to span the entire 24 h cycle. For experiments requiring that slices be prepared during the dark phase, the rats were kept in the dark and slice preparation was performed in the presence of dim red light.

2.2 | Immunocytochemistry

Rats were deeply anesthetized with isoflurane and intracardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.5. For immunocytochemistry the rats were perfused at ~ZT7-ZT9. The brain was extracted and kept in 4% paraformaldehyde (PFA) until coronal sections (50 µm thick) were cut using a vibratome (VT1200, Leica Biosystems Inc., Concord, Ontario). A series of 13 consecutive sections containing the SCN were collected from each animal. Free floating sections were permeabilized using 0.1 M PB with 0.3% Triton X-100 (PBT) for 20 min followed by blocking in 2% normal goat serum (catalog no. G9023; Sigma Aldrich, Ontario) for 1 h. Sections were then incubated for 20 h at 4°C in primary antibody (mouse monoclonal anti-VP-neurophysin 1:100; kindly provided by Dr Harold Gainer, NIH). The sections were then washed with PBT followed by the incubation at room temperature in secondary antibody: Alexa Fluor Goat anti-mouse 1:500 (Invitrogen, Thermo Fisher Scientific, catalog no. A-11004, RRID AB_2534072, Waltham, Massachusetts) for 1 h. Finally, sections were washed with PBT, mounted and coverslipped using Prolong Gold antifade reagent (Invitrogen). Immunostaining of male and female sections was performed concurrently to minimize differences in signal intensity.

2.3 | Imaging, colocalization, quantification

Images were captured using an FV 3000 inverted confocal laser scanning microscope (Olympus Canada Inc., Richmond Hill, Ontario) using excitation wavelengths of 488 nm for GFP and 561 nm for Alexa 568. The images were acquired using a 10x (1.4 NA) objective (wide views shown in Figure 1A, B) or a 30x (1.05NA)

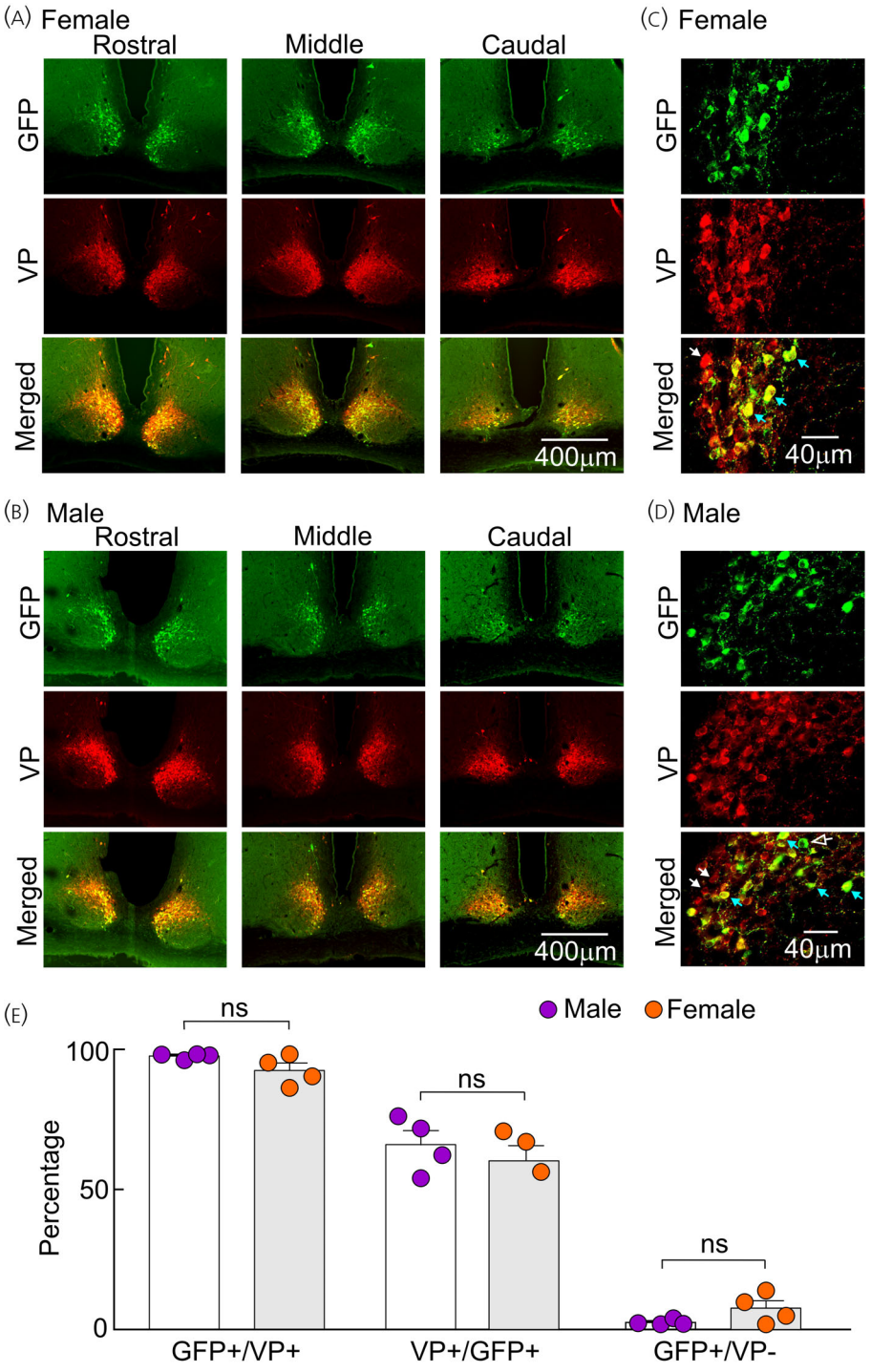

FIGURE 1 Specificity of green fluorescent protein (GFP) as a vasopressin (VP) reporter in the suprachiasmatic nucleus (SCN). (A, B) Low power images of coronal sections taken in the rostral, middle and caudal parts of the SCN show expression of GFP (green), VP (red) and merge (yellow indicates overlap) in female (A) and male (B) rats. (C, D) Higher power images taken through the middle of the SCN show GFP, VP and merge in females (C) and males (D). White arrows show VP + GFP− cells, blue arrows show GFP +/VP+ cells and open arrow shows GFP +/VP− cell. (E) Bar graphs show the percentages of GFP positive cells that also expressed VP (GFP+/VP+), the percentages of VP positive cells that also expressed visible GFP (VP+/GFP+) and the percentages of cells that expressed GFP alone (GFP+/VP−). Each point shows the percentage determined by analyzing four SCNs in a single animal and each bar shows values determined for four males and four females. Note that differences between males and females are not significant (ns): GFP+/VP+, $p = .2$, Mann–Whitney rank sum test; VP +/GFP+, $p = .462$ *t*-test; GFP+/VP−, $p = .2$, Mann–Whitney rank sum test.

oil-immersion objective (close up views in Figure 1C, D). To quantify the numbers of neurons expressing GFP or VP, stacks of 10 confocal images (30x) were collected individually from the left and right SCNs in two slices from each animal. For each animal numerical data collected from four SCN stacks was used to calculate the percentage of GFP positive neurons that also expressed VP (GFP+/VP+), the percentage of VP positive neurons that also expressed GFP (VP+/GFP+) and the percentage of cells that expressed GFP but not VP (GFP+/VP-). Each data point in Figure 1E represents the average value in a single animal determined from analyzing between 353 and 793 cells in four SCN stacks.

The analysis was performed using Image J software (NIH) and first involved determining a threshold intensity value for each channel. This value was determined arbitrarily as the average cytoplasmic fluorescence intensity observed in five of the dimmest cells considered to be positive. Colocalization between the GFP expression (green) and immunolabeled VP (red) was considered to occur when a minimum of six pixels of one of the channels was also visible in the other channel in a high-resolution confocal image.

2.4 | Rat estrous cycle assessed by vaginal cytology

Female rats display an estrous cycle period of 4–5 days comprising four phases: diestrus, proestrus, estrus and metestrus. In our experiments, stage was identified by vaginal cytology.[27] Vaginal smears were collected every days between ZT2 and ZT3 during at least three consecutive estrous cycles prior to the experiment. For this purpose a moistened (dH_2O) cotton swab was inserted into the vagina and gently rotated against the vaginal wall without applying any pressure. The smear was then transferred onto a glass slide and air dried. A metachromatic stain was then applied using Toluidine blue O 0.1% and inspection was done with an upright microscope using a 4× or 10× objective. Estrous cycle stage was evaluated by the presence and proportions of neutrophils, nucleated and/or keratinized epithelial cells, and anucleated epithelial cells. Proestrus was characterized by a predominance of round nucleated epithelial cells appearing individually or as clusters. Estrus was associated with an abundance of large, densely stained anucleated epithelial cells with irregular shape. Metestrus was characterized by a low density of large, lightly stained anucleated cells with occasional nucleated cells. Diestrus was recognized by presence of cellular debris and rare large cells. A swab test repeated immediately after decapitation was used to confirm the stage assessed by monitoring on previous days.

2.5 | Slice electrophysiology

Rats were killed by decapitation and the brains were rapidly extracted and placed in ice cold oxygenated ($95\%O_2/5\%CO_2$) artificial cerebrospinal fluid (ACSF). Coronal slices 300 µm thick were cut using a vibratome and incubated in oxygenated ACSF at 32°C for 1 h prior to being transferred to a recording chamber where they were perfused (2 mL/min; 30°C) with oxygenated ACSF composed of (mM): 120 NaCl, 3 KCl, 1.23 NaH_2PO_4, 1.48 $MgCl_2.6H_2O$, 10 glucose, 25.95 $NaHCO_3$, 2 $CaCl_2$. Glass electrodes filled with ACSF (5 MΩ, A-M Systems Inc., Everett, Washington) were used to make visually guided cell-attached recordings of action potential firing from identified (GFP positive) SCN VP neurons at different subjective CT defined as the time extrapolated from the rat's ZT prior to slice preparation. For example, for a slice prepared at 9:00 AM (i.e., ZT 2), the activity of a slice subsequently recorded at 11:00 AM was taken as a measure of activity at CT4. For each CT, recordings were obtained from a minimum of three different rats.

2.6 | Statistical analysis

Groups of data were compared using parametric or nonparametric statistical tests as appropriate using Sigmaplot 12 (Systat Inc., Palo Alto, California) or Prism version 5.01 (Graphpad Software, San Diego, California). Parametric tests were restricted to cases where the assumption of equal variance could be verified. Differences in proportions were assessed using Chi-square or Fisher's exact statistical tests. Prism 5.01 was used to analyze the circadian data on firing rates. Hourly average firing rates for males and females were fitted separately using a sigmoidal equation where the wavelength parameter was constrained at 24 h, leaving the amplitude and phase parameters to be determined by best-fit using the sum-of-squares approach. Prism uses a standard method to compute the standard error and confidence interval for each parameter fit with nonlinear regression (https://www.graphpad.com/guides/prism/7/curve-fitting/reg_how_standard_errors_are_comput.htm). The Extra sum of squares F test was then used to compare the fits. The F test compares the difference in sum-of-squares with the difference expected by chance. The result is expressed as the F ratio, from which a p-value is calculated. All values are reported as mean ± standard error of the mean (s.e.m) and differences were considered statistically significant when $p < .05$. Plots were constructed using either Sigmaplot or Prism.

3 | RESULTS

3.1 | Distribution of VP-containing neurons in the SCN of male and female rats

The use of VP-GFP Wistar rats allows a clear visualization of VP-expressing neurons in different areas of the brain; however, it has been reported that not all immunoreactive VP cells express GFP in this strain.[26] We therefore compared the fraction of SCN VP neurons identified by immunocytochemistry that also expressed visible GFP in tissue sections from females and males. As illustrated in Figure 1, GFP fluorescence observed at low magnification was strongly colocalized with endogenous VP. However, immuno-labeled VP was more broadly distributed than GFP throughout the rostrocaudal extent of the SCN (Figure 1A, B). High magnification images (Figure 1 C, D) were used to quantify colocalization at the cellular level. Our analysis revealed that the vast majority of GFP expressing neurons were also immunoreactive for VP in both females (92.3% ± 2.6%; $n = 4$ rats) and males (97.4% ± 0.5%; $n = 4$ rats; Figure 1E). [Correction added on 27 July 2023, after first online publication: The last 3 sentences of this paragraph have been removed.]

3.2 | Sex differences in the electrical activity of SCN-VP neurons

To determine the circadian activity profile of SCN VP neurons, we obtained recordings from acute coronal hypothalamic slices containing the SCN (Figure 2A, B). As shown in Figure 2C, GFP-expressing neurons were clearly visible in this preparation and could be easily targeted for cell-attached recordings (Figure 2D). Although most cells displayed spontaneous action potential firing, other cells did not (Figure 2 E). To confirm that inactive cells were functionally silent, we verified that our recording configuration was operative and that the cells being recorded were alive by applying a strong pulse of

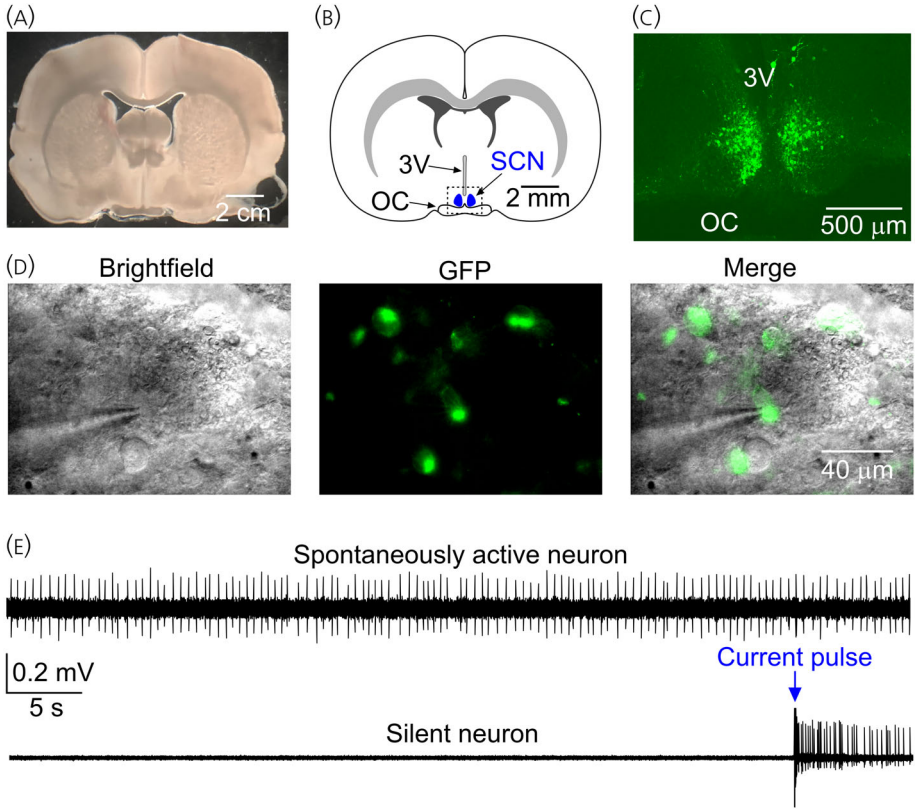

FIGURE 2 Cell-attached recordings from suprachiasmatic nucleus (SCN) vasopressin (VP) neurons. (A) Brightfield photo shows living coronal brain slice obtained from a female transgenic rat expressing green fluorescent protein (GFP) driven by the VP gene promoter. (B) Schematic drawing of the same slice highlighting the SCN. OC, optic chiasma, 3 V third ventricle. Dotted line indicates area shown in C. (C) Fluorescence image shows GFP expression in the SCN area indicated in B. (D) High power (60×) image (Brightfield, left) shows patch-clamp pipette targeting a GFP positive SCN-VP neuron (green, middle panel; merge, right panel). (E) Example traces of cell-attached recordings from actively firing (top) and silent SCN-VP neurons. Presence of the silent neuron was confirmed by spiking induced by delivery of a current pulse (5 nA, 5 ms).

depolarizing current which normally elicit the firing of action potentials. Inactive cells responding in this way were counted as "silent" (arbitrarily defined as <0.1 Hz) whereas those that did not respond to the current pulse were discarded from the analysis.

A total of 1070 identified SCN VP neurons were recorded at various subjective CT across the 24 h cycle in both males (403 neurons from 14 rats; Figure 3A) and females (667 neurons from 23 rats; Figure 3B). As expected, visual inspection of the triple-plotted data (the same data is re-plotted for 24–48 and 48–72 h) revealed that SCN VP neurons are prominently active during the light phase and show much less activity during the dark phase. However, many SCN VP neurons in males showed persistent activity during the early part of the dark phase, whereas this appeared to be less common in females. To quantify the parameters of this circadian activity, the data collected every hour were averaged, triple plotted against CT and fitted using a 4−parameter sinusoid equation [$y(x) = y_0 + a^*\sin(2\pi x/b + c)$], where a and c define amplitude and phase, y is the firing rate, and where the oscillation period (b) was fixed at 24 h (Figure 3C). This analysis revealed that average hourly firing rates were lower in females across the entire 24 h cycle compared with males. Moreover, the firing rate rhythms observed in males and females appeared to be slightly out of phase. In males peak firing rate (5.72 Hz) was observed at CT 8 whereas peak firing rate in females (3.5 Hz) occurred at CT7. Similarly, the nadir occurred later in males (0.9 Hz; CT 20) than females (0.1 Hz; CT 19). When considering 2-h periods centered on the acrophase (i.e., CT 6.0–8.0 for females and CT 7.0–9.0 for males), the mean firing rate was significantly greater in males (5.39 ± 0.29 Hz, n = 30, 2 rats) than females (3.55 ± 0.20 Hz, n = 171, 15 rats; Mann–Whitney Rank sum test $p < .001$; Figure 3D). Similarly, when considering 2-h periods centered on the nadir (CT 18.0–20.0 for females and CT19.0–21.0 for males), the mean firing rate was significantly lower in females (0.09 ± 0.04 Hz, n = 41, 3 rats) compared to males (0.90 ± 0.40 Hz, n = 27, 4 rats; Mann Whitney $p < .001$; Figure 3E).

In order to statistically compare the amplitudes and phases of the firing rate rhythms in males and females, the data for Figure 3C were replotted with the ordinate offset (y_0) subtracted (Figure 4A), allowing a nonlinear regression comparison of the data using a three parameter sinusoid fit [$y(x) = a^*\sin(2\pi x/b + c)$]. This procedure revealed statistically significant differences ($F_{(2, 140)} = 12.37$; $p < .0001$). Namely, the amplitude of the rhythm was greater in males (2.40 ± 0.58 Hz) compared to females (1.70 ± 0.32 Hz; Figure 4B) and the phase was significantly delayed in males (1.8 ± 0.2 h) compared to females (1.0 ± 0.2 h; Figure 4C). Moreover, visual inspection of the raw data also suggested that higher proportions of neurons were active in males compared to females. We therefore calculated the proportion of neurons that were active (>0.1 Hz) on an hourly basis for males and females and compared these proportions statistically using a Fisher test (Table 1). As illustrated in Figure 5, there was a clear 24 h pattern in the proportions of SCN VP neurons that were active in both females and males. Strikingly, high proportions of neurons were active throughout the light phase and into the early part of the dark phase in both females and males (until ∼CT15.5), such that a clear reduction in the proportion of active neurons was only seen between ∼CT17 and ∼CT 22. At most time points, the proportions of active neurons were significantly lower in females compared to males (Figure 5 and Table 1; $p < .05$; Fisher's exact test statistic).

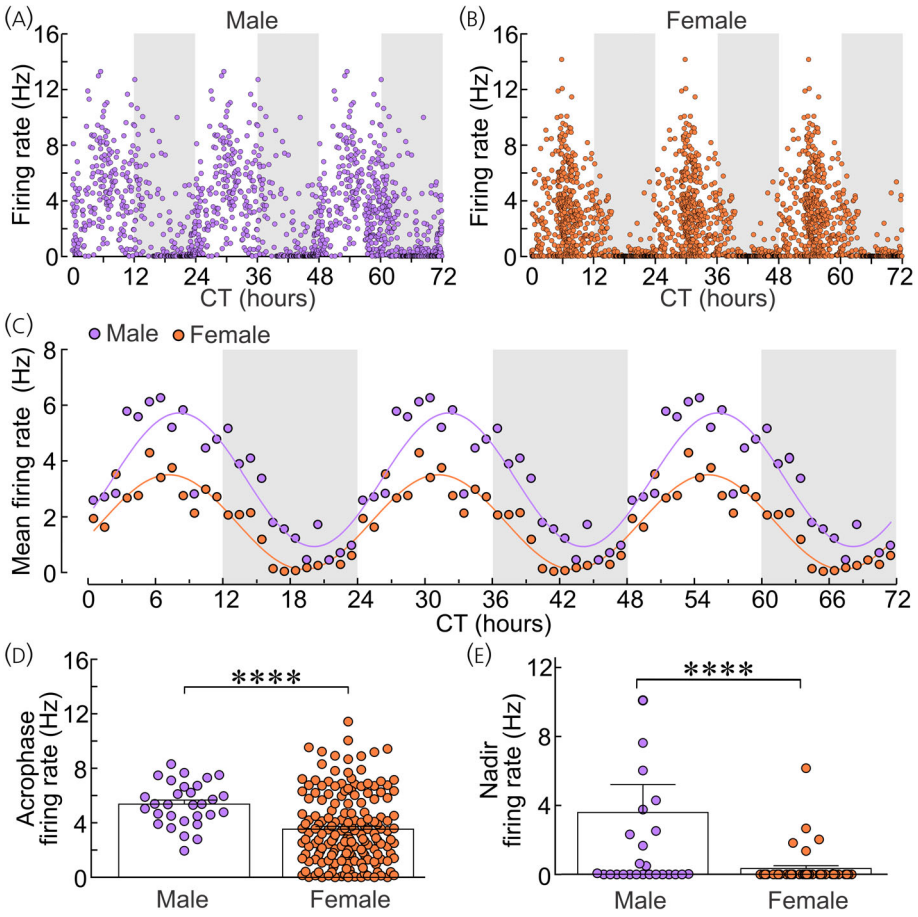

FIGURE 3 Circadian firing rates in suprachiasmatic nucleus (SCN) vasopressin (VP) neurons of females and males. (A, B) Firing rates of single SCN VP neurons recorded at different circadian times (CT) in slices prepared from males (A, $n = 403$ neurons in slices from 14 rats) and females (B, $n = 667$ neurons in slices from 23 rats). Data from CT0 to CT24 are replotted as CT24−CT48 and again as CT48−CT72 (i.e., triple plotted) to facilitate visualization of the circadian profile (dark phase represented by gray shading). (C) Plot showing the average hourly firing rates of SCN VP neurons in males (purple) and females (orange) as a function of CT (triple plotted; dark phase represented by gray shading). Curves are best fits of a 4−parameter sinusoid with the period parameter fixed at 24 h. Female data $a = 1.699$, $b = 24$, $c = -0.31$, $y_o = 1.80$; $r^2 = 0.8522$; Male data $a = 2.395$, $b = 24$, $c = -0.558$, $y_o = 3.326$; $r^2 = 0.7848$. (D) Bar graphs compare the firing rates of all cells recorded during the acrophase in males (CT7.0−CT9.0; $n = 30$ neurons in 2 rats) and females (CT6.0 − CT8.0, $n = 171$ neurons in 15 rats). ****$p < .001$ Mann–Whitney rank sum test. (E) Bar graphs compare the firing rates of all cells recorded during the nadir in males (CT19.0 − CT21.0; $n = 27$ neurons in 4 rats) and females (CT18 − CT20, $n = 41$ neurons in 3 rats). ****$p < .001$ Mann–Whitney rank sum test.

3.3 | Estrous cycle and firing rates in females

The coefficient of variation (CV) of firing rates observed during a 2 h period centered on the acrophase was greater in females (CV = 0.73) than males (CV = 0.29). This higher CV in females was associated with a greater number of cells firing in the 0–2 Hz range compared to males (Figure 3A, B). We therefore examined if the lower average firing rate of SCN VP neurons at acrophase in females might be caused by prevalence of lower firing rates during a specific stage of the estrous cycle. To this end, several recordings performed between CT 6 and CT8 were obtained from slices prepared from animals whose estrous cycle stage had been determined by vaginal cytology. As illustrated in Figure 6A, there were no significant differences in the mean rates of action potential between CT6 and CT8 across different stages of the estrous cycle, including stages associated with low circulating estrogen (i.e., diestrus 4.9 ± 0.4 Hz, $n = 41$ from 3 rats and estrus 4.0 ± 0.5 Hz, $n = 38$ from 4 rats) or high estrogen (proestrus 3.5 ± 0.4 Hz, $n = 33$ from 3 rats; $F_{(2,\ 109)} = 2.974$, $p = .055$; One-way ANOVA). Similarly, the proportions of active SCN VP neurons were not significantly different across the three stages as analyzed by a Chi-square test ($p = .1392$; Figure 6B).

4 | DISCUSSION

Previous work in rats has shown that peak levels and amplitudes of the circadian CSF VP rhythm are both greater in males than females.[22] Because VP release from SCN neurons is activity dependent,[19,20] these observations suggested there might be differences in the firing pattern of SCN VP neurons in the two sexes. Indeed, sexually dimorphic features of the SCN have been reported in several species,[28–34] including humans,[35] and sex

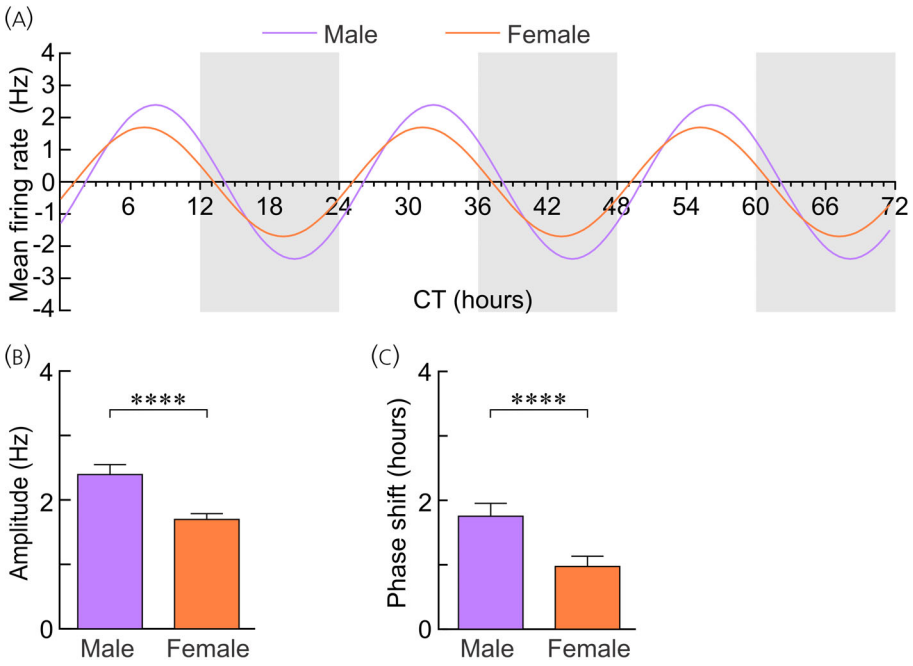

FIGURE 4 Sex-specific differences in circadian activity in female and male suprachiasmatic nucleus (SCN) vasopressin (VP) neurons. (A) plot shows best 3−parameter sinusoid fits of average firing rates in SCN VP neurons in females (orange) and males (purple) with period set at 24 h (dark phase represented by gray shading). Prior to fitting the data (from Figure 3A, B) were adjusted by subtracting the offset determined by the 4 − parameter fit, setting the center of the oscillation at y = 0 Hz and allowing a nonlinear regression comparison of the amplitude and phase parameters using an Extra sum of squares F-test. (B) Bar graphs show the mean (± s.e.m.) amplitude of the rhythm in females and males. ****$p < .0001$. (C) Bar graphs show the mean (± s.e.m.) phase shift of the rhythm in females and males. **** $p < .0001$.

differences in circadian physiology are well documented.[35–40] Therefore, the goal of this study was to characterize and compare the circadian profiles of electrical activity in identified SCN VP neurons of male and female rats.

Our experiments were performed on transgenic Wistar rats that have been engineered to express GFP driven by the VP gene promoter.[26] In this model, GFP is strongly expressed in the dorsomedial shell of the SCN, and our analysis indicated that GFP expression reliably identifies neurons expressing VP-GFP fusion protein in the SCN of both females and males. However, GFP expression was not visible in all immunolabeled VP neurons, including ∼40% and ∼34% of the population in females and males, respectively. It should be noted that this analysis was performed on tissue harvested at a time when overall GFP expression was strong (ZT7−ZT9) and it remains possible that the percentages of SCN VP neurons expressing GFP are lower at other circadian times. Although it is unclear why the transgene is not expressed by all SCN VP neurons, this finding raises the possibility that GFP expression may be restricted to specific subsets of SCN VP neurons while leaving others inaccessible by this method. Therefore, caution should be exerted when interpreting data obtained from GFP identified cells as these might not be representative of the entire population of SCN VP neurons. Indeed, there is good reason to believe that several subsets of VP neurons exist within the SCN based on the fact that small groups of SCN VP neurons can be found to project to distinct anatomical sites,[19] on the identification of distinct transcriptomic clusters of SCN neurons that express VP,[41] and on the existence of topographic differences in the timing of clock gene expression across the entire population of SCN VP neurons.[42] Whether the sex differences in firing patterns reported here apply broadly to all subsets of SCN VP neurons remains to be established.

Notwithstanding these limitations, recordings obtained from 1070 GFP-identified SCN VP neurons revealed significant differences in the global circadian profile of electrical activity displayed by such neurons in females and males. While both sexes displayed higher average action potential firing rates during the subjective light phase, the mean firing rate during the acrophase was significantly greater in males and the overall amplitude of the rhythm was significantly lower in females. Moreover, the proportions of active neurons (those with a rate >0.1 Hz) were also significantly greater in males than females across most of the 24 h cycle. Because VP secretion by SCN neurons is activity-dependent,[19,20] these findings provide a possible explanation for the higher levels of CSF VP achieved during the daytime in males and the lower CSF VP rhythm amplitude in females.[22] It remains to be determined if the higher firing rates of SCN VP neurons leads to greater VP release at central target sites in males.

Our analysis revealed a small but significant difference in the phase of circadian activity (∼1 h earlier in females). Although there was considerable overlap in firing rates observed at light onset, firing rates in males remained higher and decreased more slowly in males during the light–dark transition. In principle, differences in the amplitude and phase of firing rates in SCN VP neurons could play a role in the generation of sex-linked differences in circadian rhythms[35,37–40] and possibly sex-specific behaviors.[43,44] It is interesting to note that a previous study has reported women display a slightly earlier timing of various rhythms, including the peak of melatonin secretion and body temperature.[45] Additional work is required to examine the functional impact of sex differences in circadian firing by SCN VP neurons.

The mechanisms responsible for the differences in circadian activity of female and male SCN VP neurons remain to be determined. Sex differences are commonly attributable to the periodic effects of circulating estrogen whose concentration peaks during proestrus in females. The variability of firing rates observed during the acrophase was greater in females than in males (Figure 3A, B), due in part to a

TABLE 1 Percentage of active suprachiasmatic nucleus (SCN) vasopressin (VP) neurons (>0.1 Hz) per circadian times (CT) in females and males. Table shows data used to compare proportions of active neurons at different subjective CT in SCN VP neurons in slices from males and females. For each sex and CT (h), the table shows the number of neurons (n) and the percentage of these that were spontaneously active (firing rates >0.1 Hz).

	Males		Female		
CT	% active	n	% active	n	*p*-value
0	100	28	35.71	23	<.00001
1	100	12	82.61	23	<.00001
2	92.3	13	88.89	18	.6306
3	92.86	14	77.78	18	.0043
4	92.31	13	70.59	17	.0002
5	100	24	88.16	76	.0003
6	100	25	95.4	87	.0594
7	100	20	94.05	84	.0289
8	100	10	91.11	45	.0032
9	100	14	90.48	21	.0015
10	93.75	16	93.33	15	1
11	88.89	18	84	25	.4083
12	100	21	95.65	23	.1212
13	100	14	86.67	15	.0002
14	83.33	6	80	15	.7161
15	71.43	7	60	10	.1366
16	40	10	27.27	11	.0718
17	54.54	11	0	14	<.00001
18	45.45	11	5	20	<.00001
19	41.18	17	19.05	21	.0011
20	50	10	33.33	21	.0214
21	26.67	30	26.67	15	1
22	47.62	21	23.08	26	.0004
23	78.95	38	37.5	24	<.00001

Note: The *p*-value comparing the percentage of active cells at each CT (determined by individual *t*-tests) is also shown.

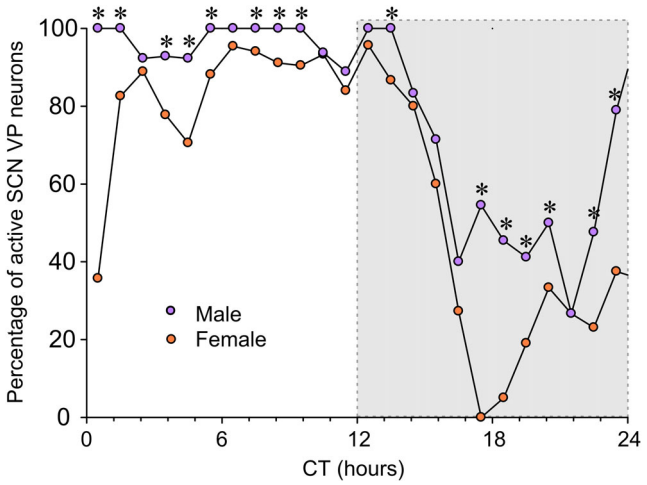

FIGURE 5 Circadian changes in proportions of active suprachiasmatic nucleus (SCN) vasopressin (VP) neurons. Plots show the proportions of spontaneously active SCN VP neurons (firing rates >0.1 Hz) as a function of CT in females (orange) and males (purple). Gray shading indicates subjective night. $^{*}p < .05$; Fisher's exact test, see details in Table 1).

significantly greater proportion of silent cells (Figure 5) and many cells firing in the 0–2 Hz range, which were much less common in males. This feature suggested the possibility that the rates of firing by SCN VP neurons might vary across the estrous cycle and include a stage when firing rates are much lower than at other times, thereby lowering the average firing rate and increasing overall variance. However, as shown in Figure 6, this hypothesis was dismissed by the observation that acrophase firing rates were not significantly different on days when estrogen levels were high (proestrus) or generally low (estrus and diestrus). Although, we found no evidence of changes associated with the estrous cycle, it remains possible that the sex differences observed in our study are due to persistent effects of estrogen pulses that occur every 4–5 days. An alternate possibility is that the difference in firing rates observed in females and males reflect a genetic dimorphism in the expression or regulation of ion channels that are important for the modulation of excitability in SCN VP neurons. Additional studies are required to test these hypotheses.

The circadian rhythmicity of the SCN is known to be affected by astrocytes.[46–49] Therefore, one possibility is that sex differences in the density or morphology of astrocytes[33,50] contribute to differences in the firing rates of female and male SCN VP neurons. Previous work has also revealed sex differences in the density and ultrastructure of synapses in the SCN of female and male rats.[32,34] Interestingly, a previous study has shown that the average daytime firing rate of unidentified neurons in the dorsal shell of the SCN is higher in males than females and reported this difference was not linked to estrous phase.[25] Notably, this previous study showed that the difference in firing rate could be abolished by bath application of a $GABA_A$ receptor antagonist, implying a difference in GABAergic tone in the two sexes. Whether sex differences in the circadian activity of SCN VP neurons reflect differences in synaptic function, or in the activity of ion channels that promote circadian changes in membrane potential and excitability,[51] remains to be determined.

Finally, another possibility is that there could be a greater degree of asynchrony among VP neurons in the SCN of females. A recent study has indicated that the expression of clock related genes in mouse SCN VP neurons is spatially and temporally asymmetric, traveling as a wave in the rostrocaudal axis.[42] As such, momentary sampling at different spatial positions during a given CT could have included cells in different phases of their individual cycle, thereby dampening the amplitude of the population's average oscillation. Further experiments are required to investigate the basis for sex differences in the circadian activity of SCN VP neurons, to establish if such differences prevail in other species, and to define the functional role of this dimorphism.

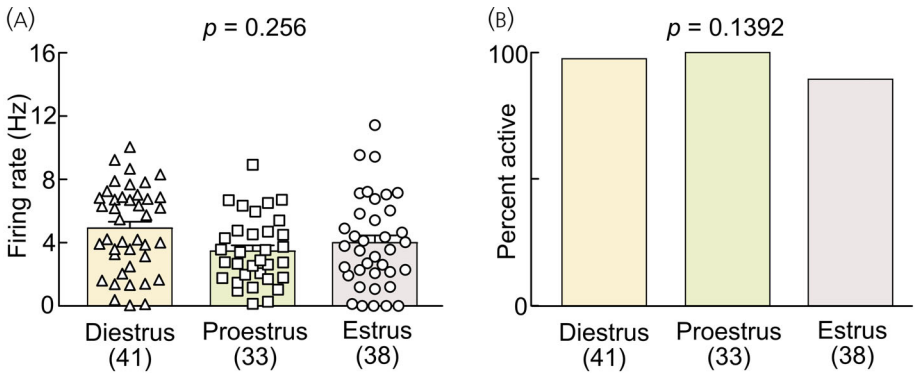

FIGURE 6 Firing rates of suprachiasmatic nucleus (SCN) vasopressin (VP) neurons at various stages of the estrous cycle in females. (A) Scatterplot (all data) and bar graphs (mean ± s.e.m.) show firing rates of SCN VP neurons (n shown in brackets) recorded during the acrophase (CT6–CT8) in slices prepared from female rats sampled during low estrogen (estrus [3 rats] and diestrus [4 rats]) and high estrogen (proestrus, 3 rats) stages of the estrous cycle (*p*-value from one-way ANOVA shown above). (B) Bars illustrate the percentage of active SCN VP neurons (firing rate >0.1 Hz) for the grouped data shown in (A) (*p*-value from chi-squared test shown above).

AUTHOR CONTRIBUTIONS

Zahra S. Thirouin: Conceptualization; data curation; formal analysis; investigation; writing – original draft. **Claire Gizowski:** Data curation; formal analysis; investigation. **Anzala Murtaz:** Data curation; formal analysis; visualization. **Charles William Bourque:** Conceptualization; formal analysis; funding acquisition; methodology; project administration; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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