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Quantitative Peptidomics for Discovery of Circadian-Related Peptides from the Rat Suprachiasmatic Nucleus

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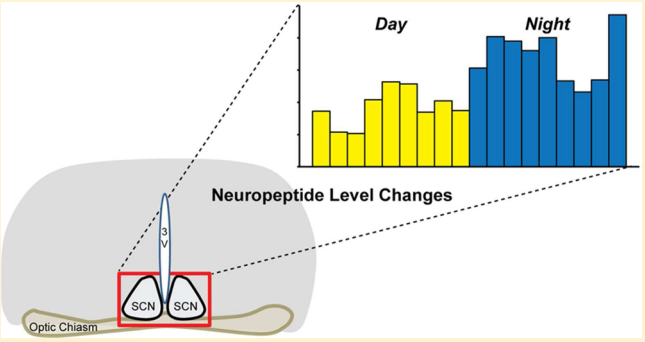
Supporting Information

ABSTRACT: In mammals the suprachiasmatic nucleus (SCN), the master circadian clock, is sensitive to light input via the optic chiasm and synchronizes many daily biological rhythms. Here we explore variations in the expression levels of neuropeptides present in the SCN of rats using a label-free quantification approach that is based on integrating peak intensities between daytime, Zeitgeber time (ZT) 6, and nighttime, ZT 18. From nine analyses comparing the levels between these two time points, 10 endogenous peptides derived from eight prohormones exhibited significant differences in their expression levels (adjusted p-value <0.05). Of these, seven peptides derived from six prohormones, including GRP, PACAP, and CART, exhibited ≥30% increases at ZT 18, and the VGRPEWWMDYQ peptide derived from proenkephalin A showed a >50% increase at nighttime. Several endogenous peptides showing statistically significant changes in this study have not been previously reported to alter their levels as a function of time of day, nor have they been implicated in prior functional SCN studies. This information on peptide expression changes serves as a resource for discovering unknown peptide regulators that affect circadian rhythms in the SCN.



KEYWORDS: *circadian rhythms, suprachiasmatic nucleus (SCN), neuropeptides, endogenous peptides, label-free quantification, quantitative peptidomic analysis*

INTRODUCTION

Circadian rhythms are biological cycles that operate over a 24-h period, acting to adjust the timing of an organism's behavior, physiology, and metabolism to the environmental light-dark cycles.[1] In mammals, circadian rhythms are regulated by paired brain nuclei located at the base of the hypothalamus, directly above the optic chiasm, known as the suprachiasmatic nuclei or nucleus (SCN).[2−4] The bilateral SCN, consisting of approximately 20,000 neurons, integrates photic and non-photic signals from the light-sensing retina and numerous brain regions to achieve synchrony with environmental or behavioral states. Neuropeptides, along with neurotransmitters, are key elements in mediating cell-to-cell signaling in SCN circadian functions.[5−7]

Radioimmunoassay (RIA), based on the interaction of antigens and antibodies, has been the classical method used for quantitative analysis of endogenous peptides.[8−10] However, because RIA techniques are targeted to specific analytes, they detect only known peptides. Furthermore, antibodies used for RIA may cross-react with N- and/or C-terminally extended forms and with post-translationally modified peptides, complicating the interpretation of the data generated. In contrast, mass spectrometry (MS) can detect and identify the precise form of a peptide without prior knowledge of its identity, allowing the detection of a large number of peptides in a single experiment.[11−14] Moreover, MS-based quantification performed in discovery mode, in which either the peptide peak intensities or the number of peptides detected in MS/MS spectra are compared for complex mixtures instead of targeted peptides lists, can screen for changes in the expression of multiple peptides in a high-throughput manner.

Our recent comprehensive peptidomic analysis of the rat SCN used nanocapillary reversed-phase liquid chromatography (LC) coupled to Fourier-transform mass spectrometry (FTMS).[15] The high resolution MS data generated in that study resulted in a robust list of 102 endogenous peptides, including 33 previously unidentified peptide forms, 12 of which

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were peptides with post-translational modifications (PTMs), such as amidation, phosphorylation, pyroglutamylation, and acetylation. Our prior work focused on endogenous peptide identification within the SCN. Here we add a functional context by performing peptidomic studies at two different ZTs in order to characterize changes in peptides as a function of circadian time and thus uncover novel SCN regulators and improve our understanding of the SCN timekeeping mechanisms.

Quantitation of peptides based on MS is normally achieved either by stable isotope labeling or label-free approaches.[12,16−21] Stable isotope labeling is an effective method for accurately determining changes in peptide or protein levels but requires additional chemistry or sample preparation. In contrast, in label-free methods each sample is separately prepared and then directly subjected to individual LC−MS or LC−tandem MS (MS/MS) runs, followed by comparisons of either the mass-spectral peak intensities of the detected peptides or the number of peptides identified in the MS/MS spectra, called spectral counting.[17,22−25] Since label-free quantification allows for the comparison of multiple experiments and provides a high dynamic range of quantitation, the approach has recently gained prominence in biomarker discovery studies for finding candidate peptides or proteins related to a specific biological phenomena or disease. As examples, Rossbach et al.[16] and Scholz et al.[19] demonstrated that label-free quantitations based on peptide ion intensities were able to successfully examine the expression changes of endogenous peptides present in rat and Japanese quail brains, respectively. Tian et al.[26] recently performed a label-free quantitative analysis of the light-responsive proteome of the murine SCN to find proteins, including Ras-specific guanine nucleotide-releasing factor 1, ubiquitin protein ligase E3A, and X-linked ubiquitin specific protease 9, involved in circadian regulation.

Here, by optimizing our prior approach used to study proteins, we examined the complement of endogenous peptide transmitters and hormones. We modified the sample preparation, collection, and analysis process to concentrate on the peptides generated from prohormones, which required quantitation of each chemical species at the peptide, not protein, level. In order to achieve our objective, three biological pools, each consisting of 24 rat SCN tissue punches, were collected at daytime (ZT 6), and three similar pools were collected at nighttime (ZT 18), over 3 days (requiring a total of 144 SCN punches). Each pooled sample was divided into three nanoLC−MS/MS injections (for a total of 18 separations). Our automated approach using the SIEVE software from Thermo Fisher Scientific (San Jose, CA) for quantitative peptidomic analysis revealed a total of 10 endogenous peptides derived from eight prohormones that exhibited significant changes in abundance between day and night. The manual mode of SIEVE analysis produced several additional peptides that were not found using automated SIEVE analysis; these additional peptides also exhibited significant changes between daytime and nighttime. Peptides derived from gastrin releasing peptide (GRP), pituitary adenylate cyclase-activating polypeptide (PACAP), and vasoactive intestinal peptide (VIP), which are well-known prohormones involved in SCN circadian functions, and the protachykinin 1-derived peptide, substance P, which is a known circadian peptide, exhibited >30% increases at nighttime. In addition to these expected peptides, peptides derived from cocaine- and amphetamine-regulated transcript protein (CART), proenkephalin A, prothyroliberin, and secretogranin 2 exhibited ≥30% increases in their peptide levels at nighttime, although these peptides have not been previously implicated in SCN circadian functional studies. This represents the first quantitative peptidomic effort to explore day−night changes in peptide expression profiles for the mammalian SCN; additional studies are needed to understand the roles of the peptides detected here and to further understand how these peptide dynamics contribute to the SCN's ability to orchestrate circadian rhythms.

EXPERIMENTAL SECTION

Chemicals

The reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), unless otherwise noted.

Animals and SCN Brain Punch Sample Collection

The vertebrate animal procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee, University of Illinois at Urbana−Champaign, and consistent with the *Principles of Laboratory Animal Care* (NIH Publication no. 85-23). Long-Evans male rats (LE-BluGill, an inbred strain from the University of Illinois at Urbana−Champaign), 8−10 wks old, were used in these studies. Animals were fed *ad libitum* and housed under 12:12 h light/dark cycle conditions as previously described.[15] Animals were sacrificed via rapid decapitation, and *ex vivo* SCN tissue samples collected at two circadian time points: (1) daytime or ZT 6, approximately 6 h following onset of lights-on conditions, and (2) nighttime or ZT 18, approximately 6 h following onset of lights-off conditions. Brain tissues collected at ZT 6 were harvested in a room with fluorescent lighting, while tissues collected at nighttime were harvested in a dark, light-sealed room with dim red lighting to prevent any phase resetting caused by illumination. For accessibility during nighttime tissue collection, rats were pair-housed prior to lights-off (ZT 12) and placed into circadian activity monitoring systems.

SCN brain punch samples were collected as previously described.[15] Each sample for peptidomic analysis contained SCN brain punches pooled from 24 rats (n = 24), at both ZT 6 and ZT 18. Samples were collected on three consecutive days (n = 3 samples, with each containing 24 SCN punches, for a total of 144 SCN punches harvested for both circadian time points). Tissue samples were collected and maintained in siliconized microcentrifuge tubes (Thermo Fisher Scientific, Waltham, MA) submerged in powdered dry ice and then boiled for 10 min after 300 μL of water was added and centrifuged at 14,000 × g for 10 min. The supernatant was saved, and the resulting tissue pellet stored in a −80 °C freezer until use. The collected tissue samples were subjected to multistage peptide extraction consisting of ice-cold acidified acetone (40:6:1 acetone/water/HCl) and ice-cold 0.25% acetic acid in order to minimize variations resulting from sample handling.[15] All of the extracts were combined and filtered through Microcon YM-10 centrifugal filter devices (Millipore, Billerica, MA).

Mass Spectral Analysis (LC−FTMS/MS)

Each prepared sample was divided into three equivalent peptide samples that were handled identically and sequentially run on a 12 T ion trap-based mass spectrometer (LTQ-FT Ultra, Thermo Fisher Scientific, San Jose, CA), alternating between daytime and nighttime biological samples (n = 18 peptide samples). More specifically, each extracted peptide sample was analyzed with the LTQ-FT instrument interfaced with a 1D NanoLC pump from Eksigent Technologies (Dublin, CA). The

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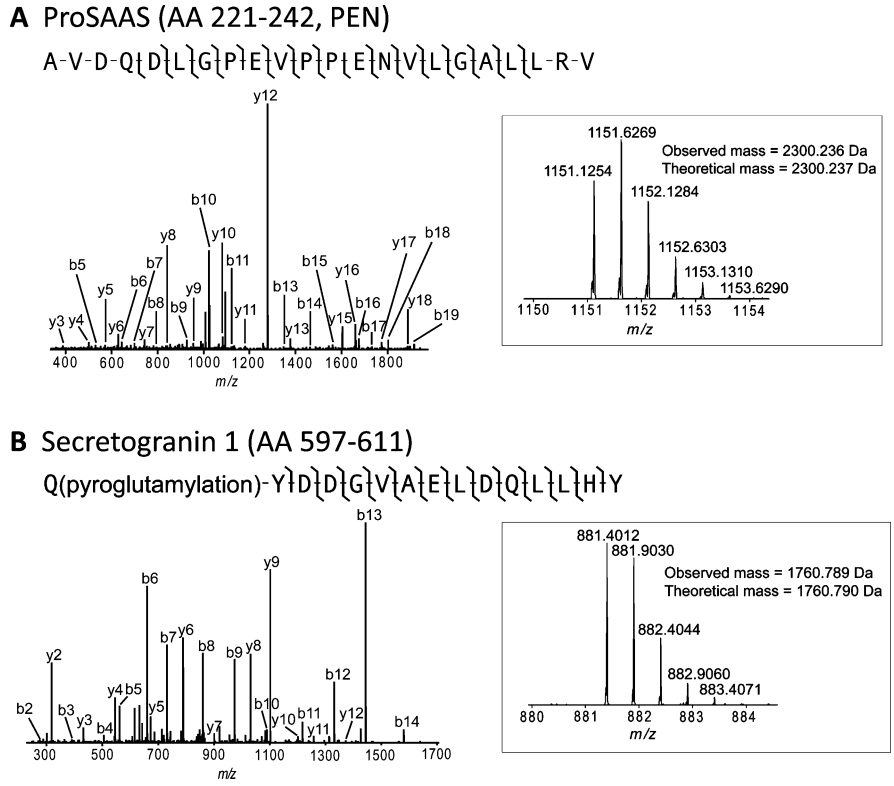

Figure 1. FTMS with high accuracy and ion trap MS/MS data allow the identification of endogenous peptides present in the SCN. The peptides derived from (A) proSAAS and (B) secretogranin 1 were identified with 14 b-ion and 15 y-ions, and 13 b-ions and 11 y-ions, respectively.

prepared peptide sample was helium bomb pressure-loaded (500 psi) into a trap column (75 μm inner diameter), 5 cm of which was fritted with LiChrosorb (EM Separations, Gibbstown, NJ) and packed with a C18 material (10 μm, YMC Co., Ltd., Allentown, PA). The nanocapillary column (10 cm × 75 μm inner diameter) containing ProteoPep II media (C18, 300 Å, 5 μm) was purchased from New Objective (Woburn, MA). The operating flow rate was 300 nL/min by use of buffer A (95% water, 4.8% acetonitrile, and 0.2% formic acid) and buffer B (95% acetonitrile, 4.8% water, and 0.2% formic acid) with the following gradient conditions: 0−10 min 0−10% B, 10−75 min 10−30% B, 75−100 min 30−45% B, 100−120 min 45−60% B, 120−125 min 60−85% B, 125−126 min 85−5% B, 126−132 min 5−85% B, 132−136 min 85−5% B, 136−150 min 5% B.

Data acquisition on the LTQ-FT mass spectrometer consisted of a full scan event (m/z 300−1500, resolving power, $m/\Delta m_{50\%}$ = 90 K in which $\Delta m_{50\%}$ is the mass spectral peak full width at half-maximum peak height) and data-dependent collision-induced dissociation (CID) ion trap MS/MS scans of the 10 most abundant peaks from the previous full scans. MS/MS settings were as follows: isolation width = m/z 3; minimum signal threshold = 1000 counts; normalized collision energy = 35%; activation Q = 0.25; activation time = 50 ms.

Peptide Identification

The raw LTQ-FTMS data were first converted to a Mascot generic format using the msconvert tool from ProteoWizard 1.5.2.[27] The Open Mass Spectrometry Search Algorithm (OMSSA) 2.1.1[28] was used to search the converted LTQ-FTMS data. While ProSightPC 2.0 software (Thermo Fisher Scientific, San Jose, CA) was used for peptide identification in our previous SCN peptidomic analysis,[15] OMSSA was used here because the ProSightPC software is not optimized for processing ion trap MS/MS spectra. The MS data were searched against a database consisting of manually curated rat proteins from UniProt 15.15[29] and known rat neuropeptides from the Neuropeptide Database (http://www.neuropeptides.nl/). The following criteria were applied for the peptide identification: enzyme, nonspecific; peptide m/z tolerance, 0.01 Da; MS/MS m/z tolerance, 0.5 Da; variable modifications, acetylation of protein N terminus, amidation of peptide C terminus, oxidation of Met, pyroglutamylation of peptide N-term Gln and Glu, phosphorylation of Ser, Thr, or Tyr; product ion types to search, b and y; precursor search type, monoisotopic; product search type, monoisotopic; at least one peak must match among the top six most intense peaks; lower bound of precursor charge, 2; upper bound of precursor charge, 20; charge at which to start considering multiply charged products, 3; precursor charge statement, assigned by instrument; allow N-terminal Met cleavage. Peptides with two or more PTMs were removed from the analysis unless they were manually validated. The resulting peptide spectral matches were filtered leading to a 5% false identification rate using a separate decoy database of reversed target sequences (i.e., less than 5% of the peptide annotations are expected to be incorrect).[30]

SIEVE Software Analysis for Label-Free Quantification

The SIEVE software, version 1.2 (Thermo Fisher Scientific), was used for label-free relative quantification based on integrated peptide peak intensities without using internal standards from a total of 18 nanoLC−MS/MS runs. The time base for each chromatographic run was first aligned to establish a common time coordinate for between-sample comparison. Non-overlapping, two-dimensional (m/z and

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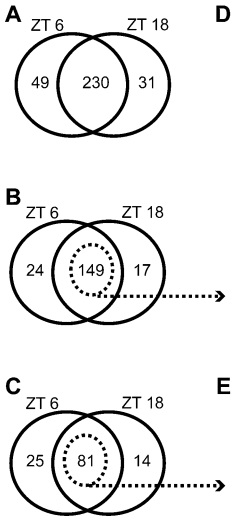
retention time, RT) frames were automatically generated from full MS scans by order of intensity, with an RT width of 2.5 min and an m/z width of 0.02. Within each run, the SIEVE software calculated the intensity within each frame over the m/z dimension by integrating response over the RT dimension using the trapezoid rule. Integrated intensities from each frame were exported and analyzed using the statistical computing software R.[31] A frame's abundance ratio was calculated as the ratio of the average integrated intensity of the nine ZT 18 samples to the average integrated intensity of the nine ZT 6 samples. The standard deviation of the frame's abundance ratio was calculated by the delta rule.[32] The significance of the difference between ZT 6 and ZT 18 for each frame was calculated using a t test between the mean integrated intensity at ZT 6 and ZT 18. Frames were identified by dispatching MS/MS data from each raw file, where available, to OMSSA. For direct congruence to stand-alone analyses, a custom interface to OMSSA was constructed, and frames were mapped to peptides, taking into account nonspecific cleavage and potential acetylations, pyroglutamylations, phosphorylations, and amidations. Only peptides identified with an E-value of $<1 \times 10^{-3}$ were included for reasons of efficiency. Frames assigned to the same peptide by the search engine had their ratios pooled by means of a variance-weighted average to produce an overall peptide ratio. The significance of whether a peptide was differentially expressed was determined by combining its constituent frames' p-values by means of Fisher's Combined Probability. The resulting combined p-value was subsequently adjusted for a false discovery rate (FDR) using the Benjamini and Hochberg method.[33]

RESULTS

A total of 310 peptides were identified across the ZT 6 and ZT 18 samples (Supplemental Table S1). Of these, 190 identified peptides were so-called endogenous peptides, derived from 28 previously characterized neuropeptide prohormones that are known to produce bioactive peptides; these included 25 peptides with PTMs (i.e., amidation, phosphorylation, pyroglutamylation, or acetylation) and six with oxidation. The remaining 120 identified peptides originated from 68 proteins (Supplemental Table S2), most of which are cytosolic proteins. Approximately one-third (67) of the endogenous peptides, including Q*(pyroglutamylation)PVVPVEAVDPMEQRAEEAP (AA 21−40) derived from cholecystokinin, have not been previously reported. Of the 190 endogenous peptides, eight, including QYDDGVAELDQLLHY (AA 597−611) derived from secretogranin 1, were homologues of the peptides found in a mouse peptidomic study,[34] but these peptides have not been previously reported in the rat peptidome. Figure 1 represents examples of FTMS and ion trap MS/MS spectra for PEN peptide (AA 221−242) derived from ProSAAS, and pyroglutamylated peptide (AA 597−611) produced from secretogranin 1. This figure demonstrates the high mass accuracy of the peptide ion detection in combination with tandem fragment ions obtained from the ion trap scans that allowed our successful identification of the endogenous peptides derived from prohormones. Approximately 75% of the identified peptides were found in both ZT 6 and ZT 18 samples, 49 peptides were only identified from ZT 6 samples, and 31 were identified just from ZT 18 samples (Figure 2A). Figure 2, panels B and C, show the distribution of the endogenous peptides derived from prohormones, and peptides originating from proteins at ZT 6 and ZT 18, respectively.



D

Peptides from SIEVE analysis (FDR-adjusted p-value <0.05)	ZT 6	ZT 18
CART (AA 37-54, AA 37-54 with phosphorylation, AA 37-55)	↓	↑
Cholecystokinin (AA 21-44)	↓	↑
Gastrin-releasing peptide (AA 24-41)	↓	↑
PACAP (AA 111-128)	↓	↑
PEBP-1 (AA 9-25)	↑	↓
Proenkephalin A (AA 219-229)	↓	↑
Prothyroliberin (AA 178-199)	↓	↑
Secretogranin 2 (AA 344-356)	↓	↑

E

Peptides from SIEVE analysis (FDR-adjusted p-value <0.05)	ZT 6	ZT 18
Cytochrome c oxidase 5A (AA 74-87)	↑	↓
Vimentin (AA 444-466)	↑	↓

Figure 2. Venn diagrams showing the distribution of a total of 310 peptides identified from ZT 6 and ZT 18 in which each measurement time consists of nine nanoLC−MS/MS runs: (A) combined, (B) prohormone-derived, and (C) non-prohormone protein-derived numbers of peptides identified at each time point. The tables list the identified peptides from (D) known prohormones and (E) non-prohormone proteins that exhibited statistically significant changes (FDR-adjusted p-value <0.05), with the arrows denoting the direction of change.

Specifically, TVGDVNTDRPGLLDL (AA 36−50) derived from Acyl-CoA-binding protein, DDGVAELDQLLHY (AA 599−611) derived from secretogranin 1, and TNEIVEEQYTPQSLATLE (AA 184−201), QELGKLTGPSNQ (AA 205−216), and IPAGSLKNEDTPNRQ (AA 571−585) derived from secretogranin 2, were identified more than or equal to 10 times each, only from ZT 6 samples. In contrast, PPENVLGALLRV (AA 231−242) derived from ProSAAS, FLDSWFSDVPQV (AA 140−151) derived from prothyroliberin, GRPEWWMDYQ (AA 220−229) and YGGFMRGL (Met-enkephalin-Arg-Gly-Leu, AA 188−195) derived from proenkephalin A, VQLAGTQESVDSAKPRV (AA 151−167) derived from provasopressin, GALFNPYFDPLQWKNSDFE (AA 514−532) derived from secretogranin 1, and LLERPLDSQSIYQLIEI (AA 340−356) derived from secretogranin 2, were identified ≥10 times, only in ZT 18 samples.

The high accuracy of the FTMS data (≪10 ppm mass accuracy) allowed accurate peptide assignments (Figure 1) and facilitated the extraction of peak signals for intact peptides. As the next step, we examined changes in the peptidome expression profile of the SCN between ZT 6 and ZT 18 using the automated mode of the SIEVE software algorithms and based on peptide peak intensities. Figure 3 represents the relative abundance of the integrated intensities obtained from the two frames for a proenkephalin A-derived peptide, VGRPEWWMDYQ (AA 219−229), generated by SIEVE analysis from 18 LC−MS runs. This example clearly shows that the level of this peptide significantly increased at ZT 18. The SIEVE analysis identified 173 peptides with an E-value cutoff of 1×10^{-3}. Among the 173 peptides that were generated from the SIEVE analysis, 113 endogenous peptides were derived from prohormones, and 60 peptides mainly originated from cytosolic proteins (data not shown). After adjusting their p-values to control for multiple tests, 12 peptides derived from known prohormones and proteins had statistically significant

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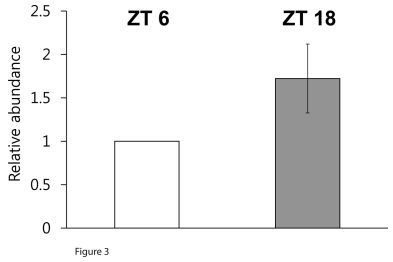

Figure 3. Results of the SIEVE analysis of VGRPEWWMDYQ peptide (AA 219−229) derived from proenkephalin A showing significant changes between ZT 6 and ZT 18. The integrated intensities of VGRPEWWMDYQ peptide from two frames exhibited a 1.7-fold increase (FDR-adjusted *p*-value = 0.00027) in levels at ZT 18.

(FDR-adjusted *p*-value <0.05) abundance ratios between ZT 18 and ZT 6; in Figure 2D and E, these are indicated with up and down arrows indicating at which time they were observed at higher and lower levels, respectively. The results of the SIEVE analysis for 10 endogenous peptides derived from eight prohormones are shown in Table 1, and the results of the SIEVE analysis for two peptides originating from two proteins are shown in Supplemental Table S3. Compared to ZT 6, seven endogenous peptides derived from six prohormones, including proenkephalin-A, exhibited significant, ≥30% increases at ZT 18 (Table 1), and two of these peptides, VGRPEWWMDYQ (AA 219−229, 1.7-fold) derived from proenkephalin A and FIDPELQRSWEEKEGEGVLMPE (AA 178−199, 1.5-fold) derived from prothyroliberin, exhibited ≥50% increases in their levels at this time point. In addition, all three of the peptides derived from CART exhibited significant increases at ZT 18. In contrast, there was only one peptide, AGPLSLQEVDEPPQHAL (AA 9−25, 0.8-fold) derived from phosphatidylethanolamine-binding protein-1 (PEBP-1), exhibited a decrease in the level at ZT 18 compared to ZT 6 (Table 1). Of the 10 endogenous peptides showing significant changes between the two time points, two peptides derived from CART and cholecystokinin were post-translationally modified and exhibited increases in expression at ZT 18. As for the CART-derived peptide ALDIYSAVDDAS*-(phosphorylation)HEKELP (AA 37−54), it was found along with its non-phosphorylated form and exhibited a similar extent of increased expression at ZT 18.

In addition to the endogenous peptides derived from prohormones identified from the SIEVE results, two peptides originating from two cytosolic proteins, cytochrome *c* oxidase subunit 5A (VGYDLVPEPKIIDA (AA 77−87)) and vimentin (IKTVETRDGQVINETSQHHDDLE (AA 444−466)), showed significant changes between daytime and nighttime (see Supplemental Table S3). In contrast to the changes in levels for peptides derived from prohormones as seen in Table 1, these two peptides exhibited >30% decreases in their peptide levels at ZT 18. While these peptides could be the result of post-mortem degradation during sample preparation, it has been recently found that peptides such as hemopressins, originating from cytosolic proteins, are produced from intracellular processing and function in cell−cell signaling; these peptides are classified as non-classical neuropeptides.[35−38]

The automated SIEVE software algorithms discussed above appeared to miss some of the peptides identified in our previous study.[15] The missed peptides were therefore not subjected to CID fragmentation either because they were missed due to their low abundance or they did not generate enough fragment ions to be used for identification in the ion trap MS/MS scans performed in this study. Of the 26 peptides identified here and in our prior study using FTMS/MS data acquisitions, several were detected only in the FTMS scans performed in the current SCN peptidomic analysis. While 16 peptides did not show consistent elution profiles or were inconsistently detected, 10 peptides exhibited consistent elution patterns over the 18 LC−MS runs. These 10 peptides were manually identified and subjected to the manual mode of SIEVE analysis by following the same procedure that automated SIEVE analysis implements. Specifically, the elution peaks of the peptides of interest were identified based on 10 ppm of mass accuracy and the peak intensity for each peptide in each sample, and each peak was manually integrated over the eluted time window using the trapezoid rule. The ratios of mean integrated intensities between ZT 6 and ZT 18 were then calculated, as well as the standard deviations of the ratios using the delta rule and the *p*-values of the unpaired *t* tests of the mean integrated intensities. Table 2 shows the results of the ratios for these 10 peptides, generated from the manual mode of SIEVE program analysis. While the *p*-scores of many of the peptides generated from manual mode were below significance (higher than 0.01), substance P with C-terminal amidation

Table 1. Results for the Ten Detected Endogenous Peptides Derived from Prohormones Reported As Being Significant by the Automated SIEVE Analysis

peptide	sequence	obsd mass (Da)	mass diff (ppm)	ratio of night/day	std dev	*p*-value	FDR adjusted *p*-value	prohormone Uniprot accession number
CART [37−54]	ALDIYSAVDDASHEKELP	1971.94	−1.0	1.27	0.19	9.4×10^{-5}	4.3×10^{-3}	P49192
CART [37−54]	ALDIYSAVDDAS*(phosphorylation) HEKELP	2051.90	−1.9	1.35	0.25	1.2×10^{-4}	4.3×10^{-3}	P49192
CART [37−55]	ALDIYSAVDDASHEKELPR	2128.04	−0.5	1.30	0.15	3.4×10^{-7}	6.1×10^{-5}	P49192
Cholecystokinin [21−44]	Q*(pyroglutamylation) PVVPVEAVDPMEQRAEEAPRRQL	2726.38	−1.8	1.24	0.17	3.7×10^{-4}	8.3×10^{-3}	P01355
Gastrin-releasing peptide [24−41][b]	APVSTGAGGGTVLAKMYP	1675.86	−0.6	1.41	0.24	4.4×10^{-6}	2.7×10^{-4}	P24393
PACAP [111−128][b]	GMGENLAAAAVDDRAPLT	1770.86	−0.6	1.32	0.23	2.2×10^{-4}	5.8×10^{-3}	P13589
PEBP-1 [9−25]	AGPLSLQEVDEPPQHAL	1799.90	−1.1	0.83	0.10	2.2×10^{-4}	5.8×10^{-3}	P31044
Proenkephalin A [219−229]	VGRPEWWMDYQ	1465.64	0.7	1.72	0.40	3.1×10^{-7}	2.7×10^{-4}	P04094
Prothyroliberin [178−199]	FIDPELQRSWEEKEGEGVLMPE	2617.24	0.0	1.49	0.48	1.6×10^{-3}	2.6×10^{-2}	P01150
Secretogranin 2 [344−356]	PLDSQSIYQLIEI	1517.80	−1.3	1.41	1.18	1.9×10^{-3}	2.8×10^{-2}	P10362

[a]Asterisk (*) denotes an amino acid having a post-translational modification. [b]Peptides derived from prohormones that are known to be involved in SCN circadian functions.

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Table 2. Results for the Ten Detected Peptides Derived from Prohormones Using the Manual Mode of SIEVE Analysis

peptide	peptide name	sequence[a]	obsd mass (Da)	mass difference (ppm)	ratio of night/day	std dev	*p*-value	prohormone Uniprot accession number
Cerebellin-1 [57−71]		SGSAKVAFSAIRSTN	1494.78	−1.3	1.03	0.34	0.80	P63182
ProMCH [131−143]	Neuropeptide E-I	EIGDEENSAKFPI*(amidation)	1446.70	−0.7	1.25	0.36	0.043	P14200
Neurosecretory protein VGF [489−507]		NAPPEPVPPPRAAPAPTHV	1914.01	−1.0	1.11	0.44	0.45	P20156
POMC [141−162]		RPVKVYPNVAENESAEAFPLEF	2505.25	−0.8	1.34	0.68	0.077	P01194
Proenkephalin A [143−185]		DADEGDTLANSSDLLKELLGTGDNRAKDSHQQESTNNDEDSTS	4592.01	−0.4	1.43	1.14	0.15	P04094
Proenkephalin B [235−248]		SQENPNTYSEDLDV	1609.67	−1.2	1.27	0.63	0.20	P06300
Protachykinin 1 [58−68]	Substance P	RPKPQQFFGLM*(amidation)	1346.73	−0.7	1.50	0.61	0.0056	P06767
Secretogranin 1 [585−594]		SFAKAPHLDL	1097.59	−0.9	1.37	0.44	0.015	O35314
Secretogranin 2 [529−568]	Manserin	VPSPGS*(phosphorylation) SEDDLQEEEQLEQAIKEHLGQGSSQEMEKLAKVS	4446.039	−0.8	1.27	1.27	0.45	P10362
VIP peptides [125−137][b]		HSDAVFTDNYTRL	1537.72	−0.2	1.53	0.48	0.0016	P01283

[a]Asterisk (*) denotes an amino acid having a post-translational modification. [b]Peptides of prohormones that are known to be involved in SCN circadian functions.

(RPKPQQFFGLM*(amidation), AA 58−68, 1.5-fold, *p*-value = 0.0056) and the truncated VIP peptide (HSDAVFTDNYTRL, AA 125−137, 1.5-fold, *p*-value = 0.0016) showed significant increases in peptide levels at ZT 18 (nighttime).

DISCUSSION

As the site of the master biological clock, the SCN controls circadian rhythms. Multiple peptides in the SCN act as critical synchronizers and mediators of the SCN. Changes in peptide expression within the SCN across the 24-h day appear to mediate time-of-day-specific circadian functions. While antibody-based approaches have been previously used with success for measuring changes in peptide expression in the SCN,[39−42] these methods provide information only on preselected peptides and can lack sufficient specificity to identify an exact peptide form due to cross-reactions with longer peptides or peptides containing distinct PTMs. MS-based quantification offers the benefit of being able to examine changes in the expression of multiple peptides without preselecting the peptides, and without prior knowledge of their structure.

Using high resolution FTMS, we expanded our previous identification of rat SCN endogenous peptides at ZT 6[15] to include nighttime, ZT 18. In comparison with the previous SCN study, 63 peptides, including APVSTGAGGGTVLAKMYP (AA 24−41) derived from GRP, were identified in both our previous and current SCN peptidomic studies. We found another 26 peptides, including the truncated VIP (HSDAVFTDNYTRL (AA 125−137)), that were identified in the previous SCN peptidomic analysis and also in the current SCN mass spectral data by use of manual validation based on the precursor peptide masses within 10 ppm of mass accuracy (data not shown). We surmise that most of these peptides either had molecular weights of ≥3 kDa and, thus, did not generate sufficient fragment ions for confident identification in the ion trap MS/MS scans or may not have been subjected to CID fragmentation due to low abundances.

In this study, we performed a label-free quantitative peptidomic analysis of rat SCN tissue samples collected at daytime (ZT 6) and nighttime (ZT 18), respectively. The daytime and nighttime samples contained distinct peptides, and so factors arising from these differences such as distinct coeluting analytes may have affected the quantitative accuracy (for example, from differences in ion suppression). However, ion suppression from complex mixtures is reduced by using nanoliter volume/minute flow rates and increased separation gradient lengths.[43−45] Therefore in the current study, peptides were analyzed using a 300 nL/min flow rate and separated over 100 min of the gradient. In addition, as seen in Figure 2, approximately 75% of the identified peptides were found in both the ZT 6 and ZT 18 samples, suggesting that the composition of the samples generated from ZT 6 and ZT 18 are similar. On the basis of these two factors, ion suppression effects were minimized.

Integrated mass spectrometric peak intensities, acquired from high resolution FTMS and CID ion trap data, were analyzed between circadian time points. While substance P derived from protachykininn 1 is known to play a role in the generation and entrainment of circadian rhythmicity,[46,47] we observed that substance P peptide exhibited about a 1.5-fold increase in its peptide level at ZT 18. In addition, many of the peptides derived from prohormones that are known to be involved in circadian functions of the SCN exhibited significant changes between daytime and nighttime. For example, truncated forms

of GRP (AA 24−41, 1.4-fold) and VIP (AA 125−137, 1.6-fold) were found to significantly increase at ZT 18 compared to ZT 6. Full-length GRP (AA 24−52) and VIP (AA 125−152) peptides are known to have phase-shifting properties in the SCN.[48−52] According to a previous study, GRP and VIP mRNA levels in mice exhibited circadian rhythms, with the highest levels during the middle of subjective night.[7] In an enzyme immunoassay-based study in rats, VIP immunoreactivity increased over the course of the dark period, whereas GRP immunoreactivity increased during the light period.[42] Our observation that the elevated expression of the truncated form of VIP is significantly higher at ZT 18 than ZT 6 correlates with previous studies using antibodies against full-length VIP.[7,42] This may imply that the truncated form of VIP could work as a bioactive peptide. In the case of the truncated form of GRP, in this study we found the level was higher at nighttime. This could indicate that the truncated form and full-length form of GRP may have different modes of action for SCN circadian functions, or may indicate that the immunoreactivity studies were not set up to distinguish such closely related peptides. The truncated peptide is predicted by our general mammalian[53] and rat-specific[54] prohormone cleavage models. While the truncated GRP peptide could be generated from the sample collection and handling process rather than endogenous intracellular processing, our approaches were designed to minimize such effects. Lastly, the full-length form of GRP may not have been detected by our current LC−MS analysis platform because it was present below the MS detection limit. A previously unidentified short form of PACAP-related peptide, GMGENLAAAAVDDRAPLT (AA 111−128), generated by cleavage of PACAP-related peptide at a monobasic Arg site, had a significant 1.3-fold increase at ZT 18 compared to ZT 6; a similar peptide has been detected via MS in other animal models.[55] PACAP is known to transmit photic signals to the SCN via the retinohypothalamic tract for SCN functions.[56−58] However, discrepancies between photic resetting in the SCN from mouse PACAP knockout models, with and without the additional deletion of the PACAP-related peptide, have been noted.[59,60] PACAP 38 (AA 131−168) peptide levels have been reported to be low during the light period and high during the dark period,[40] which correlates with our observation of the altered level for the truncated form of PACAP-related peptide between daytime and nighttime. In addition to the endogenous peptides that are well-known to be involved in SCN circadian rhythmicity, the peptides derived from proenkephalin A, CART, prothyroliberin, and secretogranin 2 that exhibited ≥30% increases in their peptide levels at nighttime have not been previously implicated in SCN circadian functional studies. Proenkephalin A-derived peptide, VGRPEWWMDYQ (AA 219−229), exhibited a significant increase of more than 1.5-fold at ZT 18 compared to ZT 6. It was reported that the proenkephalin gene interacts with cAMP response-element binding protein (CREB), which is a key regulatory transcription factor influencing timing and entrainment of the *Drosophila* circadian clock.[61] In our previous analysis,[62] VGRPEWWMDYQ (AA 219−229) was observed as one of the released peptides from the SCN stimulated via the optic tract; however, the functional roles of the proenkephalin-A peptides in SCN circadian functions have not been elucidated yet, and deserve further study. Two of the three CART-derived peptides in Table 1 exhibited a ≥30% higher level at ZT 18 than ZT 6. While we first showed that CART-derived peptides are present in the SCN in our previous peptidomic analysis,[15] these peptides have not been yet reported to be related to SCN function. CART is involved in appetite and drug addiction.[63] CART-derived peptides exhibit a diurnal rhythm in several brain regions, including the hypothalamus, depending on food intake; peptides are lower in the morning compared to evening hours under normal feeding regimens.[64]

There are few studies of the direct involvement of prothyroliberin-derived peptide in SCN circadian regulation. Thyrotropin-releasing hormone (TRH) derived from prothyroliberin is known to be expressed in the SCN[65] and has a diurnal rhythm in the rat hypothalamus.[66] While secretogranin 2 gene is reported to be highly expressed in the SCN,[67] it was first reported in our previous study[15] that several endogenous peptides derived from secretogranin 2 were actually present in the SCN. As yet, there are no reports on the involvement of secretogranin 2-derived peptides in SCN circadian functions.[15,67]

While most of the peptides derived from prohormones in the present study exhibited higher levels at nighttime over daytime, two peptides (listed in Supplemental Table S3), VGYDLVPEPKIIDA (AA 77−87) derived from cytochrome *c* oxidase subunit 5A, and vimentin, IKTVETRDGQVINETSQHHDDLE (AA 444−466), exhibited significant increases of more than 30% at daytime compared to nighttime. Although cytochrome *c* oxidase-derived peptide has not been previously reported in the direct involvement of SCN circadian functions, it was found that metabolic activity in the SCN is known to be higher during the daytime than at night, and that mitochondrial cytochrome *c* oxidase activity was higher during the light period.[68−70] In the case of vimentin, there are no reports that it is directly involved in SCN regulation.

■ CONCLUSIONS

Using high resolution FTMS, we performed label-free quantitative analysis of the peptides present in the SCN, many of which have not been previously reported. Based on the integrated peak intensities, we were able to profile the peptide level changes between ZT 6 and ZT 18, representing daytime and nighttime, respectively, in a high-throughput manner. The remarkable differences between these two time points and the high degree of reproducibility of the results we report here support the notion that multiple peptides play important functional roles in SCN circadian rhythmicity. Moreover, the quantitative information and definitive identifications of the SCN peptides detected in this study serve as a basis for discovering novel regulators of SCN circadian functions.

■ ASSOCIATED CONTENT

Supporting Information

Supporting tables S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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