

# Comparing Label-Free Quantitative Peptidomics Approaches to Characterize Diurnal Variation of Peptides in the Rat Suprachiasmatic Nucleus

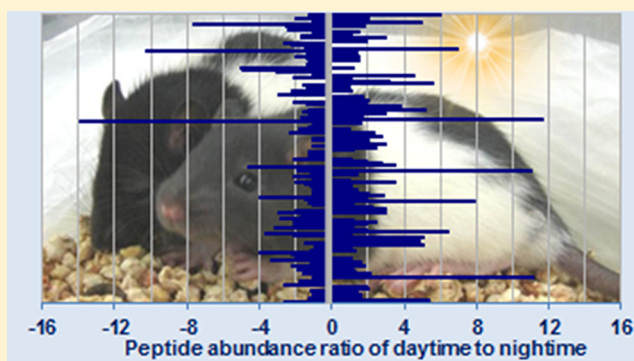
Bruce R. Southey,<sup>†</sup> Ji Eun Lee,<sup>‡,▼</sup> Leonid Zamdborg,<sup>▲</sup> Norman Atkins, Jr.,<sup>||</sup> Jennifer W. Mitchell,<sup>⊥</sup> Mingxi Li,<sup>▲</sup> Martha U. Gillette,<sup>§,||,⊥</sup> Neil L. Kelleher,<sup>▲</sup> and Jonathan V. Sweedler<sup>\*,‡,§,||,¶</sup>

<sup>†</sup>Department of Animal Sciences, <sup>‡</sup>Department of Chemistry, <sup>§</sup>Institute for Genomic Biology, <sup>||</sup>Neuroscience Program, <sup>⊥</sup>Department of Cell and Developmental Biology, and <sup>¶</sup>Beckman Institute, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, United States

<sup>▲</sup>Department of Chemistry, Department of Molecular Biosciences, and Chemistry of Life Processes Institute, Northwestern University, Evanston, Illinois 60208, United States

## S Supporting Information

**ABSTRACT:** Mammalian circadian rhythm is maintained by the suprachiasmatic nucleus (SCN) via an intricate set of neuropeptides and other signaling molecules. In this work, peptidomic analyses from two times of day were examined to characterize variation in SCN peptides using three different label-free quantitation approaches: spectral count, spectra index and SIEVE. Of the 448 identified peptides, 207 peptides were analyzed by two label-free methods, spectral count and spectral index. There were 24 peptides with significant (adjusted  $p$ -value < 0.01) differential peptide abundances between daytime and nighttime, including multiple peptides derived from secretogranin II, cocaine and amphetamine regulated transcript, and proprotein convertase subtilisin/kexin type 1 inhibitor. Interestingly, more peptides were analyzable and had significantly different abundances between the two time points using the spectral count and spectral index methods than with a prior analysis using the SIEVE method with the same data. The results of this study reveal the importance of using the appropriate data analysis approaches for label-free relative quantitation of peptides. The detection of significant changes in so rich a set of neuropeptides reflects the dynamic nature of the SCN and the number of influences such as feeding behavior on circadian rhythm. Using spectral count and spectral index, peptide level changes are correlated to time of day, suggesting their key role in circadian function.



The circadian rhythm is a physiological process that occurs in an approximately 24-h cycle controlled by biological clocks. In mammals, the suprachiasmatic nucleus (SCN), located in the hypothalamus, acts as a “master clock” that links the other biological clocks to time of day. Many body functions are synchronized, including energy homeostasis components such as the sleep-wake cycle, thermoregulation, metabolism, and feeding behavior.<sup>1,2</sup> Disruptions to biological clocks have been associated with many disorders, such as excessive sleepiness, depression, and obesity. Research has uncovered a molecular link between altered circadian rhythms and addiction, with both molecular systems sharing common signaling pathways.<sup>3,4</sup> As just one example, vasoactive intestinal peptide (VIP) is an essential peptide in the regulation of the biological clock<sup>5–7</sup> and plays a prominent role in morphine treatment and withdrawal-induced acute hormonal changes in rat.<sup>8</sup>

The basic biological clock involves a set of genes that maintains an almost constant rhythm that is influenced by

different stimuli or zeitgebers. Although light is the important zeitgeber, other stimuli, such as body temperature, and exercise and feeding schedules, can entrain the biological clock.<sup>1,2</sup> Various neuropeptides, notably VIP, pituitary adenylate cyclase-activating polypeptide (PACAP), arginine vasopressin (AVP), and gastrin-releasing peptide (GRP), are critical messengers in maintaining SCN synchrony and circadian rhythm in other tissues.<sup>5,9</sup> Recently Atkins et al.<sup>10</sup> observed that little SAAS, derived from proprotein convertase subtilisin/kexin type 1 inhibitor (PCSK1N) prohormone, is also involved in the SCN light response. Gene and protein expression patterns of appetite-related prohormones and neuropeptides, such as neuropeptide Y and Agouti-related protein (AGRP) that stimulate appetite, and cocaine and amphetamine regulated transcript (CART) peptide and pro-opiomelanocortin

Received: July 26, 2013

Accepted: December 6, 2013

Published: December 6, 2013

(POMC)-derived peptides that reduce appetite, exhibit circadian rhythm.<sup>1,2</sup> Rodents with a mutant clock circadian regulator (*Clock*) gene have abnormal patterns of food intake and metabolic process neuropeptides such as ghrelin, orexin, and CART.<sup>11</sup>

Considerable SCN peptide identification work has been done using indirect approaches, such as in situ hybridization and immunohistochemistry.<sup>12</sup> However, the specific peptide forms detected by these approaches remain unknown without extensive validation studies. Alternatively, mass spectrometry (MS) is a direct approach that allows large numbers of endogenous peptides, including associated PTMs, to be characterized.<sup>13–15</sup> MS can also be used to monitor peptide release, providing a functional context for detected peptides.<sup>10,12,16</sup> A mass spectrometric survey of SCN tissue samples collected 6 h after lights on led to the identification of 102 endogenous peptides, including many known neuropeptides associated with the biological clock.<sup>17</sup> This survey also identified shortened forms of peptides from VIP, GRP, and somatostatin (SST) prohormones that are unlikely to be distinguished from longer forms by immunohistochemistry, leading to uncertainty about the actual biologically active forms of these peptides.<sup>12</sup>

Quantitative MS-based proteomics, which can be used to characterize relative peptide abundance across different conditions, can be undertaken using either label-based or label-free methods.<sup>18–20</sup> Label-based methods typically involve distinct isotopic labeling of each sample and require extensive sample preparation and analysis. Several label-free methods have been shown to have a high correlation (0.99) to relative protein abundance.<sup>18</sup> One method involves identification and comparison of chromatographic precursor ion intensity from single stage MS, and another, called spectral count, uses the number of times that a peptide is identified with tandem MS (MS/MS). Another label-free alternative, a variation of spectral count, is spectral index, which is the cumulative intensity of product ions in the MS/MS spectrum of an identified peptide.<sup>21</sup>

Previously Lee et al.<sup>22</sup> studied 18 rat SCN peptide samples collected ~6 h after lights on (daytime) and ~6 h after lights off (nighttime). A total of 310 peptides were identified, with 230 peptides detected at both daytime and nighttime, 49 peptides detected only at daytime, and 31 peptides only detected at nighttime. Their results confirmed 63 peptides and 26 related peptides from the 102 hyperconfident peptide list reported in an earlier survey of SCN daytime peptides.<sup>17</sup> The differential peptide abundance between daytime and nighttime for 173 peptides was assessed based on label-free integrated precursor ion intensity from chromatographically aligned spectra (i.e., SIEVE analysis). Twelve peptides were found to have a significantly differential abundance between daytime and nighttime (adjusted *p*-value < 0.05), with a further 14 peptides having a marginally significant differential abundance (adjusted *p*-value < 0.1).<sup>22</sup>

Here, we reanalyze this rich data set<sup>22</sup> using the spectral count and spectral index approaches to extract relative quantitative data from the SCN peptide samples. This allows a comparison of three label-free approaches for a high dynamic range neuropeptide application and also provides a much larger and distinct list of peptides that change in a time-of-day dependent manner. First, the MS data was annotated independently from the workflow used for chromatographic alignment using the same annotation criteria for both new

approaches. The spectral count and spectral index for each peptide were obtained from every sample and analyzed for the differential abundance between daytime and nighttime. The current analysis reveals several issues with the SIEVE method and demonstrates that both spectral count and spectral index provide a richer characterization of differential peptide abundance. Moreover, this investigation confirms the roles of PCSK1N peptides and peptides associated with feeding behavior in the SCN.

## ■ EXPERIMENTAL SECTION

**Sample and Data Collection.** We used the data set previously described.<sup>22</sup> Briefly, 8–10 week old male Long–Evans rats (LE-BluGill, an inbred strain from the University of Illinois at Urbana–Champaign) housed under a 12:12 light–dark cycle, fed ad libitum, were used in this study. Samples were collected over three days at approximately 6 h after lights on (Zeitgeber time 6, or daytime) and approximately 6 h after lights off (Zeitgeber time 18, or nighttime); each sample contained individual SCN brain punches pooled from 24 rats (*n* = 24). We collected 6 samples (2 time points × 3 samples [days]). Overall this resulted in a total of 144 SCN punches harvested (24 rats × 2 time points × 3 samples [days]). Each of the 6 biological samples were subjected to multistage peptide extraction and then separated into 3 equivalent technical replicate samples, resulting in 18 total samples for analysis (2 time points × 3 biological samples × 3 technical replicate samples). The samples were collected in compliance with the National Institutes of Health guidelines (NIH Publication no. 85-23) using approved vertebrate animal protocols approved by the Institutional Animal Care and Use Committee, University of Illinois at Urbana–Champaign.

The technical replicate samples, alternating between time of collection, were sequentially run on a 12 T ion trap-based mass spectrometer (LTQ-FT Ultra, Thermo Fisher Scientific, San Jose, CA) under the same experimental conditions. Data acquisition included a full scan event (*m/z* 300–1500), and data-dependent collision-induced dissociation (CID) ion trap MS/MS scans of the 10 most abundant peaks from the previous full scans. The MS/MS settings included a 3 *m/z* isolation width and minimum signal threshold of 1000 counts.

**Peptide Identification.** We validated our prior peptide identifications using the MS/MS data and the Open Mass Spectrometry Search Algorithm (OMSSA) 2.1.1<sup>23</sup> with manually curated rat peptide sequences derived from UniProt<sup>24</sup> (15.15 release) that included known rat neuropeptides. Simulation of neuropeptide-based data has demonstrated that OMSSA is able to accurately identify neuropeptides over a wide range of scenarios.<sup>25</sup> The search criteria used the program defaults except for the following settings: nonspecific enzyme, 0.01 Da peptide *m/z* tolerance, 0.5 Da MS/MS *m/z* tolerance, monoisotopic search using both *b* and *y* ions, the instrument precursor charge state was used, charge state was bounded between 2 and 20, and a minimum charge state of 3 was used to detect multiply charged products, and required that at least one of the top six most intense peaks must match an ion from the assigned sequence. The PTMs permitted were acetylation, amidation, Met oxidation, pyroglutamylation of Gln/Glu, Ser, Thr, and Tyr phosphorylation, and N-terminal cleavage of Met. For peptides with more than two PTMs, only manually verified identified peptides were kept. The identified peptides were defined as a combination of amino acid sequence and PTM. The false identification rate (FIR) of peptide identification was

**Table 1. Number of Peptides Present in One Condition and Significant for Spectral Count and Spectral Index Using Different Thresholds**

	unanalyzed peptides	spectral count $p$ -value < 0.001	spectral count $p$ -value < 0.01	spectral count $p$ -value < 0.05	spectral count $p$ -value < 0.1	total peptides
only present at daytime	0	0	0	0	0	129
only present at nighttime	0	0	0	0	0	78
unanalyzed peptides spectral index	0	1	0	1	24	26
spectral index $p$ -value < 0.001	19	4	8	6	26	63
spectral index $p$ -value < 0.01	0	1	2	2	9	14
spectral index $p$ -value < 0.05	0	1	4	2	8	15
spectral index $p$ -value < 0.1	0	0	1	0	4	5
nonsignificant peptides	1	3	6	3	105	118
total peptides	20	10	21	14	176	448

determined by using a separate decoy search using the reversed sequences since the OMSSA  $E$ -value is determined by database size. Only peptides that surpassed a 5% FIR were used, which corresponded to a 2.7% false discovery rate (FDR)<sup>26</sup> threshold on the parametrically derived OMSSA  $E$ -Value. This implied that the calculated FIR threshold based on the separate decoy search may be more stringent than initially assumed. We used the nomenclature as listed for the official mouse and rat nomenclature guidelines.<sup>27</sup>

**Spectral Count.** The peptide spectral count was determined as the number of times that a specific peptide (defined by sequence and PTMs) was detected in each technical replicate sample. Under the spectral count definition, any undetected peptide would have a zero spectral count. However, a zero count can arise from a peptide being truly absent in a condition, that is, missing data, or from a failure to detect the presence of the peptide. Differentiation between missing data and zero counts for each peptide was determined by comparing technical replicate samples from the same biological sample. If a peptide was detected in at least 1 technical replicate sample (of the 3 from each biological sample), then all technical replicate samples from the same biological sample where the peptide was not detected were recorded as a zero count. However, if a peptide was not detected in any technical replicate samples from the associated biological sample, then that peptide was recorded as undetected in all technical replicate samples of that biological sample.

**Spectral Index.** On the basis of Griffin et al.<sup>21</sup> and Wu et al.,<sup>28</sup> the peptide spectral index was calculated as the total number of ions measured in the MS/MS spectra for a peak, divided by the total number of ions measured in all of the MS/MS spectra, and divided by the length of the peptide.

**Statistical Analysis.** The spectral count data on each peptide identified at both time points was analyzed using a Poisson model where the spectral count was modeled with collection time (daytime or nighttime) and the biological samples were nested within the time of collection. Similarly, the log-transformed spectral index was modeled as time of collection (daytime or nighttime), and the biological samples were nested within time of collection under the assumption of normality. An FDR multiple test adjustment was performed for collection times. These analyses were conducted using SAS (Statistical Analysis Systems, Cary, NC).

## RESULTS

**Peptide Identification.** A total of 47 554 spectra (22 832 or 48% at daytime and 24 722 or 52% at nighttime) were

identified with 72.5% of the identified spectra surpassing the 5% FIR threshold. There was little difference in the proportion of spectra surpassing the 5% FIR threshold between daytime (72.6%) and nighttime (71.4%) or individual biological samples at daytime (range 69.6–75.9%) or nighttime (range 69.1–73%). Identified spectra consisted of 448 peptides from 24 prohormones (167 peptides) and 167 from other proteins (281 peptides). The majority of the peptides from prohormones were derived from the same prohormones. For example, 43, 30, and 16 peptides were derived from secretogranin II (SCG2), PCSK1N, and preproenkephalin (PENK), respectively. Most of the peptides from the other proteins were uniquely derived from a single proteins (129 peptides from 129 proteins), with 15 and 33 peptides derived from cytochrome *c* oxidase, subunit Va (COX5A), and phosphatidylethanolamine binding protein 1 (PEBP1), respectively.

The 448 peptides included 167 peptides derived from prohormones and 281 peptides derived from other proteins (Supporting Information Table S1). Approximately half of the peptides (241 peptides) were identified in both sampling times across multiple biological samples. This resulted in 62 prohormone peptides and 48 other protein peptides being detected in all daytime and nighttime biological samples with spectral counts greater than one. Eighteen peptides were only detected in a single biological sample for both time points, with only 8 peptides detected once in both time points. This indicated that most peptides were being identified in multiple samples across time points rather than an overrepresentation of a few technical replicate samples.

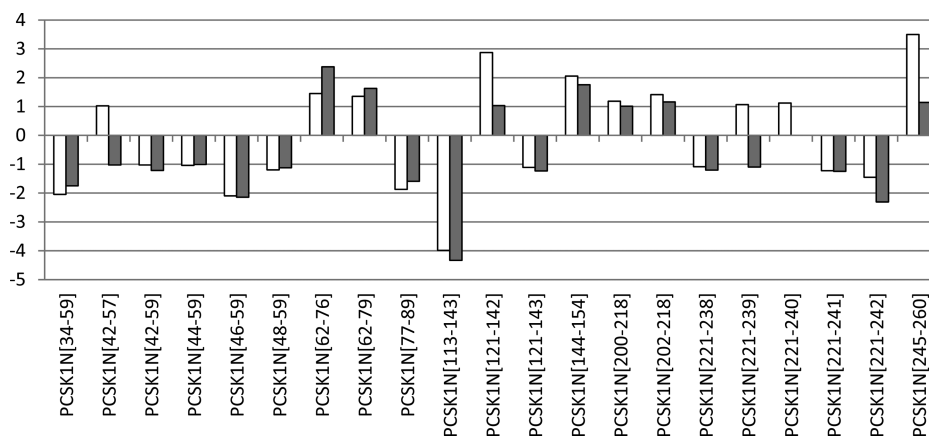
Many of the 180 peptides detected at a single time point were in low abundance, with 118 peptides (74 daytime and 44 nighttime peptides) only detected once. The 129 peptides only detected at daytime included 27 peptides derived from prohormones and 102 peptides derived from other proteins. The SCG2 prohormone provided the highest number of peptides only detected at daytime (12 peptides), and the two most frequent peptides were SCG2[184–201] (64 spectral counts in 5 samples) and SCG2[571–585] (34 spectral counts in 3 samples). Other peptides only detected at daytime with more than 10 spectral counts from two biological samples were from pyruvate kinase (PKM), N-myc downstream regulated gene 2 (NDRG2), COX5A, diazepam binding inhibitor (DBI), and  $\beta$ -actin (ACTB). The 78 peptides only detected at nighttime were derived from a large number of proteins that included 27 and 51 peptides from prohormones and other proteins, respectively. The most frequent peptides were from VGF nerve growth factor inducible (VGF) (5 peptides) and



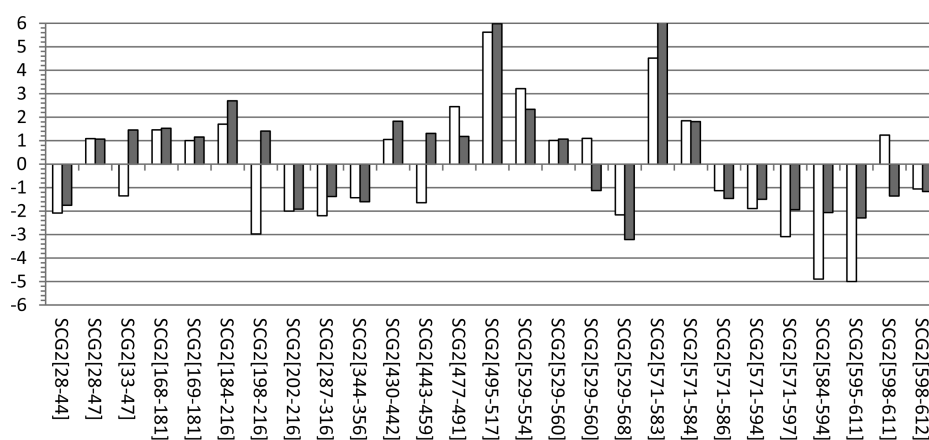
Table 2. Peptides with Differential Abundance for Both Spectral Count and Spectral Index

UniProt Accn <sup>b</sup>	location <sup>c</sup>	peptide	sequence and PTM	NS <sup>e</sup>				spectral count				spectral index			
				D	N	D/N <sup>d</sup>	SE <sup>e</sup>	p-value	aPV <sup>f</sup>	D/N	SE	p-value	aPV		
P49192	CARTPT[37–54]	ALDIYSAVDDASHEKELP		9	9	0.56	0.07	1.66 × 10 <sup>-06</sup>	1.22 × 10 <sup>-21</sup>	0.59	0.03	2.85 × 10 <sup>-05</sup>	2.19 × 10 <sup>-20</sup>		
P49192	CARTPT[37–54]	ALDIYSAVDDAS[phos]HEKELP		9	9	0.62	0.08	1.80 × 10 <sup>-04</sup>	1.46 × 10 <sup>-06</sup>	0.60	0.06	1.97 × 10 <sup>-03</sup>	6.39 × 10 <sup>-06</sup>		
P49192	CARTPT[60–79]	APGAVLQEAALQEVLLKLLKS		3	4	11.13	5.74	3.03 × 10 <sup>-06</sup>	1.53 × 10 <sup>-40</sup>	11.25	2.04	4.59 × 10 <sup>-05</sup>	1.10 × 10 <sup>-38</sup>		
P11240	COXSA[56–66]	FNKPDIDAWEL		6	7	4.89	1.51	2.60 × 10 <sup>-07</sup>	1.13 × 10 <sup>-09</sup>	3.15	0.59	5.23 × 10 <sup>-06</sup>	8.13 × 10 <sup>-09</sup>		
P14200	MCH[32–55]	NVEDDIVENTFRMGKAFQKEDTAE		6	9	0.59	0.09	7.41 × 10 <sup>-04</sup>	2.73 × 10 <sup>-03</sup>	0.68	0.59	6.38 × 10 <sup>-03</sup>	8.05 × 10 <sup>-03</sup>		
P30904	MIF[11–27]	PRASVPEGFSELTQQL		6	8	2.23	0.48	1.64 × 10 <sup>-04</sup>	2.99 × 10 <sup>-10</sup>	3.05	0.54	1.88 × 10 <sup>-03</sup>	2.38 × 10 <sup>-09</sup>		
P19804	NDKB[2–16]	[acetyl]ANLERTFAIKPDGV		9	4	7.88	3.52	3.69 × 10 <sup>-06</sup>	2.44 × 10 <sup>-07</sup>	2.80	0.56	5.23 × 10 <sup>-05</sup>	1.28 × 10 <sup>-06</sup>		
Q9QXU9	PCSK1[113–143]	WGSPRASDPLAPDDDDPDAPAAQLARALLRA		6	3	0.25	0.09	2.14 × 10 <sup>-04</sup>	2.37 × 10 <sup>-92</sup>	0.23	0.02	2.24 × 10 <sup>-03</sup>	5.10 × 10 <sup>-90</sup>		
Q9QXU9	PCSK1[221–242] (PEN)	AVDQDLGPEVPPENVLGLLRV		9	9	0.69	0.04	2.19 × 10 <sup>-13</sup>	7.42 × 10 <sup>-29</sup>	0.43	0.03	5.28 × 10 <sup>-11</sup>	1.59 × 10 <sup>-27</sup>		
P06300	PDYN[211–218]	PKLKWQDQ		7	9	0.22	0.06	1.44 × 10 <sup>-07</sup>	5.26 × 10 <sup>-09</sup>	0.36	0.06	3.46 × 10 <sup>-06</sup>	3.53 × 10 <sup>-08</sup>		
P31044	PEBP1[170–187]	[pyro]QAEWDDVSPKLDHDLQAGK		9	6	2.77	0.67	2.44 × 10 <sup>-05</sup>	5.80 × 10 <sup>-07</sup>	1.69	0.18	2.94 × 10 <sup>-04</sup>	2.71 × 10 <sup>-06</sup>		
P31044	PEBP1[93–120]	KGNDISSGTVLSEYVGGPPKDTGLHRY		6	5	2.37	0.59	5.52 × 10 <sup>-04</sup>	2.22 × 10 <sup>-05</sup>	2.70	0.63	5.12 × 10 <sup>-03</sup>	8.69 × 10 <sup>-05</sup>		
P04094	PENK[263–269] (Met-enkephalin-Arg-Phe)	YGGFMRF		4	9	0.07	0.04	1.23 × 10 <sup>-07</sup>	5.54 × 10 <sup>-19</sup>	0.10	0.03	3.30 × 10 <sup>-06</sup>	7.44 × 10 <sup>-18</sup>		
Q9R063	PRDX5[204–213]	SLAPNLSQL		9	7	5.22	1.52	1.44 × 10 <sup>-08</sup>	1.39 × 10 <sup>-06</sup>	4.13	1.21	6.93 × 10 <sup>-07</sup>	6.22 × 10 <sup>-06</sup>		
Q9R063	PRDX5[54–67]	PIKVGDTIPSVEVF		6	8	3.84	1.11	3.05 × 10 <sup>-06</sup>	1.65 × 10 <sup>-32</sup>	2.72	0.23	4.59 × 10 <sup>-05</sup>	5.07 × 10 <sup>-31</sup>		
O35314	SCG1[597–611]	[pyro]QYDDGVAELDQLLHY		9	9	0.63	0.06	3.98 × 10 <sup>-07</sup>	2.34 × 10 <sup>-06</sup>	0.58	0.07	7.38 × 10 <sup>-06</sup>	1.01 × 10 <sup>-05</sup>		
P10362	SCG2[495–517]	PYDNLNDKQDELGEYLARMLVKY		3	4	5.62	1.81	8.59 × 10 <sup>-08</sup>	4.64 × 10 <sup>-13</sup>	5.98	1.48	2.59 × 10 <sup>-06</sup>	5.25 × 10 <sup>-12</sup>		
P10362	SCG2[529–554]	VPSPGSEDDDLQEEEQLEQAIKEHLG		9	6	3.22	0.70	8.24 × 10 <sup>-08</sup>	2.34 × 10 <sup>-04</sup>	2.34	0.54	2.59 × 10 <sup>-06</sup>	8.00 × 10 <sup>-04</sup>		
P10362	SCG2[571–583]	IPAGSLKNEPTN		6	6	4.52	0.98	4.40 × 10 <sup>-12</sup>	1.90 × 10 <sup>-08</sup>	15.12	7.30	3.53 × 10 <sup>-10</sup>	1.20 × 10 <sup>-07</sup>		
Q6PDU1	SFRS2[2–21]	[acetyl]SYGRPPDVEGTMSTLKVVDNL		3	8	0.10	0.05	7.28 × 10 <sup>-06</sup>	3.58 × 10 <sup>-19</sup>	0.18	0.03	9.75 × 10 <sup>-05</sup>	5.14 × 10 <sup>-18</sup>		
P06767	TKN1[72–94]	DADSSIEKQVALLKALYGHGQIS		9	9	0.66	0.07	2.15 × 10 <sup>-05</sup>	3.85 × 10 <sup>-38</sup>	0.47	0.03	2.72 × 10 <sup>-04</sup>	2.07 × 10 <sup>-36</sup>		
P08435	TKNK[95–115]	NSQPDTPADVVEENTPSPFVGL		8	9	0.45	0.07	2.21 × 10 <sup>-07</sup>	3.10 × 10 <sup>-05</sup>	0.41	0.09	4.84 × 10 <sup>-06</sup>	1.19 × 10 <sup>-04</sup>		
Q5PPNS	TPPP3[2–17]	[acetyl]AASTDIAGLEESFRKF		9	9	2.97	0.46	1.37 × 10 <sup>-12</sup>	4.71 × 10 <sup>-08</sup>	3.15	0.66	1.66 × 10 <sup>-10</sup>	2.81 × 10 <sup>-07</sup>		
P20156	VGF[285–309] (neuroendocrine regulatory peptide-1)	LEGSFLGGSEAGERLLQQGLAQVEA		4	9	0.13	0.04	5.72 × 10 <sup>-11</sup>	2.22 × 10 <sup>-10</sup>	0.30	0.06	3.45 × 10 <sup>-09</sup>	1.84 × 10 <sup>-09</sup>		

<sup>a</sup>Number of daytime (D) and nighttime (N) samples where the peptide was detected. <sup>b</sup>Uniprot accession number. <sup>c</sup>Peptide location within in the prohormone: CARTPT, CART prepropeptide; COX5A, cytochrome c oxidase, subunit Va; MCH, pro-melanin-concentrating hormone; MIF, macrophage migration inhibitory factor (glycosylation-inhibiting factor); NDKB, NME/NM23 nucleoside diphosphate kinase 2; PCSK1N, proprotein convertase subtilisin/kexin type 1 inhibitor; PDYN, prodynorphin; PEBP1, phosphatidylethanolamine binding protein 1; PENK, proenkephalin; PRDX5, peroxiredoxin 5; SCG1, chromogranin B (secretogranin 1); SCG2, secretogranin II; SFRS2, serine/arginine-rich splicing factor 2; TKN1, tachykinin, precursor 1; TKNK, tachykinin 3; TPPP3, tubulin polymerization-promoting protein family member 3; VGF, VGF nerve growth factor inducible; and peptide name in parentheses if known. <sup>d</sup>Ratio of peptide abundance at daytime compared to nighttime. <sup>e</sup>Standard error of the peptide abundance ratio at daytime compared to nighttime. <sup>f</sup>False discovery rate adjusted *p*-value.



**Figure 1.** Ratio of peptide abundance as determined by spectral count (white bars) and spectral index (gray bars) for different PCSK1N peptides. Positive values indicate peptides more abundant at daytime than nighttime and negative values indicate peptides less abundant at daytime than nighttime.



**Figure 2.** Ratio of peptide abundance as determined by spectral count (white bars) and spectral index (gray bars) for different SCG2 peptides. Positive values indicate peptides more abundant at daytime than nighttime and negative values indicate peptides less abundant at daytime than nighttime.

myelin basic protein (MBP) (4 peptides). However, peptides from PCSK1N, PENK (2 peptides), chromogranin B (SCG1), SCG2, and thyrotropin releasing hormone (TRH) prohormones were detected in more than 1 biological sample with a spectral count greater than 10.

Most of the peptides found at a single time point were versions of more frequently detected peptides, such as single amino acid truncated forms of longer peptides. There were two peptides with spectral counts over 10 that were detected only in a single technical sample at either time point. The peptide SCG2[205–216], which was detected 18 times in a single technical sample, is a truncated form of three other SCG2 peptides that were detected at both time points. The most frequent of these peptides was SCG2[184–216], which was detected 77 and 75 times at daytime and nighttime, respectively. Similarly, a fragment of the copeptin derived from vasopressin-neurophysin 2-copeptin (NEU2) prohormone, NEU2[151–167], which was only detected in a single nighttime sample (10 spectral counts), is a single amino acid truncated form of the peptide NEU2[151–168], and was detected in almost all samples (9 daytime and 7 nighttime).

**Analysis of Spectral Count and Spectral Index.** Table 1 summarizes the different analyses provided in Supporting Information Table S1 for all peptides analyzed by spectral

count and spectral index, including 207 unanalyzed peptides because of their detection at a single time point, and 26 peptides unanalyzed by spectral index. There were 19 peptides significant (adjusted  $p$ -value < 0.001) for both spectral count and spectral index and, at a less stringent threshold (adjusted  $p$ -value < 0.01), a further 5 peptides significant for both analyses. This increased to a total of 50 peptides significant for both spectral count and spectral index at a 10% adjusted threshold. Only 4 peptides were significant for spectral count (adjusted  $p$ -value < 0.01) but nonsignificant for spectral index. There were 35 peptides significant for only spectral index (adjusted  $p$ -value < 0.01) but not for spectral count. There were 105 peptides nonsignificant (adjusted  $p$ -value > 0.1) in either analysis.

There were 24 peptides from 10 prohormones and 7 other proteins with significantly differential abundance between daytime and nighttime at an adjusted  $p$ -value of < 0.01 for both spectral count and spectral index (Table 2). The most peptides came from SCG2 (3 peptides), CART prepropeptide (CARTPT) (3 peptides), PEBP1 (2 peptides), peroxiredoxin 5 (PRDX5) (2 peptides), and PCSK1N (2 peptides). Most of the peptides from prohormones (11 peptides) were significantly more abundant at nighttime than daytime. For example, PCSK1N peptides showed more peptides that were more abundant at nighttime than daytime (Figure 1). In contrast, all

of the peptides from other proteins, except for 1 peptide, were more abundant at daytime than nighttime. Except for the CARTPT-derived peptides, all peptides from the same prohormone or protein had similar abundance patterns. Only SCG2[198–216], which was significant for both spectral count (adjusted  $p$ -value < 0.05) and spectral index (adjusted  $p$ -value < 0.001), had a different direction of the relative abundance between the spectral count and spectral index analyses (Figure 2). This peptide was only detected in a single daytime technical replicate sample and 5 nighttime samples, such that the average ion intensity was higher in the daytime sample than the nighttime samples.

The 5 peptides that were only significant (adjusted  $p$ -value < 0.01) for spectral count were derived from 2 prohormones and 3 proteins. The 47 peptides that were only significant for spectral index were from 10 prohormones and 17 proteins. The most frequent prohormones were PCSK1N (5 peptides from the prohormone), PENK (4 peptides), and SCG2 (3 peptides); the most frequent proteins were PEBP1 (8 peptides) and COXSA (3 peptides). In contrast to the spectral count analyses, the 26 peptides unanalyzed for spectral index included a ribosomal protein S21 (RS21) peptide (RS21[74–83]) and PENK[85–96], which were marginally significant for spectral count. Both of these peptides were highly frequent at daytime (spectral count of 40 and 15, respectively) but were only detected once at nighttime. However, VGF[587–600], which was detected 21 times at daytime and 3 times at nighttime, was not significant for either analysis. The few peptides only significant for spectral count implies that peptides with significant differential abundance, as determined by spectral count, are also differentially abundant with spectral index. This suggests that spectral index is more sensitive than spectral count for infrequently observed peptides.

**Influence of PTMs.** The majority (72.58%) of peptides identified had no PTMs; of those peptides with PTMs, phosphorylation (10.56%) and acetylation (8.31%) were the most common. There were 425 unique peptide sequences, with only 23 peptides having two PTMs. There were 8 peptide sequences from 5 prohormones (CARTPT, POMC, PENK, SCG1, and TRH) and 1 protein (PEBP1) where at least the modified or unmodified form was significant for either spectral count or spectral index. Except for unmodified SCG1[597–611], all peptides were found in at least 2 biological samples per time point. In all cases, both forms exhibited the same direction of difference, implying that the modification process was not related to the peptide abundance. This included the phosphorylated forms of CARTPT[37–54] (ALDIYSAVDDAS[phos]HEKELP) and CARTPT[37–55] (ALDIYSAVDDAS[phos]HEKELPR), which differed by an N-terminal Arg. The CARTPT[37–54] peptide was significantly more abundant for both spectral count and spectral index than the CARTPT[37–55] peptide. Different modifications of three peptide sequences showed differential sequences between spectral count and spectral index. Both the unmodified POMC[141–162] peptide and phosphorylated POMC[141–162] peptide (RPVKVYPNVAENES[phos]AEAFPLEF) were found in at least 17 samples and had significant differential abundance for spectral index (adjusted  $p$ -value < 0.01). The phosphorylated POMC[141–162] was nonsignificant for spectral count but the unmodified peptide was slightly significant (adjusted  $p$ -value < 0.05). A similar trend was observed with pyroglutamated PEBP1[170–187] ([pyro]-QAEWDDSVPKLHDQLAGK) peptide, where the pyrogluta-

mated peptide was significant for both spectral count and spectral index (adjusted  $p$ -value < 0.01), but the unmodified peptide was only significant for spectral index. However, sampling variation may have limited any differences because the unmodified PEBP1[170–187] peptide was only detected in 4 daytime and 5 nighttime technical replicate samples. The unmodified SCG1[597–611] peptide sequence was only detected in 1 spectral index daytime sample, whereas the pyroglutamated SCG1[597–611] ([pyro]-QYDDGVAELDQLLHY) was detected in all 18 spectral index samples. This differential abundance associated with the PTMs may reflect a biologically related, time-dependent modification of these peptides.

## DISCUSSION

Perhaps not surprisingly, of the 448 peptides reported in this study, the vast majority (310 peptides) were also reported in our previous study using SIEVE.<sup>22</sup> The remaining 138 peptides had low abundance, including 118 peptides with a single spectral count, which were not analyzed in the current work. There were 164 peptides analyzed in both studies, 77 peptides only analyzed in this study, and 9 peptides only analyzed in the prior study. Most of the differences in the number of peptides analyzed were attributed to the  $1 \times 10^{-3}$  OMSSA E-value criterion used in the prior study compared to the 5% FIR used in this study. However, many of the peptides missing in the prior study had a spectral count of at least 20, and 11 of these peptides were present in all 18 technical replicate samples. This indicates a fundamental failure of the general SIEVE method to detect and analyze many of the peptides, even though many peptides had multiple annotated MS/MS spectra from multiple technical replicate samples. We had previously observed that peptides with long elution times were problematic for the automatic alignment of SIEVE,<sup>22</sup> which may have also been a contributing factor to the differences in peptides analyzed between the two studies.

One of the more interesting outcomes is the lack of agreement between the results of the prior SIEVE analysis and the spectral count and index analyses performed in the current study for the 164 peptides analyzed in both efforts. Only 7 peptides, CARTPT[37–54], phosphorylated CARTPT[37–54], a secretogranin III (SCG3) fragment (SCG3[38–57]), TRH[178–199], a vimentin (VIME) fragment (VIME[444–466]), SCG2[340–356], and PENK[263–269], were found to be significant (adjusted  $p$ -value < 0.1) using all three methods. However, 24 peptides that were marginally significant for both spectral count and spectral index were nonsignificant in the SIEVE analysis; 54 peptides were nonsignificant for all three methods. But there were 6 peptides significant in the SIEVE analysis that there were nonsignificant for both spectral count and spectral index. Differential peptide detection was not a contributing factor because 3 of these peptides, an adenylate cyclase activating polypeptide 1 (ADCYAP1) fragment (ADCYAP1 [111–128]), SCG1[435–450], and PCSK1N[44–59], were detected in all 18 technical replicate samples with similar average spectral counts and spectral indexes between daytime and nighttime. A possible reason is the asymmetrical sensitivity of Fisher's method combined probability test, such that a high significance in one frame will dominate the test, even though there is no overall difference between conditions. In addition, there were 9 peptides that were marginally significant for both the spectral index and the SIEVE analysis but not for the spectral count. This may be

because the spectral index and SIEVE analyses both involve ion intensities from different levels and could support the differential abundance of those 9 peptides.

A potential advantage of the SIEVE method is that all frames can be analyzed for a peptide, provided that at least one frame has an annotated peptide. This was evident by the vast majority of the frames having 18 technical replicate samples with integrated peak intensities greater than zero. As a result, the SIEVE analysis provided some quantitation of the differences between daytime and nighttime for some of the peptides that could not be analyzed by the spectral methods. Sixteen and 14 peptides that were only detected at daytime and nighttime, respectively, were analyzed by SIEVE, but could not be analyzed in this study. A 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMCS1) fragment, HMCS1[159–179], that was only detected in a single nighttime technical replicate sample, was marginally significant (adjusted  $p$ -value < 0.1) in the SIEVE analysis. This indicates that there is no benefit from the SIEVE analysis for peptides only detected in one condition.

Unlike the analysis of the overall peptide abundance between time points provided by the spectral count method, the SIEVE method only provides the analysis of differential peptide abundance within a frame. SIEVE ignores variations between frames and requires similar differential peptide abundances across frames in order to provide a reliable estimate of overall peptide abundance. An important underlying assumption of the SIEVE method is that integrated intensity is only attributed to one peptide. When more than one peptide is annotated within a frame, the integrated intensity is replicated for each identified peptide and the analysis ignores the presence of other peptides annotated in the same frame. Consequently, the integrated intensity will be overestimated when more than one peptide is present in the frame. In the previous study,<sup>22</sup> 96%, 3%, and 1% of frames were assigned to 1, 2, and 4 peptides, respectively, suggesting that any bias due to multiple peptides would be small for most peptides. This ignores any bias resulting from the presence of chimeric spectra, where two or more peptides are present in the same MS/MS spectrum, since OMSSA is generally able to detect only one of the peptides present in simulated spectra.<sup>25</sup>

This study confirms the presence of a number of different neuropeptides in the SCN<sup>17,22</sup> and highlights limitations in our knowledge of the role many of these peptides play in SCN function. While some detected peptides are processed from prohormones corresponding to known and predicted cleavage sites, other peptides may be degradation products of larger peptides or proteins, and, potentially new bioactive peptides. Evaluation of the differences between an expected full-length peptide and a shorter, potentially degraded form at the two time points studied, suggests a circadian role for that peptide. Specific examples of known peptides and proteins that were detected in the current study are discussed below.

Several peptides from ADCYAP1 and VIP prohormones, which are associated with SCN synchronization and light entrainment,<sup>5,6</sup> were detected. The ADCYAP1[111–128] peptide, a truncated form of the PACAP-related peptide, was detected in all daytime and nighttime samples but showed no difference in spectral count or spectral index between daytime and nighttime. A few peptides from the C-terminal region of the VIP prohormone and a shortened form of the intestinal peptide PHI-27 region of VIP were detected. Most of the peptides from the VIP prohormone were irregularly detected except for VIP[156–169] peptide from the VIP C-terminal

region, which was detected in all samples. While VIP[156–169] was frequent at both daytime and nighttime, it had significantly (adjusted  $p$ -value < 0.001) higher abundance at nighttime than daytime. This pattern indirectly supports the increased expression of the VIP peptides at nighttime compared to daytime<sup>6</sup> because this terminal peptide is produced by one of the cleavages necessary to produce the full length VIP[125–152]. The automatic annotation failed to identify short VIP[125–137] (HSDAVFTDNYTRL), which was observed to be more abundant at nighttime compared to daytime using manual integration.<sup>22</sup> Only the terminal VIP propeptide without the C-terminal lysine was detected in all technical replicate samples in only 2 nighttime samples. This peptide was significantly higher at nighttime than daytime for spectral index, with a similar but nonsignificant tendency for spectral count.

PCSK1N, a potent inhibitor of proprotein convertase subtilisin/kexin type 1 and a regulator of feeding behavior, body weight and composition,<sup>29–31</sup> was recently identified as having a role in SCN function.<sup>10,16</sup> Here we identified 30 PCSK1N peptides that are mapped into three known regions: the SAAS region (6 peptides including the known big and little SAAS peptides), PEN-LEN region (8 peptides including the known PEN, PEN-20 and big LEN peptides), and the intermediate GAV-containing region (16 peptides). The SAAS region consists of big SAAS, little SAAS, and 4 truncated little SAAS peptides, which were detected in 17 samples. Most of the peptides detected by spectral count were little SAAS (average 51 spectral counts per sample) and a previously truncated little SAAS peptide (average 26 spectral counts per sample).<sup>17,32,33</sup> Although big SAAS was marginally significant for both spectral count and spectral index (adjusted  $p$ -value < 0.05), little SAAS exhibited a significantly higher spectral index at nighttime compared to daytime. The PEN-LEN region mainly consisted of the PEN peptide (averaged spectral count of 80 and 109 at daytime and nighttime, respectively) that was significantly more abundant at nighttime than daytime for both spectral count and spectral index. Similar to mouse studies<sup>31,34–36</sup> but in contrast to Morgan et al.,<sup>30</sup> big LEN was detected in 3 daytime samples and 1 nighttime sample. The GAV-containing region, which involves the PCSK1N region between the SAAS and PEN-LEN regions, was detected by Sayah et al.<sup>37</sup> Although the full-length GAV (PCSK1N[62–89]) was not detected here, 5 truncated GAV peptides were detected. The truncated GAV peptide PCSK1N[62–79] was significantly more abundant at daytime than nighttime, even though it was only detected in 6 daytime samples and 9 nighttime samples. A total of 9 peptides, including the previously reported PCSK1N[111–143],<sup>32</sup> were detected from the middle of this region. The PCSK1N[121–143] peptide was detected in all 18 samples but was only significantly more abundant at nighttime than daytime for spectral index. The GAV-containing region contains no known bioactive peptides and lacks consistent NeuroPred-predicted<sup>38</sup> cleavage sites across models, suggesting that the peptides from the GAV-containing region identified in this study may not be formed during intracellular processing of the prohormone.

Peptides from CARTPT, POMC, SCG1, SCG2, tachykinin 1 (TKN1), tachykinin 2 (TKNK), VGF, prodynorphin (PDYN), and PENK prohormones that are known or suspected to influence feeding behavior and energy homeostasis were found to be significant using both spectral count and spectral index. This study is the first to provide direct evidence for diurnal variation of these peptides. A review of prior studies of these



prohormones, and known peptides produced from these prohormones, provides evidence to support the circadian rhythm or diurnal variation, and the results of our current analyses. Previous studies have shown that CARTPT appears to have a circadian rhythm that is influenced by glucocorticoids and food intake. Administration of CARTPT[55–102] was able to promote wakefulness depending on dosage.<sup>39</sup> The CARTPT gene structure contains *Clock*-responsive E-box elements such that mice with a mutant *Clock* gene have significantly lower expression levels of CART than wild-type mice.<sup>40</sup> The significant CARTPT-derived peptides were two previously unreported subsequences of the known CARTPT[1–52] peptide corresponding to cleavages at Arg sites of the 'long' CARTPT isoform that produces CARTPT[55–102] peptide. Both of the identified peptides were predicted to be cleaved from the CARTPT[1–52] peptide by NeuroPred. Additional evidence that CARTPT[1–52] is further cleaved is that Faith et al.<sup>32</sup> detected the N-terminal region of CARTPT[1–52], which was not detected here, and Lee et al.<sup>17</sup> identified CARTPT[37–55]. Both unmodified and phosphorylated forms of CARTPT[37–54] and CARTPT[37–55] were detected in all 18 samples. This suggests that some of the different abundances between peptides may be related to sampling or the presence of phosphorylation. The higher spectral count and index of CARTPT[37–54] at nighttime compared to daytime was consistent with the differences reported by Vicentic et al.<sup>41–43</sup> with the CARTPT[55–102] and CARTPT[62–102] peptides.

The cleavage of corticotropin POMC peptide results in corticotropin-like intermediary peptide, POMC[141–162], which was detected, and alpha-MSH, which was undetected; both are known to exhibit diurnal variation. The enkephalins are well-known to have a diurnal variation that is modifiable by feeding.<sup>44</sup> Diurnal variation of TRH and TRH-like peptide levels in different regions of the rat brain, including the hypothalamus, have been reported.<sup>45</sup> The CARTPT and POMC prohormones are known to be coexpressed<sup>46</sup> and both are regulated by leptin.<sup>47</sup> Gene expression of *Trh* is regulated by leptin<sup>48,49</sup> and influenced by CART.<sup>50</sup>

Cleavage of TKN1 produces both substance P, a well-known peptide associated with circadian rhythm, and neurokinin A. Both peptides are released together<sup>51</sup> and are expressed together.<sup>52</sup> The fragment of TKN1 prohormone is mostly likely a secondary product of the cleavage of the larger neuropeptide K that produces neurokinin A. The terminal region of TKNK was also detected, which results from one of the cleavages necessary to produce neurokinin B. Neurokinin B is also able to bind to all the three tachykinin receptors,<sup>52</sup> although the known role for reproductive regulation related to gonadotropin release<sup>53</sup> suggests an alternative pathway. The gonadotropin-releasing hormone (GnRH) pathway is known to be involved with the SCN and circadian rhythm.<sup>54</sup>

The detected dynorphin peptide fragment, PDYN[211–218], results from the cleavage of dynorphin A(1–17) peptide (PDYN[202–218]) to form dynorphin A (1–8) peptide (PDYN[202–209]). Circadian differences in the gene expression levels of PDYN and PENK have been reported in the mouse striatum.<sup>55</sup> Somewhat similar to the simultaneous detection of peptides related to neurokinin-B and dynorphin, dynorphin is also colocalized with neurokinin-B and kisspeptin in the arcuate nucleus.<sup>56</sup> This observation may also suggest a relationship with the circadian rhythm of the GnRH pathway.

Differences between daytime and nighttime for SCG1 gene expression have been reported in the rat pineal gland.<sup>57</sup> Gene expression of VGF in the SCN has been associated with circadian rhythm<sup>58</sup> and VGF expression is regulated by light in the SCN.<sup>59</sup>

Although little is known about the role of SCG2 peptides, they have been associated with various processes including reproduction, dopamine release, and feeding.<sup>60</sup> SCG2 is highly expressed in the SCN and the importance of SCG2 in the SCN was reinforced by the successful inclusion of the SCG2 promoter in a tetracycline transactivator system that regulated *Clock* expression.<sup>61</sup> The 43 SCG2 peptides were grouped into eight distinct regions that were composed of between 2 to 12 peptides per region, including the regions containing the secretoneurin (SCG2[184–216]) and manserin (SCG2[529–568]) peptides. Little experimental evidence exists for SCG2 peptides but these regions corresponded to NeuroPred-predicted cleavage sites using models trained on mammalian data. Identification variability resulted in only 16 peptides being present in all sample periods, indicating that many peptides may be degradation products. Various peptides from the same region exhibited opposite significant differential abundances between daytime and nighttime, suggesting that SCG2 may not be associated with circadian rhythm. The distribution and expression of secretoneurin is similar to substance P and Leu-enkephalin and associated dopamine release and dynorphin B peptides that have circadian rhythm.<sup>62</sup> SCG2 expression has a diurnal rhythm in the rat pineal gland<sup>57,63</sup> that was not apparent in this study due to the specific sample times.

Several peptides from nonprohormone proteins showed significant differential abundance between daytime and nighttime using both spectral count and spectral index. There were several proteins, ATP synthase, ATP5i, NME/NM23 nucleoside diphosphate kinase 2 (NME2), PEBP1, and serine/arginine-rich splicing factor 2 (SFRS2), associated with or involving ATP and mitochondrial cytochrome subunits. While there are no prior reports of an association of these proteins with circadian rhythm, global redox state was recently shown to undergo circadian oscillations in rat and mouse SCN, with a reduced state maximal at mid-day,<sup>64</sup> suggesting a role for proteins involved in redox state in circadian rhythm.

## CONCLUSIONS

Three label-free approaches have been used to determine relative changes in peptide abundances from the same chemically complex and high dynamic range neuropeptide-rich data set. More specifically, spectral count and spectral index methods were compared to a prior SIEVE analysis. We found that SIEVE did not provide additional insights when peptides were identified in samples from only one condition, and those results were found to be inconsistent with the results using the spectral count and spectral index methods. Further, there are important statistical limitations that make it difficult to recommend SIEVE analysis for this type of data set. The spectral count and spectral index methods provided similar outcomes to each other, with spectral count being more conservative than spectral index. However, the spectral index method may suggest spurious peptides as having significant changes compared to the spectral count method when peptides are infrequently detected across samples. This study certainly demonstrates the advantages of using multiple label-free approaches to extract the most meaningful information possible from a data set.



While high resolution MS had been used to identify a surprisingly rich neuropeptide complement in the SCN, a functional context has been missing. The fact that many of these peptides change levels as a function of time of day suggests that multiple peptides are likely to have roles related to timekeeping, feeding or energy homeostasis. This analysis confirmed the importance of peptides derived from PCSK1N in SCN function. The differential abundance of numerous peptides from prohormones, notably CARTPT and PENK, further confirms the strong interrelationship between circadian rhythm, and feeding and energy homeostasis. These relationships, and the roles that specific peptides play in them, provide additional pathways to target for pharmaceutical intervention to correct altered circadian rhythms in various disorders and should lead to an improved understanding of the link between circadian rhythm, mood disorders and drug addiction.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Table S1 (Excel spreadsheet) as referenced in the text. This material is made available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### Corresponding Author

\*E-mail: [jsweedle@illinois.edu](mailto:jsweedle@illinois.edu).

### Present Address

▼ Ji Eun Lee: Center for Theragnosis, Korea Institute of Science and Technology, Seoul, Korea

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

The project described was supported by Award Nos. DA018310 (J.V.S.) and DA026672 (L.Z.) from the National Institute on Drug Abuse, and Award No. NSF IOS 08-18555 (MUG) from the National Science Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

## ■ REFERENCES

- (1) Cagampang, F. R.; Bruce, K. D. *Br. J. Nutr.* **2012**, *108*, 381–392.
- (2) Stenvers, D. J.; Jonkers, C. F.; Fliers, E.; Bisschop, P. H.; Kalsbeek, A. *Prog. Brain Res.* **2012**, *199*, 359–376.
- (3) Lynch, W. J.; Girgenti, M. J.; Breslin, F. J.; Newton, S. S.; Taylor, J. R. *Brain Res.* **2008**, *1213*, 166–177.
- (4) Albrecht, U. *Front. Mol. Neurosci.* **2011**, *4*, 41.
- (5) Aton, S. J.; Herzog, E. D. *Neuron* **2005**, *48*, 531–534.
- (6) Dragich, J. M.; Loh, D. H.; Wang, L. M.; Vosko, A. M.; Kudo, T.; Nakamura, T. J.; Odom, I. H.; Tateyama, S.; Hagopian, A.; Waschek, J. A.; Colwell, C. S. *Eur. J. Neurosci.* **2010**, *31*, 864–875.
- (7) Vosko, A. M.; Schroeder, A.; Loh, D. H.; Colwell, C. S. *Gen. Comp. Endocrinol.* **2007**, *152*, 165–175.
- (8) Domokos, A.; Mergl, Z.; Barna, I.; Makara, G. B.; Zelena, D. *J. Endocrinol.* **2008**, *196*, 113–121.
- (9) Maywood, E. S.; Chesham, J. E.; O'Brien, J. A.; Hastings, M. H. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 14306–14311.
- (10) Atkins, N., Jr.; Mitchell, J. W.; Romanova, E. V.; Morgan, D. J.; Cominski, T. P.; Ecker, J. L.; Pintar, J. E.; Sweedler, J. V.; Gillette, M. U. *PLoS One* **2010**, *5*, e12612.
- (11) Sukumaran, S.; Almon, R. R.; DuBois, D. C.; Jusko, W. J. *Adv. Drug Delivery Rev.* **2010**, *62*, 904–917.
- (12) Mitchell, J. W.; Atkins, N., Jr.; Sweedler, J. V.; Gillette, M. U. *Front. Neuroendocrinol.* **2011**, *32*, 377–386.

- (13) Fricker, L. D.; Lim, J.; Pan, H.; Che, F. Y. *Mass Spectrom. Rev.* **2006**, *25*, 327–344.
- (14) Li, L.; Sweedler, J. V. *Annu. Rev. Anal. Chem.* **2008**, *1*, 451–483.
- (15) Svensson, M.; Skold, K.; Nilsson, A.; Falth, M.; Svenningsson, P.; Andren, P. E. *Biochem. Soc. Trans.* **2007**, *35*, 588–593.
- (16) Hatcher, N. G.; Atkins, N., Jr.; Annangudi, S. P.; Forbes, A. J.; Kelleher, N. L.; Gillette, M. U.; Sweedler, J. V. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 12527–12532.
- (17) Lee, J. E.; Atkins, N., Jr.; Hatcher, N. G.; Zamdborg, L.; Gillette, M. U.; Sweedler, J. V.; Kelleher, N. L. *Mol. Cell. Proteomics* **2010**, *9*, 285–297.
- (18) Zhu, W.; Smith, J. W.; Huang, C. M. *J. Biomed. Biotechnol.* **2010**, *2010*, No. 840518.
- (19) Neilson, K. A.; Ali, N. A.; Muralidharan, S.; Mirzaei, M.; Mariani, M.; Assadourian, G.; Lee, A.; van Sluyter, S. C.; Haynes, P. A. *Proteomics* **2011**, *11*, 535–553.
- (20) Hou, X.; Xie, F.; Sweedler, J. V. *J. Am. Soc. Mass Spectrom.* **2012**, *23*, 2083–2093.
- (21) Griffin, N. M.; Yu, J.; Long, F.; Oh, P.; Shore, S.; Li, Y.; Koziol, J. A.; Schnitzer, J. E. *Nat. Biotechnol.* **2010**, *28*, 83–89.
- (22) Lee, J. E.; Zamdborg, L.; Southey, B.; Atkins, N.; Mitchell, J. W.; Li, M.; Gillette, M. U.; Kelleher, N. L.; Sweedler, J. V. *J. Proteome Res.* **2013**, *12*, 585–593.
- (23) Geer, L. Y.; Markey, S. P.; Kowalak, J. A.; Wagner, L.; Xu, M.; Maynard, D. M.; Yang, X.; Shi, W.; Bryant, S. H. *J. Proteome Res.* **2004**, *3*, 958–964.
- (24) UniProt Consortium. *Nucleic Acids Res.* **2012**, *40*, No. D71–5.
- (25) Akhtar, M. N.; Southey, B. R.; Andren, P. E.; Sweedler, J. V.; Rodriguez-Zas, S. L. *J. Proteome Res.* **2012**, *11*, 6044–6055.
- (26) Benjamini, Y.; Hochberg, Y. *J. R. Statist. Soc., Ser. B* **1995**, *57*, 289–300.
- (27) International Committee on Standardized Genetic Nomenclature for Mice. Guidelines for Nomenclature of Genes, Genetic Markers, Alleles, and Mutations in Mouse and Rat. [http://www.informatics.jax.org/mgihome/nomen/gene.shtml#gene\\_sym](http://www.informatics.jax.org/mgihome/nomen/gene.shtml#gene_sym) (accessed November 13, 2013).
- (28) Wu, Q.; Zhao, Q.; Liang, Z.; Qu, Y.; Zhang, L.; Zhang, Y. *Analyst* **2012**, *137*, 3146–3153.
- (29) Wei, S.; Feng, Y.; Che, F. Y.; Pan, H.; Mzhavia, N.; Devi, L. A.; McKinzie, A. A.; Levin, N.; Richards, W. G.; Fricker, L. D. *J. Endocrinol.* **2004**, *180*, 357–368.
- (30) Morgan, D. J.; Wei, S.; Gomes, I.; Czyzyk, T.; Mzhavia, N.; Pan, H.; Devi, L. A.; Fricker, L. D.; Pintar, J. E. *J. Neurochem.* **2010**, *113*, 1275–1284.
- (31) Wardman, J. H.; Berezniuk, I.; Di, S.; Tasker, J. G.; Fricker, L. D. *PLoS One* **2011**, *6*, e28152.
- (32) Falth, M.; Skold, K.; Svensson, M.; Nilsson, A.; Fenyó, D.; Andren, P. E. *Mol. Cell. Proteomics* **2007**, *6*, 1188–1197.
- (33) Bora, A.; Annangudi, S. P.; Millet, L. J.; Rubakhin, S. S.; Forbes, A. J.; Kelleher, N. L.; Gillette, M. U.; Sweedler, J. V. *J. Proteome Res.* **2008**, *7*, 4992–5003.
- (34) Mzhavia, N.; Berman, Y.; Che, F. Y.; Fricker, L. D.; Devi, L. A. *J. Biol. Chem.* **2001**, *276*, 6207–6213.
- (35) Morgan, D. J.; Mzhavia, N.; Peng, B.; Pan, H.; Devi, L. A.; Pintar, J. E. *J. Neurochem.* **2005**, *93*, 1454–1462.
- (36) Wardman, J. H.; Zhang, X.; Gagnon, S.; Castro, L. M.; Zhu, X.; Steiner, D. F.; Day, R.; Fricker, L. D. *J. Neurochem.* **2010**, *114*, 215–225.
- (37) Sayah, M.; Fortenberry, Y.; Cameron, A.; Lindberg, I. *J. Neurochem.* **2001**, *76*, 1833–1841.
- (38) Southey, B. R.; Amare, A.; Zimmerman, T. A.; Rodriguez-Zas, S. L.; Sweedler, J. V. *Nucleic Acids Res.* **2006**, *34*, W267–72.
- (39) Keating, G. L.; Kuhar, M. J.; Bliwise, D. L.; Rye, D. B. *Neuropeptides* **2010**, *44*, 241–246.
- (40) Turek, F. W.; Joshu, C.; Kohsaka, A.; Lin, E.; Ivanova, G.; McDearmon, E.; Laposky, A.; Losee-Olson, S.; Easton, A.; Jensen, D. R.; Eckel, R. H.; Takahashi, J. S.; Bass, J. *Science* **2005**, *308*, 1043–1045.
- (41) Vicentic, A. *Peptides* **2006**, *27*, 1942–1948.

- (42) Vicentic, A.; Jones, D. C. *J. Pharmacol. Exp. Ther.* **2007**, *320*, 499–506.
- (43) Vicentic, A.; Lakatos, A.; Jones, D. *Peptides* **2006**, *27*, 1934–1937.
- (44) McLaughlin, C. L.; Baile, C. A.; Della-Fera, M. A. *Physiol. Behav.* **1987**, *41*, 465–469.
- (45) Pekary, A. E.; Stevens, S. A.; Sattin, A. *Brain Res.* **2006**, *1125*, 67–76.
- (46) Menyhert, J.; Wittmann, G.; Lechan, R. M.; Keller, E.; Liposits, Z.; Fekete, C. *Endocrinology* **2007**, *148*, 4276–4281.
- (47) Harrold, J. A.; Dovey, T. M.; Blundell, J. E.; Halford, J. C. *Neuropharmacology* **2012**, *63*, 3–17.
- (48) Nillni, E. A.; Vaslet, C.; Harris, M.; Hollenberg, A.; Bjorbak, C.; Flier, J. S. *J. Biol. Chem.* **2000**, *275*, 36124–36133.
- (49) Pekary, A. E.; Sattin, A.; Blood, J. *Brain Res.* **2010**, *1345*, 9–18.
- (50) Fekete, C.; Lechan, R. M. *Peptides* **2006**, *27*, 2012–2018.
- (51) Debeljuk, L.; Lasaga, M. *Peptides* **1999**, *20*, 285–299.
- (52) Pennefather, J. N.; Lecci, A.; Candenas, M. L.; Patak, E.; Pinto, F. M.; Maggi, C. A. *Life Sci.* **2004**, *74*, 1445–1463.
- (53) Rance, N. E. *Peptides* **2009**, *30*, 111–122.
- (54) Williams, W. P., 3rd; Jarjisian, S. G.; Mikkelsen, J. D.; Kriegsfeld, L. J. *Endocrinology* **2011**, *152*, 595–606.
- (55) Cai, Y.; Ding, H.; Li, N.; Chai, Y.; Zhang, Y.; Chan, P. *Neuroreport* **2010**, *21*, 79–83.
- (56) Lasaga, M.; Debeljuk, L. *Peptides* **2011**, *32*, 1972–1978.
- (57) Bailey, M. J.; Coon, S. L.; Carter, D. A.; Humphries, A.; Kim, J. S.; Shi, Q.; Gaildrat, P.; Morin, F.; Ganguly, S.; Hogenesch, J. B.; Weller, J. L.; Rath, M. F.; Moller, M.; Baler, R.; Sugden, D.; Rangel, Z. G.; Munson, P. J.; Klein, D. C. *J. Biol. Chem.* **2009**, *284*, 7606–7622.
- (58) Levi, A.; Ferri, G. L.; Watson, E.; Possenti, R.; Salton, S. R. *Cell. Mol. Neurobiol.* **2004**, *24*, 517–533.
- (59) Wisor, J. P.; Takahashi, J. S. *J. Comp. Neurol.* **1997**, *378*, 229–238.
- (60) Bartolomucci, A.; Possenti, R.; Mahata, S. K.; Fischer-Colbrie, R.; Loh, Y. P.; Salton, S. R. *Endocr. Rev.* **2011**, *32*, 755–797.
- (61) Hong, H. K.; Chong, J. L.; Song, W.; Song, E. J.; Jyawook, A. A.; Schook, A. C.; Ko, C. H.; Takahashi, J. S. *PLoS Genet.* **2007**, *3*, e33.
- (62) Wiedermann, C. J. *Peptides* **2000**, *21*, 1289–1298.
- (63) Humphries, A.; Klein, D.; Baler, R.; Carter, D. A. *J. Neuroendocrinol.* **2002**, *14*, 101–108.
- (64) Wang, T. A.; Yu, Y. V.; Govindaiah, G.; Ye, X.; Artinian, L.; Coleman, T. P.; Sweedler, J. V.; Cox, C. L.; Gillette, M. U. *Science* **2012**, *337*, 839–842.