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## Vasopressin resets the central circadian clock in a manner influenced by sex and vasoactive intestinal polypeptide signaling

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### Abstract

**Background/Aims**—Circadian rhythms in behavior and physiology are programmed by the suprachiasmatic nucleus (SCN) of the hypothalamus. A subset of SCN neurons produce the neuropeptide arginine vasopressin (AVP), but it remains unclear whether AVP signaling influences the SCN clock directly.

**Methods**—Here we test that AVP signaling acting through V1A and V1B receptors influences molecular rhythms in SCN neurons. V1 receptor agonists were applied *ex vivo* to PERIOD2::LUCIFERASE SCN slices, allowing for real-time monitoring of changes in molecular clock function.

**Results**—V1A/B agonists reset the phase of the SCN molecular clock in a time-dependent manner, with larger magnitude responses by the female SCN. Further, we find evidence that both G<sub>αq</sub> and G<sub>αs</sub> signaling pathways interact with V1A/B-induced SCN resetting, and that this response requires vasoactive intestinal polypeptide (VIP) signaling.

**Conclusions**—Collectively, this work indicates that AVP signaling resets SCN molecular rhythms in conjunction with VIP signaling and in a manner influenced by sex. This highlights the utility of studying clock function in both sexes and suggests that signal integration in central clock circuits regulates emergent properties important for the control of daily rhythms in behavior and physiology.

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Author Contributions Statement

Designed research: KER, JAE, Performed research: KER, TI, JAE, Analyzed data: KER, TI, JAE, Wrote paper: KER, JAE, with approval of all authors.

Statement of Ethics

All procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Animals and were approved by the Institutional Animal Care and Use Committee at Marquette University (Protocol# AR-282).

Conflict of Interest Statement

The authors have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Keywords

Vasopressin signaling; suprachiasmatic nucleus; circadian; AVP; VIP; signal integration; sex differences

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## Introduction

Daily rhythms are pervasive, regulating everything from cell division to sleep, hormone release, and cognition. Designed to maintain temporal homeostasis, daily rhythms allow organisms to anticipate environmental changes important for survival. Circadian rhythms are generated endogenously at the cellular level, driven by a molecular clock that controls the daily timing of gene expression [1]. Nearly every type of cell in the body displays daily rhythms in clock gene expression, and these mammalian clock cells form a system of tissues orchestrated by a central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. As the central clock, the SCN coordinates tissue clocks throughout the body [2] so that tissues in the system are adjusted to one another and the solar day [3, 4]. In the SCN itself, clock neurons of different types communicate with one another and send outputs to downstream tissues [5]. Network communication is critical for SCN function, and these clock neurons appear to use multiple signals to influence one another, (i.e., GABA, neuropeptides [6–10]) in ways that remain poorly defined.

The neuropeptide arginine vasopressin (AVP) is released by a large subset of SCN neurons [11, 12], but the role of this neuropeptide in regulating network function is not clear. Long recognized as an important SCN output signal [13], recent work indicates that altering the molecular clock of SCN AVP neurons will translate into changes in locomotor rhythms [14, 15] and deficits in SCN network function [16]. This complements *ex vivo* studies suggesting that AVP signaling regulates SCN function itself [9, 17–19]. Indeed, the SCN expresses both V1A and V1B receptors [19], which are G-protein coupled receptors that activate  $G_{\alpha q}$  signaling cascades [20]. Consistent with this receptor profile, V1 signaling elevates intracellular calcium and depolarizes SCN neurons [21–24]. Further,  $G_{\alpha q}$  signaling is implicated in SCN resetting [25–27], which is important for entrainment of the circadian system. Lastly, daily rhythms in locomotor behavior are altered by the loss of AVP, V1A and/or V1B [28–30], with some effects localized to the SCN itself [29] and with sex differences in the resulting phenotype [30]. Despite this work, it remains unclear whether V1 signaling directly influences the SCN molecular clock, how it might do so, and whether this response differs by sex.

Here we test the hypothesis that AVP influences the SCN molecular clock. Using PERIOD2::LUCIFERASE (PER2::LUC) mice, we measured changes in SCN molecular rhythms in response to *ex vivo* application of a V1A/B receptor agonist cocktail. V1 signaling reset the SCN molecular clock in a dose- and time-dependent manner, with the female SCN showing greater magnitude responses relative to the male SCN. Further, we find that V1A/B-induced resetting involves both  $G_{\alpha q}$  and  $G_{\alpha s}$  signaling. Lastly, V1A/B-induced SCN resetting was occluded by genetic or pharmacological abrogation of vasoactive intestinal polypeptide (VIP) signaling. These results provide novel insights

into the role of V1 signaling in the SCN network, demonstrating that resetting by this pathway is sexually dimorphic and that circuit- or cellular-level integration of multiple peptide-signaling pathways modulates circadian timekeeping in central clock neurons.

## Results

### V1A/B agonists phase delay SCN PER2::LUC rhythms in a sex-biased manner

To test whether AVP signaling affects the SCN molecular clock, a V1A/B agonist cocktail or vehicle was applied *ex vivo* to PER2::LUC SCN slices. Three consecutive SCN slices were collected through the anteroposterior axis of the network (Figure 1), and data were analyzed with a Marginal Multilevel Model to control for repeated observations collected from a single mouse (c.f., *Statistical analyses* in Methods). Following baseline recording, V1A/B agonists were applied on the fourth cycle *in vitro* at the predicted time of peak V1A expression in the SCN. Specifically, *V1a* mRNA is rhythmically expressed, with highest expression during late subjective night [31, 19], which occurs 8–10 h after peak PER2::LUC expression and the start of locomotor activity *in vivo* (i.e., Circadian Time 12, CT12). Therefore, we applied V1A/B agonists to SCN slices 10 h after peak PER2::LUC expression (CT22) to also account for the predicted 2–4 h lag required for translation [32]. Timing of V1A/B agonist or vehicle administration was predicted based on the phase and period of PER2::LUC rhythms for each SCN slice over the first three cycles *in vitro* (Figure 1, Figure 2A).

Select properties of SCN PER2::LUC rhythms differed by sex prior to drug administration. Male and female SCN did not differ in the peak time of PER2::LUC on the first cycle *in vitro* or damping rate over the first three cycles (Table S1). However, the period of PER2::LUC rhythms was longer in the female SCN before treatment ( $t(54) = -2.5$ ,  $p < 0.05$ ,  $d = 0.67$ ), which was observed across multiple experiments (Table S1). Despite the sex difference in period, the error rate for a “mock” prediction of PER2::LUC phase for peak time on the third cycle did not differ by sex (Table S1), indicating that phase can be predicted accurately in both male and female slices. Lastly, vehicle administration tended to shift the female SCN earlier than in the male SCN (Figure S2A, Marginal Multilevel Model, Sex:  $F(1,9) = 4.68$ ,  $p = 0.06$ , Slice:  $F(2,18) = 0.18$ ,  $p > 0.8$ , Sex\*Slice:  $F(2,18) = 0.28$ ,  $p > 0.7$ ; Estimated Marginal Means Contrasts:  $p = 0.06$ ,  $d = 0.67$ ), and vehicle responses were more variable for the female SCN (Figure S1B, Levene’s test,  $F(1,31) = 6.5$ ,  $p = 0.02$ ). As a result, V1A/B-induced responses were normalized by subtraction of sex-specific vehicle data (Figure 2B) to facilitate comparison of drug effects and control for sex differences in non-specific procedural responses. Raw phase resetting data are provided in supplemental materials, and analyses of non-normalized responses produced similar results.

V1A/B agonists shifted the phase of SCN PER2::LUC rhythms later (i.e., phases delayed) in a dose-dependent manner that varied by sex (Figure 2B, Marginal Multilevel Model, Concentration:  $F(4,32) = 12.1$ ,  $p < 0.0001$ , Sex:  $F(1,32) = 15.7$ ,  $p < 0.0001$ , Concentration\*Sex:  $F(4,32) = 5.3$ ,  $p = 0.002$ ). Specifically, V1A/B agonists elicited 1–2 h phase delays in female SCN at 1, 5, and 10  $\mu\text{M}$  doses (Figure 2B, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 1.14$ – $2.28$ ), whereas this treatment elicited  $< 1$  h phase delays in the male SCN at the 1 $\mu\text{M}$  and 10 $\mu\text{M}$  doses (Figure 2B, Estimated Marginal Means

Contrasts:  $p < 0.05$ ,  $d = 1.17$ – $1.60$ ). At  $10\mu\text{M}$ , V1A/B agonists phase delayed the female SCN more than the male SCN (Figure 2B, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 1.57$ ). Similar dose response curves were displayed by the three anteroposterior SCN slices (Figure S1B, Marginal Multilevel Model, Slice\*Concentration:  $F(8,33) = 0.8$ ,  $p = 0.57$ ). However, the magnitude of V1A/B-induced SCN resetting varied across this axis in a manner that interacted with sex (Figure 3A, Marginal Multilevel Model, Slice:  $F(2,32) = 6.2$ ,  $p = 0.005$ , Sex\*Slice:  $F(2,32) = 4.8$ ,  $p = 0.01$ , Sex\*Slice\*Concentration:  $F(8,32) = 0.8$ ,  $p = 0.59$ ). Relative to vehicle controls,  $10\mu\text{M}$  V1A/B agonists reset all three female SCN slices (Figure 3A, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 1.14$ – $2.72$ ), but male responses were restricted to the posterior SCN (Figure 3A, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 0.88$ ).

Next, we examined whether SCN V1A/B-induced phase resetting would be blocked by V1A/B antagonists and mimicked by V1A and/or V1B specific agonists. For V1A/B occlusion, we selected a cocktail of  $10\mu\text{M}$  V1A/B antagonists, a dose shown previously to not alter SCN phase, period, or damping when applied alone [19]. Co-administration with V1A/B antagonists blocked  $10\mu\text{M}$  V1A/B-induced SCN resetting (Figure 3B, Marginal Multilevel Model, Drug Group:  $F(2,25) = 17.4$ ,  $p < 0.0001$ , Sex:  $F(1,25) = 12.8$ ,  $p = 0.001$ , Drug Group\*Sex:  $F(2,25) = 6.0$ ,  $p = 0.007$ , Slice:  $F(2,25) = 0.3$ ,  $p > 0.7$ , Slice\*Sex:  $F(2,25) = 1.3$ ,  $p > 0.2$ , Drug Group\*Slice:  $F(4,25) = 1.7$ ,  $p > 0.1$ , Drug Group\*Slice\*Sex:  $F(4,25) = 0.6$ ,  $p > 0.6$ , Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 0.84$ – $0.85$ ). When effects of the two V1 receptors were dissected in an independent experiment, SCN resetting responses to the V1A/B cocktail and V1A agonist differed by sex (Figure S3C, Figure S2, Marginal Multilevel Model, Sex:  $F(1,67) = 7.7$ ,  $p < 0.01$ , V1 Agonist:  $F(3,67) = 6.6$ ,  $p = 0.001$ , Sex\* V1 Agonist:  $F(3,67) = 2.0$ ,  $p > 0.1$ , Sex\*Slice  $F(2,67) = 2.4$   $p = 0.1$ , V1 Agonist\*Slice:  $F(6,67) = 0.4$ ,  $p > 0.8$ , Sex\*V1 Agonist\*Slice:  $F(6, 67) = 1.2$ ,  $p > 0.3$ ). Once again vehicle responses tended to differ by sex (Figure S2, Estimated Marginal Means Contrasts:  $p = 0.08$ ,  $d = 0.44$ ), and female SCN displayed larger shifts to V1A/B treatment relative to male SCN (Figure S3C, Figure S2, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 0.42$ ). Relative to vehicle, V1A/B agonists reset the female SCN (Figure S3C, Figure S2, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 1.09$ ), but not the male SCN (Figure S3C, Figure S2, Estimated Marginal Means Contrasts:  $p > 0.3$ ,  $d = 0.24$ ). SCN responses to the V1A agonist were also sexually dimorphic. Female SCN phase shifted in response to the V1A agonist (Figure S3C, Figure S2, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 1.04$ ), but not the V1B agonist (Figure S3C, Figure S2, Estimated Marginal Means Contrasts:  $p > 0.1$ ,  $d = 0.42$ ). Male SCN did not respond to either V1A or V1B alone (Figure S3C, Figure S2, Estimated Marginal Means Contrasts:  $p > 0.7$ ,  $d = 0.11$ – $0.14$ ).

To further test the basis of V1A/B-induced phase shifts, we examined whether other SCN clock properties were affected by V1A/B agonist treatment. SCN PER2::LUC expression was not influenced acutely by V1A/B agonist administration (Figure 1, Figure 2A), nor was the damping of PER2::LUC rhythms increased by V1A/B agonists (Table S2). Administration of  $50\mu\text{M}$  V1A/B agonists decreased PER2::LUC period in female SCN (Table S2). However,  $10\mu\text{M}$  V1A/B agonists did not alter SCN period in either sex (Table S2), indicating that the phase shift in response to this dose does not reflect a change in period. Collectively, these data indicate that the  $10\mu\text{M}$  V1A/B agonist resets the molecular

clock by shifting its phase, with larger responses in the female SCN. Further, the sex difference in 10 $\mu$ M V1A/B-induced phase resetting does not appear to be driven by effects on other clock properties. Based on this pattern of results, we selected the 10 $\mu$ M dose to test how V1A/B signaling resets the SCN molecular clock.

### V1A/B-induced resetting varies across the circadian cycle in a sexually dimorphic manner

Given that SCN responses to other stimuli vary across the circadian cycle (e.g., light, VIP, melatonin), we speculated that sex-biased responses at CT22 could reflect sex differences in the daily rhythm of V1A/B-induced resetting rather than the absolute magnitude of phase shifts. To test for sex differences in the rhythm of V1A/B-induced resetting, we constructed a phase response curve (PRC) by applying V1A/B agonists to SCN slices at one of four timepoints spanning the circadian cycle. Response to vehicle alone varied by time of day, consistent with previous work [33]. Notably, time-dependent effect of vehicle interacted with sex, but not slice position (Figure S3B, Marginal Multilevel Model, CT:  $F(3,22) = 8.9$ ,  $p < 0.0001$ , Sex:  $F(1,22) = 7.7$ ,  $p = 0.01$ , Slice:  $F(2,22) = 0.0$ ,  $p > 0.9$ , CT\*Sex:  $F(3,22) = 1.0$ ,  $p = 0.4$ , Slice\*Sex:  $F(2,22) = 0.1$ ,  $p > 0.9$ , Slice\*CT:  $F(6,22) = 2.1$ ,  $p = 0.09$ , Slice\*CT\*Sex:  $F(6,22) = 0.4$ ,  $p > 0.8$ ). Thus, data for each timepoint was normalized to sex- and phase-specific vehicle controls to evaluate effects of drug application relative to non-specific factors (Figure 4A, Figure S3).

V1A/B agonists induced time-dependent resetting responses, and the overall magnitude of the V1A/B-induced resetting rhythm was larger in female SCN (Figure 4A, Figure S3A, Marginal Multilevel Model, CT:  $F(3,39) = 20.9$ ,  $p < 0.001$ , Sex:  $F(1,39) = 9.1$ ,  $p < 0.05$ , CT\*Sex:  $F(3,39) = 3.45$ ,  $p < 0.05$ , V1 Agonist:  $F(1,39) = 14.8$ ,  $p < 0.001$ , CT\*V1 Agonist:  $F(3,39) = 8.8$ ,  $p < 0.05$ , Sex\*V1 Agonist:  $F(1,39) = 4.52$ ,  $p < 0.05$ ). In addition to CT22, the female SCN responded to V1A/B agonists with phase delays at CT16 and CT04 (Figure 4A, Figure S3A, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 0.82$ – $1.12$ ), but V1A/B treatment at CT10 induced phase advances when compared to vehicle controls (Figure 4A, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 0.94$ ). In contrast, V1A/B-induced responses in male SCN were only detected at CT22 (Figure 4A–B, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 0.66$ ). When collapsed by sex, the daily rhythm in V1A/B agonist resetting resembled that for the female SCN (Figure S3B).

The daily rhythm in V1A/B-induced resetting also varied by SCN slice position (Figure 4B, Marginal Multilevel Model, Slice\*CT:  $F(6,39) = 6.3$ ,  $p < 0.001$ , Sex\*Slice\*CT:  $F(6,39) = 2.0$ ,  $p = 0.08$ , Slice:  $F(2,39) = 1.2$ ,  $p > 0.3$ , Slice\*V1 Agonist:  $F(2,39) = 0.42$ ,  $p > 0.5$ , Slice\*Sex:  $F(2,39) = 0.1$ ,  $p > 0.9$ , Slice\*CT\*Sex\*V1 Agonist:  $F(6,39) = 1.44$ ,  $p > 0.1$ ). At CT16, V1A/B-induced responses were restricted to the posterior SCN in females (Figure 4B, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 1.5$ ). In contrast, phase delays at CT04 and phase advances at CT10 were displayed by the anterior SCN (Figure 4B, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 1.58$ – $1.92$ ). A smaller, non-significant phase advance was also observed in the anterior SCN of males at CT10 (Figure 4B, Estimated Marginal Means Contrasts:  $p > 0.1$ ,  $d = 0.47$ ). Collectively, these data indicate that the magnitude of V1A/B-induced resetting is influenced by sex, and the rhythm in V1A/B-induced resetting varies in a SCN region-specific manner.

### V1A/B-induced resetting requires both $G_{\alpha q}$ and $G_{\alpha s}$ signaling

Having found V1A/B agonists reset the molecular clock in a time-dependent manner, next we decided to interrogate the required signaling cascades. V1A and V1B are both  $G_{\alpha q}$  coupled receptors, which are blocked by the Phospholipase C (PLC) inhibitor U-73122 (10 $\mu$ M, as in [26]). Given precedent that  $G_{\alpha s}$  coupled pathways are involved in SCN resetting [26], we also used the adenylate cyclase (AC) inhibitor MDL-12,330A (2 $\mu$ M, as in [26]). PLC and AC inhibition without V1A/B agonists affected SCN phase (Figure S4A), but not period or damping of SCN rhythms (Table S2). Thus, we visualized responses specific to V1A/B agonists by normalizing to inhibitor-specific controls for each sex (Figure 5A).

PLC and AC inhibitors blocked V1A/B-induced phase delays at CT22 when administered alone and in combination (Figure 5A, Marginal Multilevel Model, Inhibitor:  $F(3,52) = 15.9$ ,  $p < 0.0001$ , Inhibitor\*V1 Agonist:  $F(2,52) = 6.7$ ,  $p < 0.05$ , V1 Agonist:  $F(1,52) = 1.1$ ,  $p > 0.2$ , Sex:  $F(1,52) = 1.2$ ,  $p = 0.27$ , Inhibitor\*Sex:  $F(3,52) = 1.5$ ,  $p = 0.24$ , Slice:  $F(2,52) = 1.3$ ,  $p = 0.28$ , Slice\*V1 Agonist:  $F(2,52) = 0.3$ ,  $p > 0.7$ , Slice\*Sex:  $F(2,52) = 0.2$ ,  $p > 0.8$ , Slice\*Inhibitor:  $F(6,52) = 0.9$ ,  $p = 0.52$ , Slice\*Inhibitor\*Sex:  $F(6,52) = 0.7$ ,  $p > 0.6$ , Sex\*V1 Agonist:  $F(1,52) = 0.5$ ,  $p > 0.4$ ). Relative to V1A/B agonists alone, the PLC inhibitor suppressed V1A/B-induced phase delays in the female SCN (Figure 5A, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 1.03$ ), which was also observed with the AC inhibitor (Figure 5A, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 0.95$ ). Interestingly, applying both PLC and AC inhibitors together with the V1A/B agonists caused the SCN to shift in the opposite direction in both sexes (Figure 5A, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 1.17$ – $1.46$ ). Overall, these data suggest that  $G_{\alpha q}$  and  $G_{\alpha s}$  pathways play an ongoing role in the unstimulated SCN and that V1A/B-induced SCN resetting involves both signaling cascades. Given that V1A and V1B are both coupled to  $G_{\alpha q}$  signaling pathways, we speculated that the effectiveness of the AC inhibitor in blocking V1A/B-induced resetting may reflect a contribution of another SCN receptor mechanism.

### VIP signaling is required for V1A/B-induced resetting

Because there is precedent for AVP and VIP to interact in the SCN [34, 9, 19, 35], we next tested whether VIP was necessary for V1A/B-induced resetting using both a pharmacological and genetic approach [36, 10, 37, 35]. The latter, genetic approach involved collecting SCN slices from VIP-IRES-Cre mice, which are VIP-deficient in a copy-dependent manner [37, 35]. As expected, there were genotype differences in SCN properties prior to treatment, which interacted with sex (Table S1). Prior to V1 agonist treatment, SCN period was shorter in female mice with at least one copy of a VIP-IRES-Cre transgene (Table S1), but the male  $Vip^{cre/cre}$  SCN had a longer period (Table S1). Further, the peak time on the first cycle *in vitro* was earlier in  $Vip^{cre/+}$  and  $Vip^{cre/cre}$  female SCN (Table S1). Importantly, the ability to predict the phase of SCN PER2::LUC rhythms did not differ by genotype (Table S1), and there were no genotype or sex differences in vehicle responses (Figure S5).

Using both approaches, we found evidence that VIP signaling is required for V1A/B-induced SCN resetting. First, V1A/B-induced resetting was blocked by VIP deficiency in



the VIP-IRES-Cre model (Figure 5B, Marginal Multilevel Model, Genotype:  $F(2,45) = 13$ ,  $p < 0.0001$ , Sex:  $F(1,45) = 7.8$ ,  $p < 0.01$ , Genotype\*Sex:  $F(2,45) = 8.6$ ,  $p = 0.001$ , V1 Agonist:  $F(1,45) = 9.4$ ,  $p < 0.005$ , Slice:  $F(2,45) = 6.7$ ,  $p = 0.005$ , Slice\*V1 Agonist:  $F(2,45) = 3.07$ ,  $p = 0.06$ , Sex\*Slice:  $F(2,45) = 0.2$ ,  $p > 0.8$ , Slice\*Genotype:  $F(4,45) = 1.2$ ,  $p = 0.35$ , Sex\*V1 Agonist:  $F(1,45) = 2.8$ ,  $p = 0.1$ , Sex\*Slice\*Genotype:  $F(4,45) = 0.5$ ,  $p > 0.7$ ). Interestingly, V1A/B-induced phase shifts were eliminated in both *Vip<sup>cre/cre</sup>* and *Vip<sup>cre/+</sup>* female mice (Figure 5B, Estimated Marginal Means Contrasts:  $p < 0.05$ ,  $d = 0.96$ – $1.40$ ). Importantly, the occlusion of V1A/B-induced resetting was not attributable to genotype differences in the period response to drug application (Table S2). Complementary results were obtained with a pharmacological approach, and co-administration of the 10 $\mu$ M V1A/B agonist cocktail with the 20 $\mu$ M VPAC2 antagonist blocked SCN resetting (Figure S6, Marginal Multilevel Model, V1 Agonist\*VPAC2 Antagonist:  $F(1,61) = 4.9$ ,  $p < 0.05$ , V1 Agonist:  $F(1,61) = 2.8$ ,  $p = 0.1$ , VPAC2 Antagonist:  $F(1,61) = 7.9$ ,  $p = 0.008$ , Sex:  $F(1,61) = 5.2$ ,  $p < 0.05$ , Sex\* V1 Agonist:  $F(1,61) = 1.2$ ,  $p > 0.2$ , Sex\*VPAC2 Antagonist:  $F(2,61) = 3.4$ ,  $p = 0.07$ , Slice:  $F(2,61) = 0.6$ ,  $p = 0.54$ , Slice\*V1 Agonist:  $F(2,61) = 0.3$ ,  $p > 0.7$ , Slice\*Sex:  $F(2,61) = 0.9$ ,  $p > 0.4$ , Slice\*VPAC2 Antagonist:  $F(2,61) = 1.1$ ,  $p = 0.35$ , Sex\*Slice\*VPAC2 Antagonist:  $F(2,67) = 0.8$ ,  $p > 0.4$ ). As expected, the VPAC2 antagonist shortened SCN period regardless of V1 agonist administration (Table S2). Overall, these data indicate that V1 and VPAC2 pathways interact to reset the SCN molecular clock.

## Discussion

Here we find that V1 agonists can reset the phase of the SCN molecular clock, with a larger magnitude response in the female SCN. Interestingly, the magnitude of the sex difference in V1A/B-induced resetting was influenced by SCN slice position, which may reflect sex differences in regional V1A and/or V1B expression. Both V1A and V1B appear to contribute to SCN resetting, with an effect of V1A alone in females but not males. At the intracellular level, both  $G_{\alpha q}$  and  $G_{\alpha s}$  appear to contribute to SCN V1A/B-induced resetting, consistent with previous work indicating SCN resetting involves both these signaling pathways [26]. Lastly, we find evidence that V1A/B-induced resetting in the SCN requires VIP signaling, which suggests that AVP and VIP signaling pathways interact at the cellular and/or network level (Figure 6). Overall, this work provides insight into how AVP signaling modulates local SCN clock function, reveals that response to V1A/B signaling is influenced by biological sex, and uncovers a novel way in which neuropeptide integration modulates central clock function.

### V1A/B agonists can reset the phase of the SCN molecular clock

We demonstrate that V1A/B agonists reset the phase of SCN PER2::LUC rhythms, with more modest effects on other properties. At the 10 $\mu$ M dose selected, V1A/B agonists did not decrease the quality of PER2::LUC rhythms or alter SCN period. This may not discount potential effects of V1A/B signaling on period since repeated or long-term drug application is often required for modulation of this parameter [26, 39]. V1A/B agonists did not reset SCN PER2::LUC rhythms when co-applied with 20 $\mu$ M V1A/B antagonists, which is a dose previously shown to not alter SCN function when applied alone [19]. This indicates V1A/B receptor signaling is required for this response, which is consistent with the high specificity

and efficacy of agonists selected here [40, 41]. An important consideration is that V1A and V1B agonists can each stimulate V1A, V1B and oxytocin receptors when applied at high doses [41], oxytocin receptors have been detected in the rat SCN [42]. The interface slice preparation used here (i.e., slice suspended above medium on membrane) is expected to reduce the agonist dose within the tissue itself. In addition to V1A and V1B. Previous work indicates that SCN responses to nonapeptide ligands are largely driven by V1-induced signaling [22], but it remains difficult to dismiss potential contributions of other receptor types to the SCN response to V1A/B agonist administration. Additional limitations of this approach include damping of SCN PER2::LUC rhythms that occurs acutely after dissection, high variability in vehicle control responses, and effects of inhibitors when applied alone. Despite its limitations, continued optimization and use of this technique may be used to delineate mechanisms by which V1 signaling modulates molecular clock function.

### SCN resetting induced by V1A/B agonists is influenced by biological sex

One of the most interesting results is that V1A/B-induced resetting was larger in the female SCN than in the male SCN. In fact, the female SCN responded to the V1A/B agonist cocktail more consistently at lower doses, across experiments, and across circadian time than seen in the male SCN. Similar to the lack of large male SCN responses to V1A/B agonists, male hamsters do not display behavioral resetting when AVP is injected into the SCN region *in vivo* [43]. A sex difference in the role of AVP in circadian timekeeping is consistent with previous work using an AVP-deficient mouse [37, 30]. In this genetic model, AVP deficiency decreased the precision of free-running locomotor rhythms and increased the duration of the active phase in both sexes, but only female AVP-deficient mice displayed longer period under free-running conditions [30]. Combined with the present results, these data indicate that AVP regulates multiple properties of circadian timekeeping and that these effects can differ by sex. In non-SCN tissues, AVP neurons play sexually dimorphic roles in the regulation of social behaviors, which are thought to reflect sex differences in AVP synthesis and V1A binding [44]. It remains to be determined what mechanisms may drive sex differences in V1A/B-induced resetting of the SCN, but this represents an exciting area for future work.

Although the mechanistic basis of sex differences in V1A/B-induced SCN resetting remains to be determined, previous work indicates that daily rhythms in *Avp* and *V1a* transcription in the SCN do not differ by sex [45, 46, 44]. Nevertheless, it remains possible that there are sex differences in the expression or function of SCN nonapeptide receptors. Sex differences in the *ex vivo* SCN response to V1A/B agonist administration may reflect hormonal and/or non-hormonal mechanisms acting *in vivo* prior to tissue collection [47]. For example, the SCN expresses both testosterone and estrogen receptors, and sex steroid signaling modulates photic phase resetting *in vivo* [47]. Further, hormonal and non-hormonal mechanisms may sculpt SCN circuits in other ways. In the present work, we observed a modest, but consistent sex difference in SCN period, consistent with a trend present in the one other study that has systematically investigated sex differences in PER2::LUC rhythms [48]. It is perhaps surprising that sex differences in SCN function can be detected in the *ex vivo* preparation used here since this approach does not fully recapitulate the *in vivo* condition (e.g., lack of gonadal sex hormones), but this suggests that mechanisms driving these sex differences



produce long-lasting changes in the central clock network. Given that larger V1A/B-induced resetting in females facilitated examination of cellular mechanisms in the present work, these results highlight the benefit of routine inclusion of females in preclinical research [49]. Further work leveraging sex as a biological variable may be useful in delineating SCN mechanisms and consequences of V1 signaling on cellular clock function. Overall, the present results indicate that SCN circuitry is influenced by sex, and a deeper understanding of SCN sexual dimorphism will benefit from more studies that include both sexes [48, 50].

### **V1A and V1B contributions to V1A/B-induced SCN resetting**

Our data indicate that both V1A and V1B are required for SCN resetting in males, but V1A agonist alone can reset the female SCN. In both male and female SCN, the largest response to V1A/B agonists was observed at CT22, which is predicted to be the phase of highest V1A expression in the SCN (based on male data; [19]). Further, similar dose response curves were observed for all three SCN slices collected here, consistent with *V1a* expression spanning the anteroposterior axis of the SCN network [19]. In the male SCN, significant phase shifts at CT22 were largest in the posterior SCN, which is the region with the densest *V1a* expression [19]. Previous work suggests that *V1a* is expressed by SCN neurons that produce *Avp-Nms*, *Vip-Nms*, and *Cck-Clq13* in male mice [51], but SCN AVP receptor expression has not been systematically studied in both sexes. Further, it remains unclear which subpopulations of SCN neurons respond to V1A/B agonists in the current study because luminometry is a population-level approach that may largely reflect rhythms from the SCN shell (e.g., AVP neurons [52]). Future work using real-time imaging techniques may be used to visualize the spatiotemporal kinetics of V1A/B-induced SCN resetting and clarify the identity of V1A/B-responsive SCN neurons in each sex [39, 16].

### **SCN response to V1A/B signaling most resembles that induced by non-photoc stimuli**

In addition, we investigated how V1A/B-induced resetting varies over the circadian cycle to test how this signal is interpreted by the SCN. Time-dependent responses is a classic feature of circadian stimuli, with the magnitude and direction of phase resetting varying over the circadian cycle. This type of daily rhythm is driven by cellular signaling mechanisms and is most often illustrated using a phase response curve [53]. There are two main types of stimuli that have been classified in the field: photic and non-photoc (e.g., arousal, food, temperature). Both photic and non-photoc resetting is characterized by time-dependent responses, although the nature of these rhythms differs based on stimulus class [53–55]. Whereas responses to photic stimuli are typically restricted to subjective night [53, 56], non-photoc stimuli often induce responses during both subjective day and night [53, 54, 56, 55]. We find here that the rhythm in V1A/B-induced SCN resetting resembles that for a non-photoc stimulus, with phase delays elicited during subjective night and phase advances elicited during subjective day. In contrast, photic stimuli induce phase delays during early subjective night and phase advances during late subjective night [53, 54, 56, 55]. With this in mind, it is also of interest that application of V1A/B agonists in the current study did not induce acute *PER2::LUC* expression. Photic stimuli up-regulate *PER* and other immediate early genes to shift the molecular clock [57–60], whereas non-photoc stimuli down-regulate immediate early genes or have no immediate effect [61–64]. Overall, the SCN response to AVP appears to be distinct from that induced by other SCN peptides. For example, VIP

and GRP are most often associated with light-like resetting responses [65, 66, 26, 67] and induction of immediate gene expression [68, 69, 39]. Given the general similar resetting response to AVP and other signaling molecules that provide feedback to the SCN (e.g., melatonin, NPY, 5HT), this suggests the interesting possibility that AVP regulates SCN function by acting like a local feedback signal.

### V1A/B-induced SCN resetting involves both $G_{\alpha q}$ and $G_{\alpha s}$ signaling

We also find that V1A/B agonists administered at CT22 failed to delay the SCN when PLC and/or AC was inhibited. These results suggest that multiple signaling cascades are required for V1A/B-induced resetting. Previous work indicates that PLC and AC inhibition blocks VIP-induced SCN resetting [26], and PLC is required for *Per1/2* induction [69]. PLC/AC inhibitors affected SCN rhythms without administration of V1A/B agonists and co-administration of V1A/B agonists with PLC+AC inhibitors reversed the direction of SCN V1A/B-induced phase resetting. The response to PLC/AC inhibitors alone makes it difficult to cleanly parse the role of these pathways in V1A/B-mediated responses, but these results suggest that  $G_{\alpha q}$  and  $G_{\alpha s}$  signaling influence SCN phase under basal conditions in ways that are independent of AVP. Consistent with an ongoing role of these intracellular signaling pathways, the SCN displays daily rhythms in the phosphorylation of PLC and CREB that are important for SCN clock function [70–72]. Previous work using these same compounds found that these inhibitors at these doses did not phase shift PER2::LUC rhythms when applied at CT12 [26], further suggesting that the ongoing activity of these pathways may be rhythmic. The identity of downstream effectors through which  $G_{\alpha q}$  and  $G_{\alpha s}$  pathways may intersect with one another to reset SCN molecular clock warrants further investigation, as does the potential role of additional signaling pathways not studied here [39, 73].

### V1A/B-induced SCN resetting requires VIP signaling

Using two complementary approaches, we find that VIP signaling is necessary for V1A/B-induced SCN resetting. Specifically, V1A/B-induced resetting was blocked by both co-administration of VPAC2 antagonists and genetic VIP deficiency. In the latter study, neither *Vip<sup>cre/cre</sup>* or *Vip<sup>cre/+</sup>* SCN responded to V1A/B agonists, suggesting that even partial loss of VIP [35] is sufficient to block V1A/B effects. Although *Vip<sup>cre/+</sup>* mice do not display a phenotype in basic circadian phenotyping assays, *Vip* and VIP expression is reduced in *Vip<sup>cre/+</sup>* mice [37, 35]. Unlike *Vip<sup>-/-</sup>* mice [19], *Vip<sup>cre/cre</sup>* and *Vip<sup>cre/+</sup>* mice do not show pronounced deficits in AVP signaling or SCN function [37, 35], making this an excellent model for use in the present studies. Collectively, these results indicate AVP and VIP signaling interact, which is consistent with their overlapping time of neuropeptide and receptor expression. Both AVP and VIP are produced during late subjective day, with peak receptor expression for each occurring during mid-to-late subjective night [31, 45, 74, 46, 51]. Similarly, both AVP and VIP when applied separately cause the SCN to phase delay during early subjective night [75, 76]. However, the idea that these two signals interact in a cooperative manner is difficult to reconcile with previous work indicating that AVP and VIP influence SCN neurophysiology in distinct ways. For instance, VIP decreases firing rate in SCN neurons [77], whereas AVP increases firing rate [22]. Likewise, AVP and VIP elicit opposite calcium responses, with VIP reducing intracellular calcium during subjective day and AVP increasing intracellular calcium during subjective day and night [24]. Further,

AVP and VIP produce distinct effects on the SCN molecular clock, with VIP producing light-like resetting, lengthened period, and acute PER induction [39]. Although opposite SCN responses to AVP and VIP signaling would be predicted to abrogate one another, there is precedent for different classes of stimuli producing antagonist or synergistic effects depending on the timing of stimuli administration [78–82].

Although little is known about how AVP and VIP may interact to reset the SCN molecular clock, the underlying cellular mechanism could involve integration at the intracellular and/or intercellular levels (Figure 6). At the intracellular level, V1 and VPAC2 signaling could interact via transcriptional (1) or cytosolic synergy (2) to phase shift the SCN molecular clock (Figure 6). At the intercellular level, AVP and VIP signaling may interact through network-level circuits (Figure 6). In this second model, V1 signaling would cause the release of VIP to shift the SCN molecular clock, and the nature of this response would be predicted to resemble that elicited by VIP alone. Thus, the observation that V1A/B-induced SCN resetting is distinct from that elicited by VIP [26, 67] may provide evidence against a model involving only network-level integration. Further, SCN electrical responses to AVP are not abolished in *Vipr2* knockout mice [83], suggesting that V1A/B-induced signaling is able to influence SCN properties in the absence of VIP signaling. Thus, intracellular integration of AVP and VIP signaling seems the more parsimonious model to account for the present results. Although it is not known if *V1a* and *Vipr2* are co-expressed in single SCN neurons, both *V1a* and *Vipr2* are expressed by *Avp-Nms* and *Vip-Nms* SCN neurons [51]. Thus, future work could test the validity of the intracellular integration model for AVP and VIP signaling by examining whether *V1a* and *Vipr2* are co-expressed in time and space at the single cell level in both sexes.

## Methods

### Mice and husbandry conditions

Homozygous PERIOD2::LUCIFERASE (PER2::LUC) mice [84] were bred and raised under a 24-hour light-dark cycle with 12 hours of light and 12 hours of darkness (LD12:12; lights off: 1800 CST, defined as Zeitgeber Time 12, i.e., ZT12). Throughout life, ambient temperature was maintained at 22°C ± 2°C, and mice had *ad libitum* access to water and food (Teklad Rodent Diet 8604). Founder homozygous VIP-IRES-Cre mice were obtained from Jackson Laboratory (*Vip*<sup><tm1(cre)Zjh>/J</sup>; Stock No: 010908) and bred to PER2::LUC mice for at least 4 generations. At weaning, mice were group housed in cages without running wheels.

### PER2::LUC recording

Coronal SCN slices (150 µm) were collected from PER2::LUC mice (10–18 weeks old) and cultured as described previously [19]. Briefly, male and female mice were anesthetized with isoflurane and sacrificed using cervical dislocation 4–6 h before lights off to minimize dissection-induced resetting [33]. Three consecutive SCN slices were collected using a vibratome (Leica VT1200S) and trimmed by hand under a dissecting microscope. After collection, SCN slices were placed on a membrane insert in a dish containing 1.2mL of luciferin-treated recording medium: air-buffered Dulbecco's modified explant medium

(DMEM, Sigma D2902) supplemented with 0.1mM beetle luciferin, 0.02% B27 (Gibco 17504), 0.01% HEPES (Gibco 15630), 0.005% NaCHO<sub>3</sub> (Gibco 25080), 0.004% Dextrose (Sigma G7021), and 0.01% penicillin/streptomycin (Gibco 15140). SCN were cultured at 37°C for at least 6 days with a luminometer (Actimetrics, LumiCycle 32).

## Pharmacology

Drugs were added to the media after three cycles *in vitro* and not washed out. To assess how AVP signaling resets the molecular clock, responses were compared between samples receiving vehicle control (<0.001% DMSO) and a drug cocktail of AVP agonists targeting both V1A and V1B receptors (V1A-[Phe<sub>2</sub>, Ile<sub>3</sub>, Orn<sub>8</sub>]-Vasopressin, Phoenix Pharmaceuticals, Inc., Cat#065–30; V1B-d[Leu<sub>4</sub>, Lys<sub>8</sub>]-VP, Tocris, Cat#3127). To test the necessity of V1 signaling, a subset of samples were co-treated with a drug cocktail of V1A and V1B antagonists at the time of V1 agonist administration (V1A-OPC21268, 10μM, Tocris, Cat# 3924; V1B-SSR149415, 10μM, Axon Medchem Cat# 1116, as in [19]). In addition, we tested involvement of different intracellular signaling cascades [26] using inhibitors of phospholipase C (U-73122, 10μM, Sigma, Cat#U6756) and adenylate cyclase (MDL-12,330A, 2μM, Sigma, Cat#M182). Lastly, the role of VIP signaling was tested using the VPAC2 antagonist [4Cl-D-Phe<sub>6</sub>,Leu<sub>17</sub>] VIP (20μM, Tocris, Cat# 3054, as in [35]). SCN slices treated with 0 and 10uM V1A/B agonists were collected at CT22 in two independent replicates each spanning a two-year period to serve as negative and positive controls for experiments conducted in parallel.

## PER2::LUC analyses

PER2::LUC rhythms were analyzed with Lumicycle- and Excel-based analyses, as in previous work [38, 10]. The PER2::LUC rhythm for each sample was detrended and fit with a damped sine wave on the three cycles before and after drug administration, and the time of peak PER2::LUC expression were determined to quantify SCN resetting. The circadian time (CT) of drug administration was projected using the average cycle-to-cycle period length and the peak time on the last cycle prior to drug application. The shift in the PER2::LUC rhythm was measured as the discrepancy between the projected peak time versus actual peak time after treatment. In addition, period, goodness of fit, and damping rate (i.e., number of days for rhythm amplitude to decrease ca. 37%) was recorded from sine wave fit to cycles before and after drug administration. Drug-induced changes in period were quantified by the difference between post-treatment period and pre-treatment period (positive values indicate period was lengthened).

## Statistical analyses

Data are represented in figures and tables as mean ± SEM. Statistical analyses were performed with JMP software (SAS Institute, Cary, NC) and SPSS software (IBM Corporation, Armonk, NY). To examine group differences in pre-treatment PER2::LUC rhythms, data from three slices/mouse were averaged, then assessed with Student's t test, one-way ANOVA or Full Factorial ANOVA as appropriate (Table S1). To examine post-treatment responses, resetting data from all three slices were included in a Marginal Multilevel Model, which is a Full Factorial Linear Mixed Model well suited to analyze datasets that lack independence, sphericity, and/or compound symmetry due to collection of

multiple observations from individuals (i.e., Slice Position, Anterior, Middle, and Posterior SCN). For each experimental dataset, the Marginal Multilevel Model included all relevant factors: 1) Biological Sex, 2) V1A/B Dose, 3) Antagonist, 4) Circadian Time, 5) VIP Genotype and/or 6)  $G_{\alpha q}/G_{\alpha s}$  Intracellular Inhibitor. Post hoc tests were performed with Estimated Marginal Means Contrasts and one-sample t tests. Effect sizes of pairwise comparisons were estimated with Cohen's d. To facilitate comparison of drug effects across sex, data from each vehicle control was subtracted in a sex-specific manner for main figures. Raw phase shifting data is provided supplemental materials. Marginal Multilevel analyses of both raw and normalization data yielded similar conclusions. Post-treatment resetting, period modulation, and damping data (averaged across slice position) are provided in Table S2 for all experiments. Statistical significance was set at  $p < 0.05$ .

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data Availability Statement

All data generated during this study are included in this article or its supplementary material files. Data can be obtained from the corresponding author upon request.

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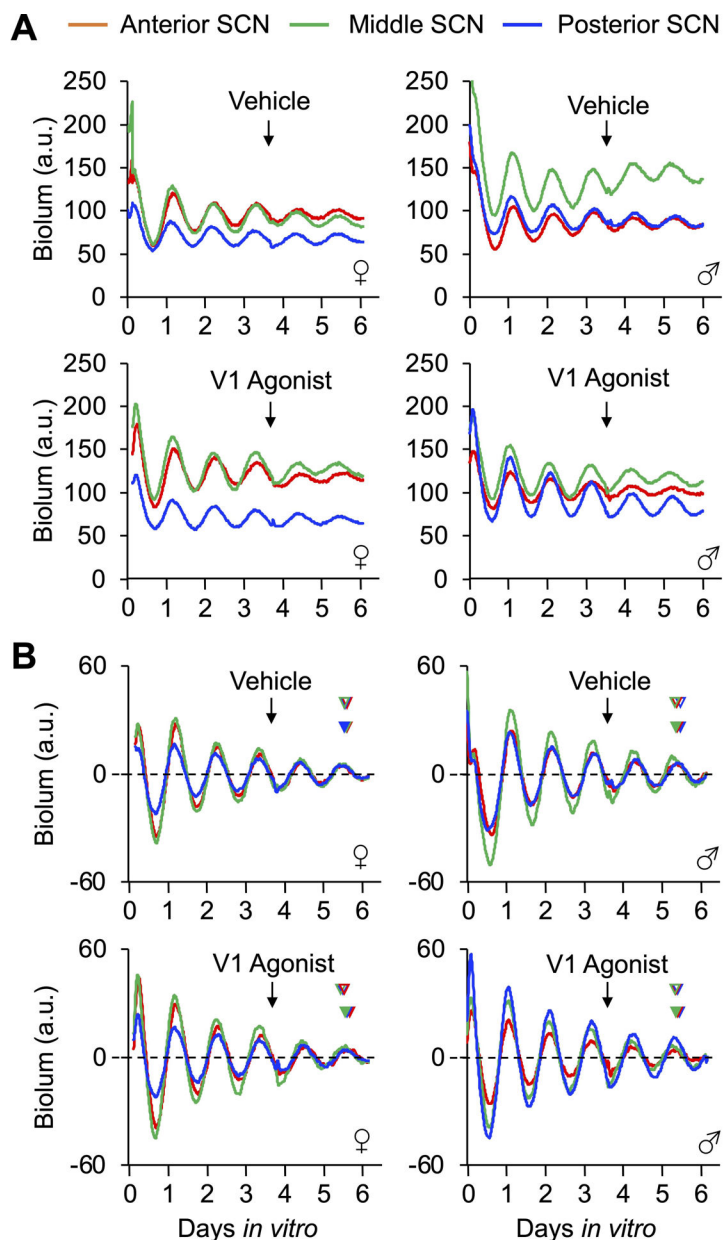


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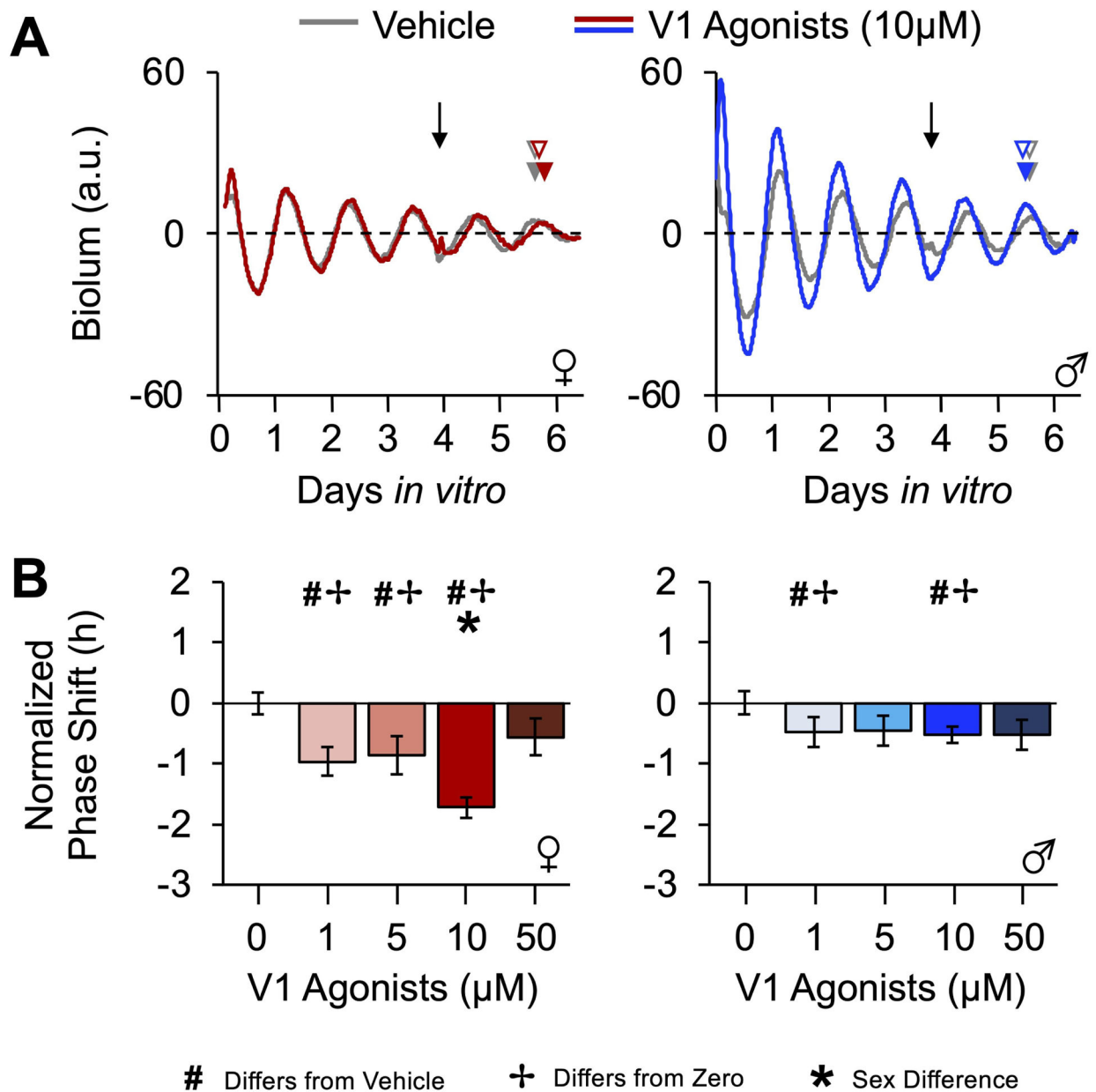
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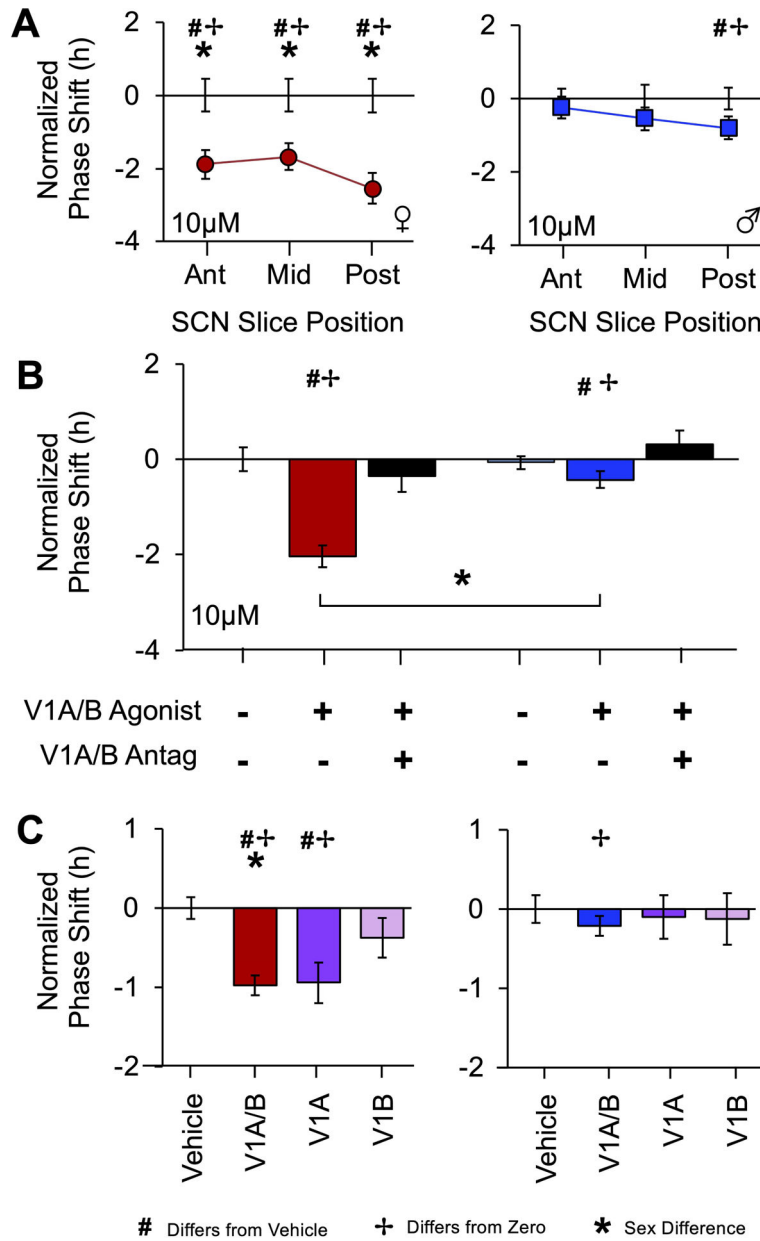
**Figure 1. PER2::LUC rhythms in vehicle- and 10 $\mu$ M V1A/B-treated slices collected from the anterior, middle, and posterior SCN of adult male and female mice.**

A. Absolute PER2::LUC levels in three slices collected from a mouse in each group. B. Baseline-subtracted PER2::LUC rhythms in the same three slices in A). Arrow represents time of treatment *in vitro* for each panel. In B, predicted and actual post-treatment peak times are indicated by open and filled arrowheads, respectively (color coded by slice).



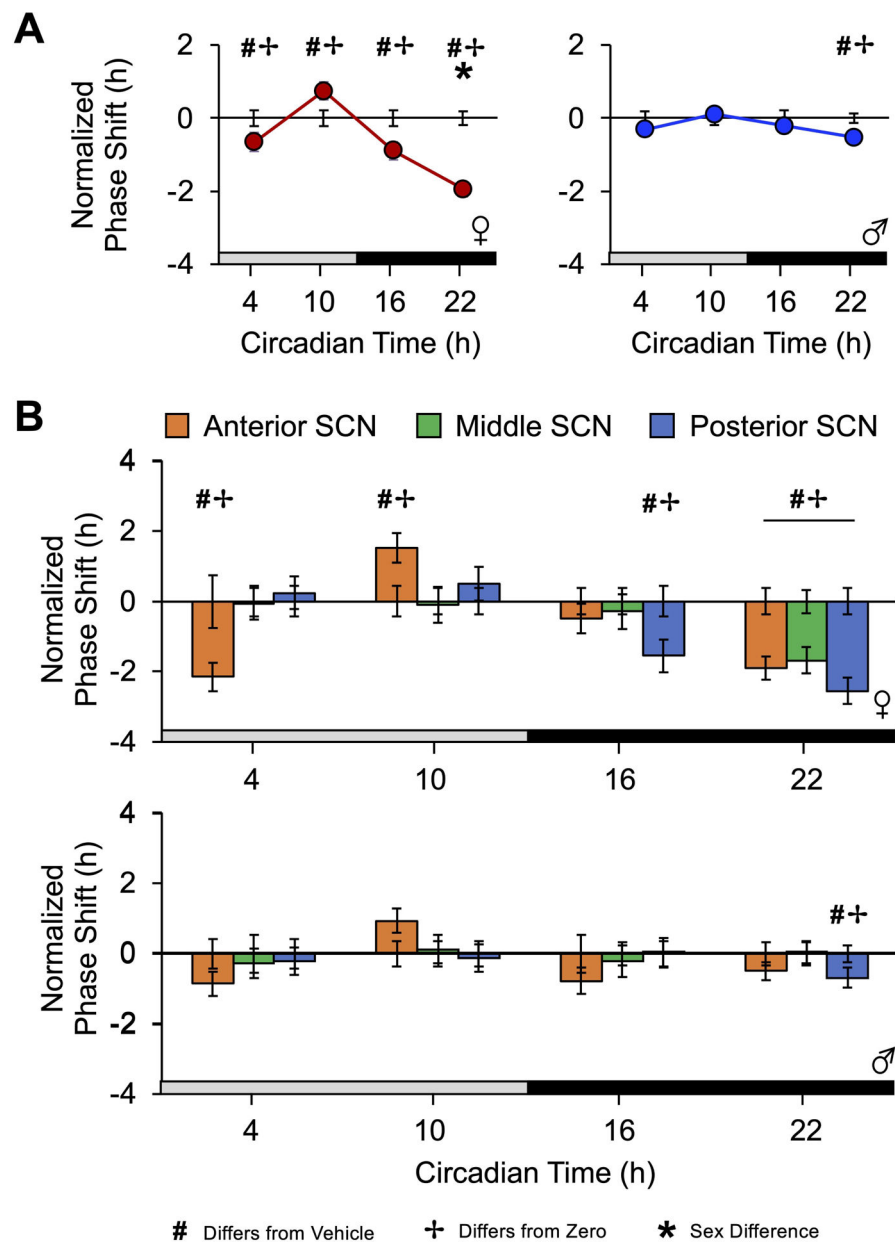
**Figure 2. V1A/B agonists reset SCN PER2::LUC rhythms in a dose- and sex-dependent manner.** A. Representative, baseline-subtracted time series illustrating PER2::LUC rhythms from posterior SCN slices treated with vehicle or 10µM V1A/B agonists. Arrow represents time of treatment for each panel. Predicted and actual post-treatment peak times are indicated by open and filled arrowheads, respectively (color coded by sample). B. SCN PER2::LUC rhythms are reset by V1A/B agonists in a dose-dependent manner influenced by sex. # Different from 0µM, Estimated Marginal Means Contrasts:  $p < 0.05$ . \* Different by sex, Estimated Marginal Means Contrasts:  $p < 0.05$ . + Different from zero, Single sample t-test:  $p < 0.05$ . For raw resetting data c.f., Figure S1.





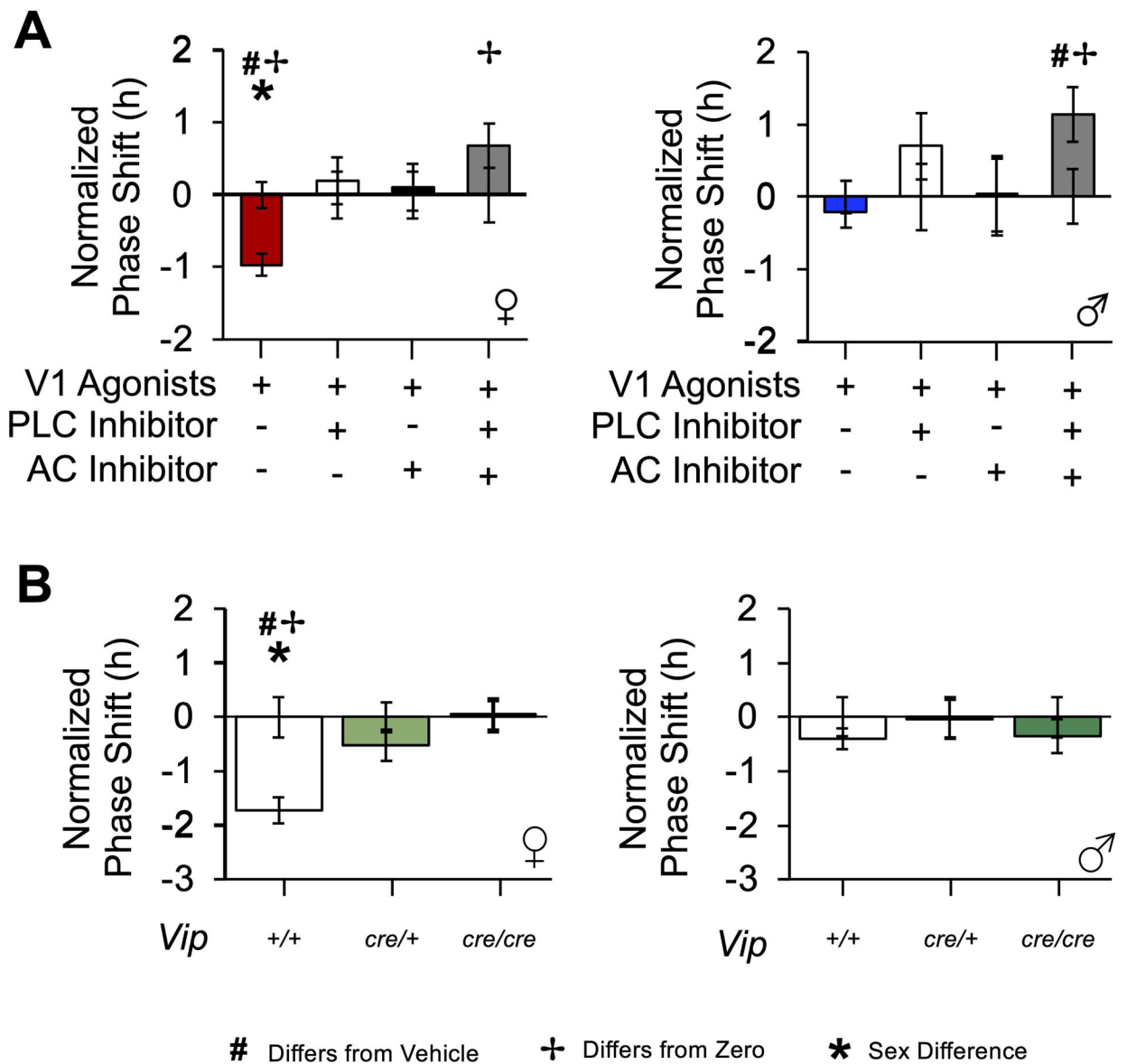
**Figure 3. SCN responses to 10µM V1A/B agonists varies by region and is blocked by co-culture with 10µM V1A/B antagonists.**

A. Resetting after 10µM V1A/B agonist administration was consistently larger in female SCN compared to males, with significant resetting responses across the anteroposterior axis in female SCN and only posterior responses in male SCN. Error bars plotted on the abscissa represent variability for vehicle controls. B. Female SCN displayed larger resetting responses to 10µM V1A/B agonists, and this response was blocked by V1A/B antagonist co-administration. C. Responses to V1A/B and single receptor agonists differ by sex. Note 0 and 10µM V1A/B data in A-B are re-plotted from Figure 2. # Different from 0µM, Estimated Marginal Means Contrasts:  $p < 0.05$ . \* Different by sex, Estimated Marginal Means Contrasts:  $p < 0.05$ . + Different from zero, Single sample t-test:  $p < 0.05$ . For raw resetting data c.f., Figures S1–2.



**Figure 4. V1A/B agonists reset SCN PER2::LUC rhythms in a time-, sex-, and region-specific manner.**

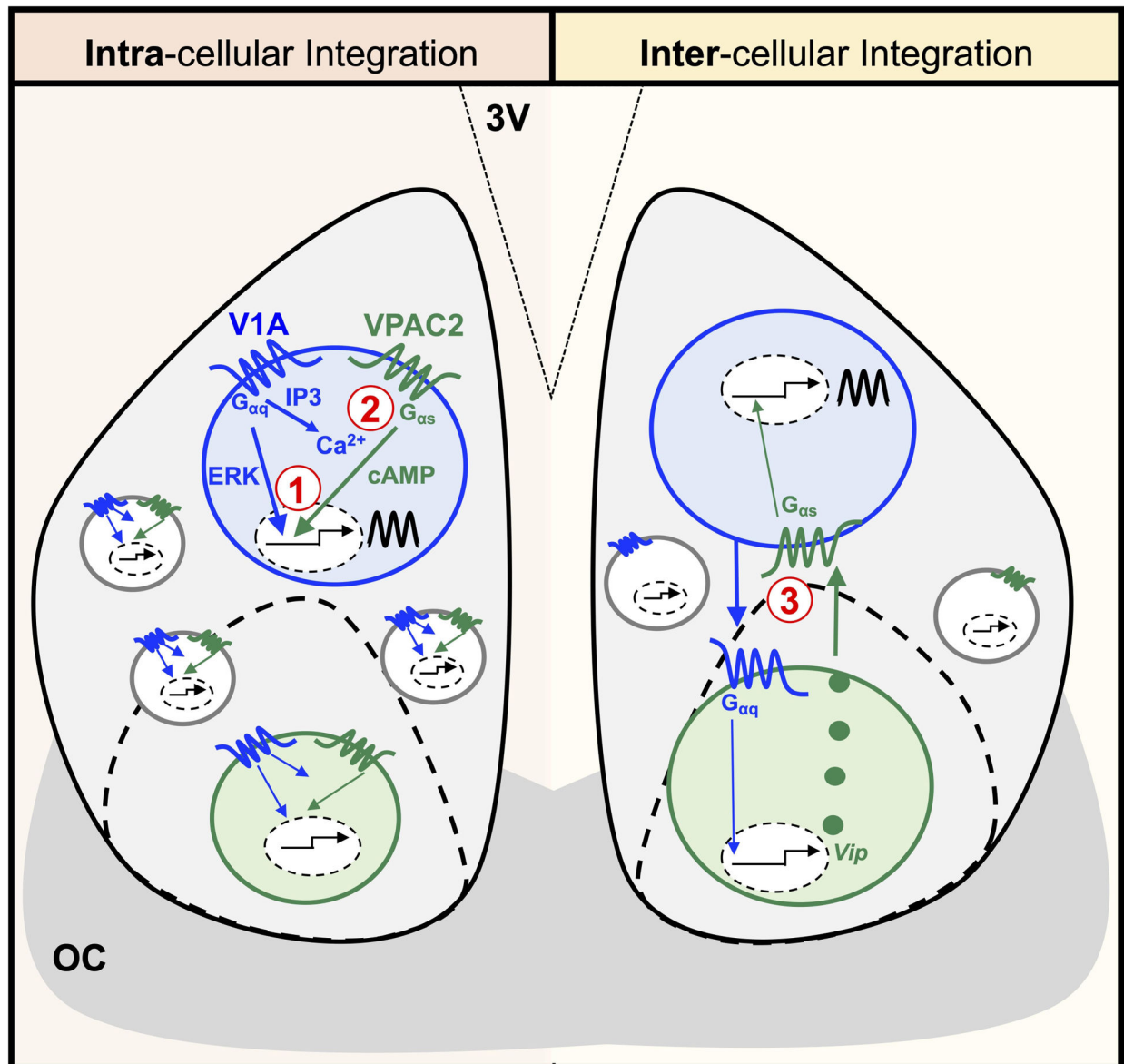
A. The V1A/B agonists resetting response across four times spanning the circadian cycle divided by sex. B. V1A/B-induced resetting varies by SCN region in each sex. Error bars plotted on the abscissa represent variability for vehicle controls. Note CT22 0 and 10 $\mu$ M V1A/B data are re-plotted from Figure 2. # Different from vehicle, Estimated Marginal Means Contrasts:  $p < 0.05$ . \* Different by sex, Estimated Marginal Means Contrasts:  $p < 0.05$ . + Different from zero, Single sample t-test:  $p < 0.05$ . For raw resetting data c.f., Figure S3.



**Figure 5. V1A/B agonists do not reset SCN PER2::LUC rhythms in the absence of  $G_{\alpha}$  signaling or VIP.**

A. V1A/B agonists do not phase delay PER2::LUC rhythms at CT22 when PLC or AC signaling is blocked. B. V1A/B-induced resetting is eliminated in the VIP-Cre SCN at CT22. Error bars plotted on the abscissa represent variability for vehicle controls. \*

Different by sex, Estimated Marginal Means Contrasts:  $p < 0.05$ . \* Different from zero, Single sample t-test:  $p < 0.05$ . For raw resetting data c.f., Figures S4-5.



**Figure 6. Putative intracellular and intercellular mechanisms by which AVP and VIP signaling interact to reset the SCN molecular clock.**

Left: At the single cell level, temporal and spatial co-expression may lead to interaction between V1- and VPAC2-induced signaling cascades. 1) Transcriptional integration: V1-activated extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) and VPAC2-activated cAMP-response element binding protein (CREB) signaling may synergistically regulate gene expression to shift the SCN clock. 2) Cytosolic integration: V1-activated diacylglycerol (DAG)/inositol triphosphate (IP<sub>3</sub>) could increase intracellular calcium to potentiate VPAC2-activated cAMP regulation of gene expression to shift the SCN clock. Right: At the network level, AVP and VIP signaling may interact through poly-synaptic circuits involving V1-induced up-regulation of *Vip* to shift the SCN clock. Note: Interactions between AVP and VIP neurons could occur either directly as shown or indirectly via connections with other SCN neurons. Further, other intracellular and

intercellular signaling pathways may also be involved (not shown). 3V = third ventricle. OC = optic chiasm.

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