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Functional peptidomics: Stimulus- and time-of-day-specific peptide release in the mammalian circadian clock

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Abstract

Daily oscillations of brain and body states are under complex temporal modulation by environmental light and the hypothalamic suprachiasmatic nucleus (SCN), the master circadian clock. To better understand mediators of differential temporal modulation, we characterize neuropeptide releasate profiles by non-selective capture of secreted neuropeptides in an optic nerve-horizontal SCN brain slice model. Releasates are collected following electrophysiological stimulation of the opticnerve/retinohypothalamic tract under conditions that alter the phase of SCN activity state. Secreted neuropeptides are identified by intact mass via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). We found time-of-day-specific suites of peptides released downstream of optic-nerve stimulation. Peptide release was modified differentially with respect to time-of-day by stimulus parameters and by inhibitors of glutamatergic or PACAPergic neurotransmission. The results suggest that SCN physiology is modulated by differential peptide release of both known and unexpected peptides that communicate time-of-day-specific photic signals via previously unreported neuropeptide signatures.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information includes both Figure S1 Stimulusevoked peptide release, and Table S1, a list of unknown m/z values detected in releasates.

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Keywords

circadian clock; mass spectrometry; neuropeptidomics; optic nerve; suprachiasmatic nucleus; SCN

INTRODUCTION

Biological processes, from tissue-specific gene ex pression to organismic behavior, display daily, near-24-h circadian rhythms[1–3]. The suprachiasmatic nucleus (SCN) of the ventral hypothalamus is the primary site that orchestrates circadian rhythms in mammals. Comprised of ~20,000 cells[4–5] ,the mammalian SCN generates timekeeping signals and regulates timing homeostasis through integration of myriad neuronal and humoral signals, including information about environmental light. Time-of-day-spe- cific responses of the SCN then align internal circadian rhythms with the natural cycle of darkness and light.

Environmental light signals are communicated directly to the SCN by the retinohypothalamic tract (RHT). Axons of intrinsically photoreceptive retinal ganglion cells (ipRGCs) project to the SCN via the optic nerve (ON), transmitting light information directly from the retina to the hypothalamus. Within the SCN, light information is processed and integrated into time-of-day- specific adjustments of the SCN time-base[6–8]. Photosensitivity of ipRGCs adapts to background light levels, which leads to differential patterns of action potentials that communicate light information to the SCN[9–11]. Glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) are the chemical messengers of light information from synapses of the ipRGC onto SCN neurons[12–14]. Mechanisms of circadian sensitivity and responses of the SCN to these two neurochemicals are well studied 15. Light has different effects on circadian timing that depend on time-of-day[15], and the RHT innervates only a subset of SCN neurons[16]. Consequently, some form(s) of inter-cellular communication must coordinate signaling and subsequent light-phase alignment among the ~20,000 cells of the SCN.

While mechanisms such as electrical coupling[17–18] and inhibitory neurotransmission[19] have some role in SCN intercellular communication, mounting evidence points to diffusible signaling as a key contributor to the SCN re sponses to photic signals[20–21]. Endogenous peptide expression among SCN neurons is heterogeneous[4,22–24] and neuropeptides have been implicated in altering the phase of the circadian oscillation generated in the SCN, *i.e.*, phase-shifting[25–28]. Gastrin-releasing peptide (GRP), vasoactive intestinal polypeptide (VIP), and little SAAS are expressed within retinorecipient SCN neurons and are involved in SCN feed-forward relay of phase shifting signals[29–31]. The roles of these peptides in intercellular SCN communication have been investigated using a combination of both genetic knockout and exogenous *in vitro* chemical stimulation,[27,30–34] however confirmatory peptide release from the SCN downstream of light signal innervation has not been demonstrated.

Mass spectrometry-based approaches are effective for identifying suites of neuropeptides, the approach known as peptidomics[35–37]. Over the past decade we have performed extensive global characterization of the peptides contained within the SCN using these approaches[35–37]. However, such global measurements do not provide functional

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information. Thus, we have measured the neuropeptides released at the SCN following time-of-day-specific and light signal-associated stimulation conditions[38]. Unlike immunobased assays, our strategy utilizes non-selective capture and unbiased identification of neuropeptides secreted into the media. Released neuropeptides are collected at specified time intervals using C18-containing ZipTips™ positioned above the tissue region of interest, and captured releasates are eluted off-line and identified by intact masses measured by MALDI-TOF mass spectrometry[38]. Thus, heterogeneous release profiles of secreted peptides can be measured directly in spatial, temporal, and stimulation-dependent contexts without *aprior* knowledge of the secretomes[15]. Limitations in releasate abundances prevent direct MS/MS measurements on the releasate. Here we characterize peptides by comparison of intact mass values in releasate mass spectra to those reported from our high-resolution proteomic/peptidomic discovery studies using isolated subhypothalamic nuclei[23–24, 39–40] These analytical chemistry protocols permit secreted peptide profiling and identification with spatial-, temporal-, and stimulation-dependent resolution[15]. We previously demonstrated endogenous peptide release could be detected from the SCN in *ex vivo* hypothalamic slice[38]. Building on this experimental paradigm, we here utilize a horizontal hypothalamic brain slice - retaining the SCN with ON and RHT innervation - to evaluate whether SCN peptide release is detected following different light signal-associated stimulus conditions that elicit circadian phase shifting of the SCN circadian clock.

RESULTS AND DISCUSSION

To determine the parameters of electrical stimulation of the ON that evoke phase-shifting of the SCN (Fig. 1, 2A), the CT of the subsequent peak of the mean spontaneous firing of the SCN neuronal population was compared with the CT under control conditions. In the unstimulated SCN, the spontaneous firing rate peaked during the mid-subjective daytime at CT 6.1 ± 0.28 (n = 5; Fig. 3A, O). When the ON was stimulated (5 V, 20 Hz, 1 ms pulse duration, Fig. 2A) at CT 6, the spontaneous firing rate peak advanced in phase compared to control (+2.85 ± 0.25 h, n = 3, $p < 0.001$; Fig. 3B, O). This phase-shift is comparable in direction and magnitude to that induced by exogenous PACAP applied to the SCN in a brain slice at the same CT[41–42].

The efficacy of various stimulus parameters was explored with regard to degree of phase shift and potential signaling pathways.[43] A 5 Hz stimulation applied at CT6 had no effect on the subsequent peak of circadian activity; however, a 20 Hz stimulation at CT 6 produced a phase advance that was blocked completely in the presence of a selective PACAP inhibitor, 10mm PACAP 6–38. PACAP 6–38 applied alone at CT6 had no effect on clock phase.

Previous research has demonstrated that exogenous glutamate, applied directly to SCN *in vitro* during the subjective nighttime, evokes phase delay when administered at CT 14 whereas at CT 19 causes phase advance[12,26]. We found that comparable time-dependent phase shifts could be induced in the horizontal SCN slice when ON stimulation is applied. Stimulation at CT 14 (1 V, 10 Hz, 0.2ms pulse duration, Fig. 2A) elicited a phase delay (−1.65 ± 0.5 h, n = 10, $p < 0.001$; Fig. 3C, O). During late subjective nighttime (CT 19), two ON stimulation parameters were effective in phase-advancing the rhythm of spontaneous SCN firing rate. Stimulating the ON at 5 V, 5 Hz, 1 ms pulse duration (Fig. 2A) triggered a

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glutamate-like phase shift in direction and magnitude (+2.18 ± 0.30 h, n = 6, $p < 0.001$; Fig. 3D, O). Increasing stimulus frequency to 20 Hz at CT 19 (Fig. 2A) generated a potentiated phase advance (+5.95 ± 0.29 h, n = 5, $p < 0.001$; Fig. 3E, O). Stimulation parameters that caused a phase advance at CT 19 were ineffective when applied at CT 14 (+0.27 ± 0.52 h, n= 3; Fig. 3F, O). Similarly, stimulus parameters effective CT 14 did not cause phase-shifting of the firing rate rhythm when applied at CT 19 (+0.18 ± 0.52 h, n = 3; Fig. 3O). This is consistent with the established time-dependency and directionality of the SCN to glutamate-induced phase-shifting during the nighttime 12. Physically crushing the ON prevented phase shifting via electrical stimulation (−0.07 ± 0.52 h, n =3; Fig. 3G, O). This demonstrates that the electrical stimuli act via neurotransmission, rather than through current spread, and their effects represent physiological correlates of time-of-day ipRGC photosensitivity[9–11] and light-stimulated activation of the retinohypothalamic tract. The electrical stimuli, therefore, evoke acute light-like responses and subsequent phase shifting in the SCN[12].

Next, we coupled electrical stimulation of the ON with ZipTip capture above the SCN region and subsequent MALDI TOF MS analysis to characterize neuropeptide release profiles following ON-stimulation paradigms that shift the phase of SCN activity. ON stimulation parameters that were effective in phase-shifting at CT 6 (Fig. 3I), CT 14 (Fig. 3J), and CT 19 (5 Hz and 20 Hz, Fig. 3K and L, respectively) were correlated with distinct peptide release profiles in a stimulus- and time-of-day-specific manner ($n \geq 3$ for all releasate sample conditions, Fig. 3B). Peptide-release profiles obtained from tissue subjected to stimulation parameters ineffective at phase shifting or ON crush (Fig. 3M and N) were comparable to the pre-stimulation releasate sample control (Fig. 3H). Previously, we have performed a number of studies on the peptides contained within SCN tissue using a variety of high resolution tandem MS approaches; the amount of peptide released from the brain slice in response to the electrical stimulation precludes the use of tandem MS 23–24’ 39–40. Here we combine our lists of previously identified SCN peptides with release profiles to assign putative masses to known SCN peptides (Table 1). Despite our detailed prior studies, we detect a number of m/z values that are not in our databases. This may be due to differences in the ionization method used. Our previous assignments were based on electrospray ionization MS whereas the current studies required the superior sensitivity of MALDI-TOF MS, which has high ion transmission rate and lowest residence time in the vacuum. Also, the previous studies analyzed tissue punches of SCN from coronal brain slices compared with the present study which analyzed SCN with attached optic nerves, wherein distinct peptides could be contained within the RHT and/or horizontally sliced SCN. These unassigned masses are listed in Supplemental Information (SI, Table 1).

What are the functions of these peptides? A number of the molecular masses identified in stimulated releasate correspond to known, physiologically relevant peptides within the SCN. A salient example is *arginine vasopression* (AVP), which displays circadian oscillations in expression and release[38, 44]. A prominent peak in release occurs spontaneously in early daytime of SCN maintained *in vitro*[45–46]. AVP is expressed in the dorsomedial SCN[6, 22], and acts as an output of the circadian clock, transmitting time-of-day information to other brain regions and the cerebral spinal fluid.

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Whereas AVP acts as an output of the SCN, several of the identified peptides are components of intrinsic SCN circuits that process incoming signals. Our previous peptidomic studies identified *little SAAS* together with other peptides derived from the prohormone, ProSAAS[26]. Little SAAS-expressing neurons are the third most abundant peptidergic class in rat SCN[26]. They localize to the retinorecipient area, and ~50% are targets of light-stimulated cFOS-induction. Little SAAS neurons relay signals downstream of the photic/glutamatergic signaling from the eye to the SCN. The action of little SAAS in altering phase of the SCN is independent of pathways involving *vasoactive intestinal peptide* (VIP) and *gastrin-releasing peptide* (GRP) action 26, which also relay light signals within the SCN. Little SAAS partially colocalizes with VIP- and GRP-expressing neurons. The functions of other ProSAAS-derived peptides, such as Big LEN and PEN, within the SCN remain unknown.

Many of the multitude of peptides identified within the SCN have established functions in other brain regions, but their contributions in the SCN are less well understood. Several of these include the following. *Angiotensin II* (ANGII), derived by the action of angiotensin-converting enzyme (ACE), along with its cognate receptor, AT1, are expressed in the SCN. ANGII-AT1 signaling is involved in the depolarization of SCN neurons[47]. Prepropeptide mRNAs encoding *opiom elanocortin* (POMC) and *secretogranin* have been localized within the SCN, but physiological roles have not been determined[48–50]. *Galanin*, an inhibitory neuropeptide with critical roles in regulation of sleep and feeding[51], has demonstrated staining that may reside in projections to the SCN from other regions of the hypothalamus[4].

Although a number of known, physiologically relevant neuropeptides were released in response to optic nerve stimulation, two anticipated neuropeptides, VIP and GRP, were not detected. VIP and GRP are established intrinsic SCN peptides with roles processing photic signals from the eye to the SCN clock[30, 52]. Several considerations may have contributed to this result. First, collection of these peptides from releasate may have been hindered a number of ways: 1) unusually strong VIP- and GRP- binding affinities to their respective receptors, VPAC2 and BB2 receptors, 2) tight synaptic junctions impeding release of VIP and/or GRP into the extracellular environment, and/or 3) rapid degradation post-release. Second, concentrations of VIP and/or GRP released in response to the brief, acute light signal-associated stimulus may be too low to capture and aggregate using SPE pipette sampling. Third, VIP and/or GRP release may occur, however, it may be downstream from the acute phase of the light response. VIP's role in SCN synchronization and entrainment is via regulation of SCN electrical activity[53], inhibitory synaptic transmission[54], intracellular signal transduction, and clock gene expression[31]. This is supported by the observation that sustained *Period1* expression (after 30 min), but not initial *Period1* gene induction, is reduced in VIP-mutant mice[31]. A required role for VIP has been proposed in timing-specific, light-induced gene expression[55]. Recent studies suggest that GRP and VIP may play redundant and intermediate roles in light-induced phase shifting[30]. Thus, neither may contribute in the initial, more acute physiological response to a phase-shifting light stimulus. This might explain why neither of these two SCN peptides were detected in samples collected during the first 20 min following ON stimulation in the horizontal SCN slice preparation.

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Glutamate and PACAP are co-stored in RHT terminals of the ipRGCs that innervate the SCN[46]. These neurochemicals are known to orchestrate the effects of environmental light signals from the retina via the RHT that induce time-of-day- specific phase shifting of the SCN circadian clock[12–14]. To assess whether the observed stimulus-induced peptide release is downstream from these known RHT neurotransmitters, we incubated the horizontal SCN slice with competitive antagonists of the NMDA glutamate receptor ((2*R*)-amino- 5-phosphonovaleric acid, APV) or the PACAP PAC1R receptor (PACAP 6–38) prior to electrical stimulation of the ON coupled with simultaneous collection of releasate for peptidomic analysis. When APV was applied during subjective daytime at CT 6, a significant suite of peptides was released in response to ON stimulation (n = 10; Supplementary Fig. 1A *vs.* B). However, the peptide composition of releasate was diminished by PACAP 6–38 administration at the same circadian time-point (n=4; Supplementary Fig. 1C). At CT 14, glutamate signaling via NMDA receptor activation has been implicated in delay of SCN clock phase[12]. No peptide release was detected at CT 14 when APV is administered during the electrical stimulus that alone effectively causes phase-shifting (n=4; Supplementary Fig. 1D). APV administration at CT 14 effectively blocks glutamate-induced phase delays[12]. Peptide release also was blocked when the selective inhibitor, PACAP 6–38 was present during both stimulus parameter conditions at CT 19 (n=4; Supplementary Fig. 1E and F). Blocking of PACAP signaling during direct glutamate stimulation has been shown to augment the glutamate-stimulated phase-advance in SCN brain slices[56].

Although previous studies have reported differential peptide expression[23–24, 57] and single peptide release[58] from the hypothalamus under physiological conditions, our work is the first to demonstrate temporally specific suites of peptide release evoked by a single environmentally relevant stimulus. Previously reported SCN peptides include molecules that are both endogenously expressed within the SCN and those delivered to the SCN via afferent innervation. These peptides may act as modulators of SCN responses, relays during intra-SCN stimulus processing, and/or feed-forward output signals from the SCN that influence timing of downstream physiological processes. Further characterization of these observed peptides is necessary to understand their mechanistic implications in circadian regulation.

CONCLUSION

Our results are significant in two ways, one being methodological and the second providing new insights on the complex interplay of peptides governing circadian rhythms. From the methodological point of view, MS analysis of peptide releasate represents a direct and untargeted discovery strategy that can be adapted to a number of brain systems. While our results demonstrate surprisingly complex peptide release profiles, a number of variables prevent this approach from being comprehensive. High-affinity peptide/receptor binding, low-concentration release, rapid extracellular degradation, and biophysical properties may result in peptide quantities below detection limits. In addition, the amount of peptide detected precludes most peptide identification approaches, so that our current study relied on matching our release profiles to more comprehensive LC-MS-based peptidomics measurements.

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From the neuroscience perspective, the results of our study highlight qualitative differences in secreted neuropeptide profiles across circadian time-points in response to stimuli that induce physiological state-changes. The peptide release profiles are surprisingly rich. The results suggest that SCN physiology is modulated by differential peptide release of both known and unexpected peptides that communicate time-of-day-specific photic signals via previously unreported neuropeptide signatures. Our findings validate the horizontal SCN slice preparation as a model system and inform future research toward exploring previously uncharacterized SCN neuropeptides and their prospective contributions to mammalian circadian-timing homeostasis.

METHODS

Animals

This study was conducted using male Long-Evans rats (BluGill), seven- to twelve-weeks old. This inbred strain has been analyzed via dense genomic scan (10 cM inter-marker interval), confirming only one allele at each locus examined[59]. Male animals were entrained to a 12h:12h light/dark cycle and provided food and water *ad libitum*. Circadian time (CT) in SCN brain slices reflects the light schedule that the animals were entrained to prior to sacrifice, in which the time of "lights on" is designated as CT 0. All animal care and procedures were approved by the University of Illinois at Urbana-Champaign Laboratory Animal Care Advisory Committee, in full compliance with federal animal care guidelines.

SCN Brain Slice Preparation

Animals were disoriented and sacrificed by decapitation during subjective daytime (CT 0 – 10). Horizontal brain slices (500 μm) with attached ONs (Fig. 1) were prepared using a combination of tissue chopper and Vibratome (Leica Microsystems). Tissue slices were maintained in a brain slice chamber perfused with phenol red-free Earle's Balanced Salt Solution (EBSS), supplemented with 24.6 mM glucose, 26.2 mM $NaHCO_3$, and 2.5 mg/l gentamicin, and saturated with 95% 02/5% CO2 at 37°C, pH 7.4.

Optic nerve stimulation

Electrical stimulation of the ON was performed at circadian time (CT) 6, 14, or 19. These circadian times in the free-running SCN *in vitro* correspond to the entrained animals' subjective daytime, early night, and late night, respectively. In animals, the circadian system is "blind' to light experienced in daytime, even in ambient darkness, whereas at the later two CTs, which occur during nighttime, light stimulates phase-resetting[41–42, 5, 60]. Non-photic signals can alter SCN phasing in daytime[41–42, 55, 60]. Accordingly, effects of stimulation applied to both optic nerves (ONs) were examined at each CT using paired suction electrodes at a distance ~ 4 mm distal to the optic chiasm. Effective voltage, frequency, and pulse duration parameters were employed at respective stimulation times and delivered for 5 min using a Grass isolated-pulse stimulator (Grass Technologies). For tissue used in electrophysiological recording, ONs were gently released from the suction electrode immediately following stimulation. When releasate was collected, suction electrodes remained attached to the ONs until the end of releasate collection (15 min following termination of stimulation).

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In some experiments, the horizontal SCN slice was incubated for 10 min with either a competitive antagonist of the NMDA glutamate receptor, (2R)-amino-5-phosphonovaleric acid (APV, 100 μM) or the competitive PAC1R antagonist, PACAP 6–38 (10 μM), before stimulating the ON and collecting releasate. Effects of these inhibitors of ON signaling on peptide release were evaluated at CT 6, 14, or 19.

Electrophysiological Recording

During the circadian cycle subsequent to stimulation, single-unit spontaneous activity of SCN neurons was recorded extracellularly 12. Activity of each neuron was averaged across a 4-min window. A 2-h binned running average of randomly sampled single-neuron firing activity was recorded using an in-house program developed in LabView (Laboratory Technologies). For each experiment, averages of firing rates and standard errors were plotted *versus* timeof-day to determine the CT of peak activity for the SCN neuronal population. Statistical analysis of electrophysiological results was performed using one-way ANOVA with Dunnett post-hoc test to compare experimental groups to control.

SPE pipette sampling of releasates

The approach employed for direct peptidomics of the SCN is outlined in Fig. 1, and has been described previously[38]. Briefly, C_{18} Zip Tip™ pipettes (Millipore Co., Billerica, MA) were “wetted” by aspiration with 50% ACN and equilibrated in EBSS immediately prior to use. The wetted pipettes were connected via Tygon tubing (Saint-Gobain Performance Plastics Co., Paris, FR) to a Harvard Apparatus (Harvard Biosience, Holliston, MA) syringe pump and Hamilton gas-tight syringe (Hamilton Company, Reno, NV). The ZipTip was mounted on a micromanipulator for precise positioning above the SCN on the surface of the brain slice. The inner diameter of the pipette tip measured 500 μm and was sufficient to cover the surface area of SCN tissue. Collection was performed by running the pump in negative mode, pulling extracellular samples across the C18 material at a rate of 0.2 μL/min. Pipette collections, both pre-stimulation and stimulation-onset, lasted 20 min unless otherwise noted.

Following sample collection, ZipTips removed, cleared of EBSS and aspirated with Millipore H_2O to rinse salts. Peptide samples were eluted with 2 μl of 70% ACN onto MALDI target plates prespotted with cyano-4 hydroxycinnamic acid (HCCA) MALDI matrix (PAC384 anchor-chips™, Bruker). Eluate droplets were maintained within pre-defined sample spot regions on the target plate and allowed to dry at room temperature prior to analyses.

MALDI TOF MS

MALDI TOF MS (MALDI time-of-flight MS) was performed using an Ultraflex II (Bruker Daltonics) mass spectrometer which was equipped with pulsed nitrogen lasers for desorption/ionization and coupled to time-of-flight tubes and detectors with high-speed digitizer/analyzers. Mass spectra were obtained using positive, reflectron mode. Mass calibration was performed using the pre-spotted calibrant mixture included with the Bruker PAC anchor-chips. Mass accuracies typically were observed to be within 100 ppm. Spectra

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represent the accumulation of 300 shots. Following data collection, the mass spectra were smoothed and baseline corrected.

Because the amount of peptide collected and measured using this approach precludes identification via tandem MS, the information on the peptides involves our accurate mass values. However, we have performed a number of studies on the peptides within the SCN using tissue punches and tandem MS that have identified detailed and accurate lists of prohormone-derived peptides[23–24, 38, 40]. Here, our neuropeptide identifications were inferred from intact mass values observed in MS1 data via MALDI TOF MS analyses and matching them to our curated lists of confirmed SCN peptides with a mass accuracy within 100 ppm.[61]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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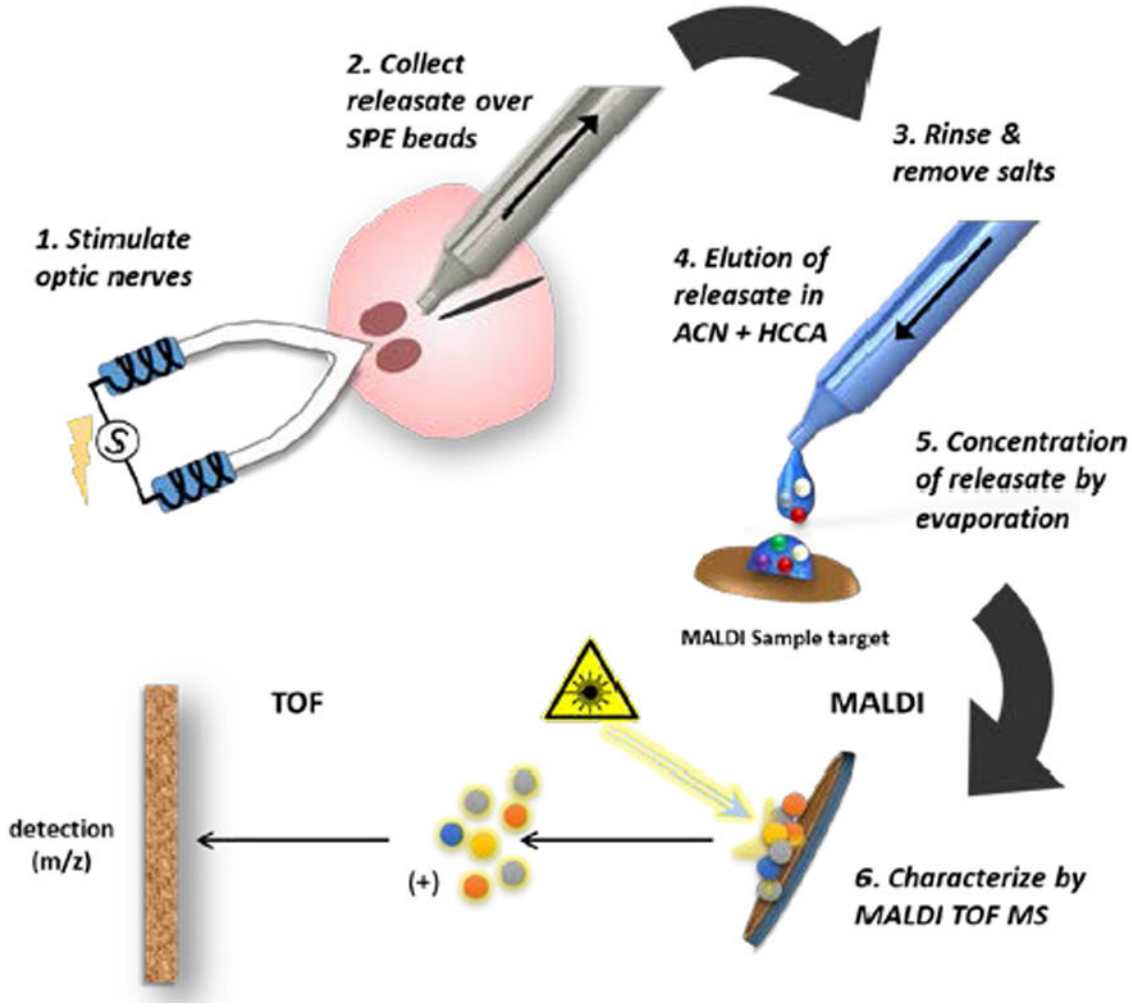

Figure 1.

Schematic of the workflow for neuropeptide collection from the SCN brain slice and MS analysis. 1. The optic nerves (ON) attached to a horizontal SCN brain slice are stimulated in tandem by suction electrodes or the SCN is treated directly by droplet containing a chemical stimulus. 2. Releasate is aspirated through a micropipette containing solid-phase extraction beads (SPEs), which bind peptides based on their charge. 3. The beads are rinsed to remove salts and then samples (represented as small colored circles) are transferred to a MALDI target surface. 4. Bound analytes are eluted with acetonitrile (ACN) and addition of a cyano-4 hydroxycinnamic acid (HCCA) MALDI matrix solution. 5. As the acetonitrile evaporates, analytes are concentrated with MALDI matrix onto discrete hydrophobic regions within the HCCA crystals on the pre-treated target plate. 6. The sample is volatized and ionized by matrix-assisted laser desorption/ionization (MALDI). Following ionization, the analytes are subjected to a mass analyzer and detector for spectrophotometric analysis where their mass/charge ratio (m/z) is determined.

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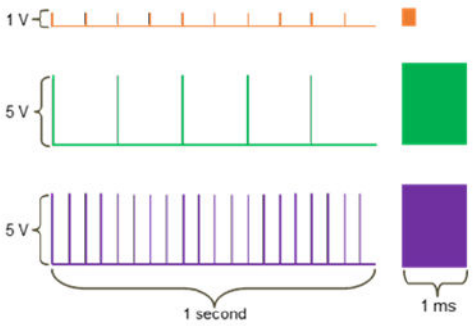
A.



B.

Time of Day	Stimulus	Peptide Release	Phase Shift (φ)		
			φD	φA	φA+
CT 6 (Mid-day)	ON stimulation (20Hz)	■		■	
	ON stimulation + APV	■			
	ON stimulation + PACAP 6-38	N.R.			
CT 14 (Early Night)	ON stimulation (10Hz)	■	■		
	ON stimulation + APV	N.R.			
	ON stimulation (CT19 20Hz)	N.R.			
CT 19 (Late Night)	ON stimulation (5Hz)	■		■	
	ON stimulation (20Hz)	■			■
	ON stimulation + PACAP 6-38	N.R.			

Figure 2.

Stimulus parameters applied to the ON to induce temporal phase shifting and peptide release in the SCN and outcomes. **(A)** Three different 5-min electrical stimulation paradigms were utilized: Orange, 1 V, 10 Hz, 0.2 ms pulse duration; Green, 5 V, 5 Hz, 1 ms pulse duration; Purple, 5 V, 20 Hz, 1 ms pulse duration. **(B)** These three stimulation parameters at distinct temporal windows caused different patterns of peptide release (■), and phase shifts of a delay of ~ 2 hr (φD), advance of ~2–3 hr (φA), or an advance of ~ 6 hr (φA+), respectively. N.R., no releasate measured.

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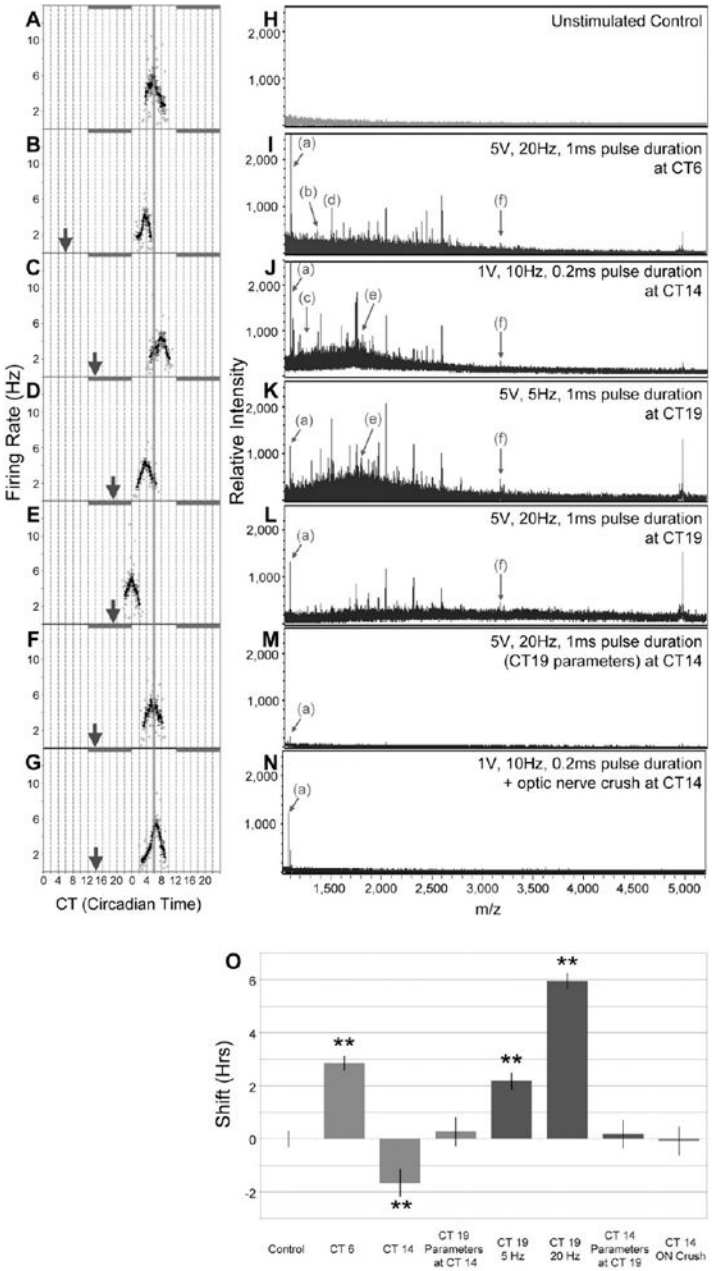

Figure 3.

Stimulus- and time-of-day specific phase-shifts of SCN neuronal activity are accompanied by stimulus- and time-of-day-specific peptide release. Horizontal suprachiasmatic brain slices of the mediobasal hypothalamus and preserving the ONs, which contain retinal afferents to the SCN, were monitored *in vitro*. **(A)** The SCN displays a circadian rhythm in neuronal firing rate that peaks at mid-subjective day, CT 6. ONs were stimulated bilaterally via suction electrodes with specific current, frequency, and pulse duration parameters at circadian time points (arrowheads). During daytime (CT 6) and late subjective nighttime (CT 19), ON stimulation evokes phase advance of the neuronal activity rhythm **(B, D**, and **E)**, while effective parameters at early subjective nighttime (CT 14) trigger phase delay of

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SCN firing rhythm **(C)**. MALDI-TOF-MS analyses of releasate collected from the SCN exhibit time-of-day and stimulus-specific peptide release profiles (H-L). Some peptide peaks, such as arginine vasopressin **(a)** and galanin **(f)**, are observed at every timepoint, while release of other peptides, such as neurokinin-B **(b)**, somatostatin-28 **(c)**, angiotensin **(d)**, and little SAAS **(e)** is restricted to specific time-of-day and stimulus conditions. Effective parameters at CT 19 are ineffective in phase shift **(F)** and peptide release **(M)** at CT 14. Transectional ON crush prevents stimulus-evoked phase shifting **(G)**and peptide release **(N)**. Phase-shifting responses to ON stimulation depend upon unique, time-of-day-effective stimulus parameters **(O)**. **, $p < 0.001$, one-way ANOVA.

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Table 1.

Peptides detected directly from stimulated releasate of the suprachiasmatic nucleus

Putative Peptide (Prohormone) Name	Observed m/z (MH^+)	Theoretical m/z (MH^+)	Ref	RHT Stimulus-Triggered Releasate Conditions				
				Pre-Stim	CT6	CT14	CT19 (5Hz)	CT19 (20Hz)
Angiotensin I (**Angiotensinogen**)	1296.62	1296.69	38		•			
Little SAAS (**Pro-SAAS**)	1784.97	1784.98	23, 38–39			•	•	
Big LEN (**Pro-SAAS**)	1745.95	1745.97	38			•		
PEN (**Pro-SAAS**)	2301.25	2301.24	38–39		•	•	•	•
Cerebellin, aa57–71 (**Cerebellin-1**)	1495.75	1495.79	23	≠	•	•	•	
Galanin (**Galanin**)	3163.59	3163.58	23, 38		•	•	•	•
P-endorphin (**POMC**)	3463.80	3463.86	61			•		
Melanotropin α (**POMC**)	1622.78	1622.79	38, 40			•		
Morphogenetic Neuropeptide (**HA**)	1141.68	1141.67	61			•		
Neurokinin-B	1210.56	1210.54	38		•			
Pro-enkephalin A, aa219–229 (**PENK**)	1466.64	1466.65	23, 38, 40			•		
ProSomatostatin 89–100	1244.58	1244.57	38			•		
Somatostatin 14	1637.73	1637.71**(ss)**	38			•		
Substance P (**Protachykinin-1** (PPT))	1347.73	1347.74	23, 38, 40			•		
Thymosin β−4 (**Thymosin β−4**)	4961.50	4961.49(a)	38	≠	•	•	•	•
Arginine-Vasopressin (**AVP-NPII**)	1084.46	1084.46(ss)	38, 40	≠	•	•	•	•
Vasopressin-Neurophysin2-copeptin, aa151–168 (**AVP-NPII**)	1948.00	1948.01	23		•			
Vasopressin-Neurophysin2-copeptin, aa154–168 (**AVP-NPII**)	1607.80	1607.80	23		•			
MBP, aa2–18 (**MBP**)	2028.10	2028.07 (a)	39		•	•		
Secretogranin 2, aa598–612 [**Secretogranin 2**]	1785.81	1785.85	23		•		•	

≠, Corresponding peaks identified in < 50% of the samples taken for the particular Releasate Condition. **(ss)**, mass w/disulfide bond; **(a)**, mass w /acetylation; (p) mass w/pyro-glu PTM at Q