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Neuropeptidomics of the Rat Habenular Nuclei

Ning Yang¹, Krishna D. B. Anapindi¹, Stanislav S. Rubakhin¹, Pingli Wei², Qing Yu³, Lingjun Li^{2,3}, Paul J. Kenny⁴, and Jonathan V. Sweedler^{1,*}

¹Department of Chemistry and the Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana Champaign, Urbana 61801, United States

²Chemistry Department, University of Wisconsin-Madison, Madison 53705, United States

³School of Pharmacy, University of Wisconsin-Madison, Madison 53705, United States

⁴Department of Pharmacology & Systems Therapeutics, Icahn School of Medicine at Mount Sinai, New York, NY 10029, United States

Abstract

Conserved across vertebrates, the habenular nuclei are a pair of small symmetrical structures in the epithalamus. The nuclei functionally link the forebrain and midbrain by receiving input from and projecting to several brain regions. Each habenular nucleus comprises two major asymmetrical subnuclei, the medial and lateral habenula. These subnuclei are associated with different physiological processes and disorders, such as depression, nicotine addiction, and encoding aversive stimuli or omitting expected rewarding stimuli. Elucidating the functions of the habenular nuclei at the molecular level requires knowledge of their neuropeptide complement. In this work, three mass spectrometry (MS) techniques – liquid chromatography (LC) coupled to Orbitrap tandem MS (MS/MS), LC coupled to Fourier transform (FT)-ion cyclotron resonance (ICR) MS/MS, and matrix-assisted laser desorption/ionization (MALDI) FT-ICR MS – were used to uncover the neuropeptide profiles of the medial and lateral rodent habenula. With the assistance of tissue stabilization and bioinformatics, a total of 262 and 177 neuropeptides produced from 27 and 20 prohormones were detected and identified from the medial and lateral habenula regions, respectively. Among these neuropeptides, 136 were exclusively found in the medial habenula, and 51 were exclusively expressed in the lateral habenula. Additionally, novel sites of sulfation, a rare post-translational modification, on the secretogranin I prohormone are identified. The results demonstrate that these two small brain nuclei have a rich and differentiated peptide repertoire, with this information enabling a range of follow-up studies.

Graphical abstract

*Corresponding author: jsweedle@illinois.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Figure S1 showing MS/MS spectra of select neuropeptides detected in the habenula; Figure S2 showing parathyroid hormone-related protein mRNA expression; Figure S3 showing putative orexin-A signals in different charge states; Table S1 summarizing neuropeptides detected and identified in the medial habenula using LC-MS; Table S2 summarizing neuropeptides detected and identified in the lateral habenula using LC-MS; Table S3 summarizing neuropeptides detected in the medial and lateral habenula by MALDI MS; Table S4 showing sulfated peptides identified in the medial and lateral habenula identified using Byonic 2.0.

Keywords

medial habenula; lateral habenula; neuropeptidomics; mass spectrometry; ESI MS; MALDI MS; FT-ICR MS

INTRODUCTION

The habenular nuclei are small structures symmetrically located above the posterior end of the thalamus, functionally linking the forebrain and the midbrain. They are phylogenetically conserved in vertebrates and part of the epithalamus.¹ The habenula nucleus has two substructures, the medial and lateral subnuclei, which interconnect with different brain regions.² The habenula had not attracted significant attention until the discovery of its involvement in the reward system.^{3–6} To date, the habenula has been found to play important roles in a wide range of physiological processes, including sleep regulation, reward-based decision-making, avoidance and mood behaviors, and drug addiction, particularly tobacco dependence.^{7–10} An important step in understanding the contribution of the habenula to these biological events and behaviors is to elucidate the cell-to-cell signaling molecules, including neuropeptides, present in this brain region. In addition, there are orphan G-protein coupled receptors (GPCRs) that are densely or exclusively expressed in the habenula;^{11,12} characterizing the peptide complement in the habenula may help to uncover the endogenous ligands for these GPCRs.

Neuropeptides are important cell-to-cell signaling molecules involved in the coordination of behavioral, cognitive, and homeostatic events involving various physiological functions, such as food intake, pain sensation, circadian rhythms, and tissue regeneration, as well as many pathophysiological processes such as drug addiction.^{13–17} These signaling peptides are produced by post-translational enzymatic processing of large precursor proteins called prohormones at conventional cleavage sites, which include mono/di-basic amino acid motifs. To modulate the biological functionality of peptides, many neuropeptides also undergo post-translational modifications (PTMs),^{18,19} resulting in formation of chemically unique peptides possessing the same sequence of amino acids.

The development of modern mass spectrometry (MS) techniques, aided by improvements in sample preparation methods, have changed the way peptide detection, identification, and quantitation are performed, allowing characterization of a fairly complete set of neuropeptides present in a biological structure.^{20–24} In the past decade, hundreds of neuropeptides have been discovered and identified from a range of animals using matrix-assisted laser desorption/ionization (MALDI) MS and electrospray ionization (ESI) MS.
15,17,25–28

A number of *in situ* mRNA hybridization and immunocytochemistry studies have suggested the presence of several neuropeptide receptors in the habenula region, such as the orexin-2 receptor in the lateral habenula, and the neuropeptide Y (NPY) Y1 receptor in both the medial and lateral habenula.^{29–31} However, there has yet to be a systematic investigation of the neuropeptide content in this small but functionally important brain region. Here, we combined a sample stabilization approach, high molecular mass accuracy and resolution MS, and bioinformatics to characterize the endogenous peptides within the rat habenula. We identified 262 and 177 peptides from 27 and 20 prohormones in the medial and lateral habenula, respectively. Of these, 38 and 36 peptides in the medial and lateral habenula, respectively, possess PTMs, including amidation, pyroglutamylation, acetylation, disulfide bonds, and phosphorylation.

In addition to characterizing these commonly found PTMs, targeted experiments were aimed at characterizing sulfated peptides. Tyrosine (Tyr)-sulfation is a well-known PTM that is involved specifically in modulating protein-protein and ligand-receptor interactions.^{32–37} Though sulfation as a modification of proteins was identified in the early 1950s,³⁸ determination of the exact site of Tyr-sulfation using MS is still a challenging task for several reasons. First, sulfation increases the mass of the modified peptide by almost the same amount (+79.9568 Da) as phosphorylation (+79.9663 Da). Moreover, both PTMs can occur on Tyr. Secondly, sulfation is a labile PTM where the SO₃ group falls off, even with soft ionization techniques such as ESI. Finally, even if sulfation remains after ESI, it does not survive during classic collision-induced dissociation (CID), making it difficult to determine the site of the modification. To overcome these difficulties, we used a custom approach utilizing beam-type CID (high-energy C-trap dissociation, or HCD) and electron transfer HCD (ET_hCD) dissociation techniques. The obtained data were manually deconvoluted, revealing neutral loss signals of the parent ion corresponding to a mass difference of 80 Da. Using this approach, we confidently localized four novel sites of modification for sulfation on the secretogranin I (SCG1) prohormone.

Together, these data represent the first comprehensive neuropeptidomics study of the vertebrate habenula. The information presented on the neuropeptide content and PTMs of these molecules in the habenular nuclei can be used as a basis for better understanding of the roles of cell-to-cell signaling molecules in different biological processes.

EXPERIMENTAL SECTION

Materials

The solvents used were of LC–MS grade and obtained from Thermo Fisher Scientific (Waltham, MA), except for triethylamine, acetone, and hydrochloric acid, which were obtained from Sigma-Aldrich (St. Louis, MA).

Habenula Isolation and Stabilization

Male Sprague Dawley rats, 8–12 weeks old, were euthanized by decapitation in compliance with animal use protocols approved by the University of Illinois Institutional Animal Care and Use Committee and in accordance with all state and federal regulations. The brain was quickly removed after fast decapitation and cooled in ice cold mGBSS buffer. Targeted brain areas were identified according to the Paxinos and Watson rat brain atlas,³⁹ surgically isolated, and stabilized as outlined below. Samples from the identical habenula regions were pooled together for analysis with individual techniques. For LC–FT-ICR MS analysis, the medial and lateral habenula-containing regions from 10 rats were collected and stabilized in boiling water for 10 min. For LC–Orbitrap MS analysis, 6 rats were euthanized. The isolated medial and lateral habenula regions were stabilized with a Denator Stabilizer T1 (ST1) (Denator, Sweden) and pooled, respectively.⁴⁰ For LC–MALDI MS analysis, entire habenula regions from 3 rats were stabilized with the ST1. For characterization of the sulfated peptides via LC–Orbitrap MS, medial and lateral habenula regions from 3 rats were isolated and stabilized by the ST1 and pooled into one sample.

Extraction of Endogenous Peptides

The isolated habenula tissues were stabilized by the ST1 or hot water, followed by a three-stage peptide extraction process, as described in our previous work.²⁸ Briefly, habenula tissues were homogenized in 200 μ L of ice cold water and incubated on ice for 1 h. Supernatants were saved in a new microcentrifuge tube after centrifugation at $14,000 \times g$ for 10 min. The tissue pellets were subjected to two more stages of peptide extraction with acidified acetone (acetone:H₂O:HCl 40:6:1) and 0.25% acetic acid solution, respectively, performed in a similar manner. The supernatants collected during these three extraction steps were combined.

The volume of combined supernatant sample was reduced to about 20 μ L using a vacuum pre-concentrator device (GeneVac, UK) leading to preferential HCl and acetone removal. Concentrated samples were desalted with a C18 spin column (Thermo Fisher Scientific). For LC–Orbitrap MS and LC–MALDI MS analysis, peptides were pre-purified on the C18 spin column. Analytes were eluted from the column using an acetic acid organic solution buffer (pH 4.0). For LC–FT-ICR MS analysis, peptides retained on the C18 spin column were sequentially eluted into different vials using a set of buffers containing 70/30, 50/50, and 20/80 H₂O/acetonitrile (ACN) solutions, with the pH adjusted to 11.0. Each eluent fraction was collected, dried using a vacuum pre-concentrator, and stored in a –20 °C freezer until LC–MS analysis.

LC–Orbitrap MS Analysis

The peptides extracted from the rat habenular nuclei regions were analyzed using a nanoLC system (Dionex UltiMate 3000, Thermo Fisher Scientific) coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). The samples were reconstituted in loading solvent (95%/5% H₂O/ACN with 0.1% formic acid (FA)) and loaded onto a trap column. H₂O with 0.1% FA, and ACN with 0.1% FA, were used as solvents A and B, respectively. The loaded peptides were separated on a 15 cm Pepmap Acclaim analytical column (Thermo Fisher Scientific) packed with 2- μ m particles (100 Å pore size) at a 300 nL/min flow rate. The gradient for the peptide elution range was as follows: 3–6 min, 1–10% B; 6–90 min, 10–70% B. Data were acquired in a top speed data-dependent mode. Other parameters included: precursor scan automatic gain control (AGC), 2e+5; MS/MS scan AGC, 5e+4; isolation window, 1.6 Da; normalized collision energy, 35%; activation Q, 0.25.

LC–FT-ICR MS Analysis

The three dried eluent fractions were reconstituted in 10 μ L of 95%/5% H₂O/ACN solution and analyzed individually using an Eksigent nanoLC 1D Plus system (SCIEX, Framingham, MA, USA) coupled to an 11 Tesla FT mass spectrometer (LTQ-FT Ultra, Thermo Fisher Scientific), as described in a prior peptidomics work.²⁷ The experimental parameters were the same as described in the previous study,²⁷ including the columns, solvents, and flowrate. Briefly, the peptides were first loaded onto a peptide trap column and then separated on the analytical column. The analytes were eluted using a gradient as follows: 0–10 min, 0–20% solvent B; 10–65 min, 20–55% solvent B; 65–75 min, 55–85% solvent B; 75–80 min, 85–85% solvent B; 80–85 min, 85–0% solvent B; 85–90 min, 0–0% B. The MS acquisition parameters included mass scans at m/z 300–2000. Data-dependent precursor selection was restricted to the top five most intense ions with the following parameters: precursor isolation window, m/z 10; activation Q, 0.25; activation time, 50 ms; collision energy 35 eV. Dynamic exclusion was enabled with a repeat count of 2 and exclusion duration of 180 s.

LC–Orbitrap MS for Characterization of Sulfation PTM

The peptide extracts were analyzed using the Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) coupled to the Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific) located at UW. A binary solvent system composed of H₂O containing 0.1% FA (solvent A) and ACN containing 0.1% FA (solvent B) was used for all analyses. Peptides were loaded and separated on a 75 μ m $\times$ 15 cm column, fabricated in the lab and packed with 1.7 μ m, 150 Å, BEH C18 material (part no. 186004661, Waters, Milford, MA). Samples were loaded with 3% solvent B, and solvent B was linearly ramped to 30% in 102 min, and ramped to 75% in another 20 min at a 300 nL/min flow rate. Data were acquired in data-dependent mode using the Orbitrap mass spectrometer with EThcD and high-energy C-trap dissociation. The precursor ions in the range of 300–1500 m/z were scanned with an AGC target set to 2e+5 and an Orbitrap resolution of 60,000. The fragment ion analysis for the precursors was divided into five different scan events based on the charge state of the precursor ion. Scan event 1: intensity>1e+4; scan event 2: intensity>1e+4 and charge state 2; scan event 3: intensity>1e+4 and charge states 3–4; scan event 4: intensity>1e+4 and charge state 5; scan event 5: intensity>1e+5 and charge states 6–8. Scan

event 1 analyzed all ions, whereas the other events only looked at specific charge states. The electron transfer dissociation reaction times were chosen appropriately for scan events 2–5, depending on the charge state. All of the scan events were performed with an Orbitrap resolution of 15,000 and AGC target value of 5e+4.

Peptide Identification: Untargeted Database Search

The raw files acquired on the LC–Orbitrap Fusion Lumos system were loaded into PEAKS software, Version 7.5 (Bioinformatics Solutions Inc. Canada). The loaded data were first processed with the *de novo* function in the software and then searched against a rat proteome database from UniProt⁴¹ (36,078 entries). The mass error tolerance was set to 20 ppm for precursor ion and 0.1 Da for fragment ions. No enzyme digestion was selected and up to three variable PTMs were allowed for each peptide from a list of seven modifications, including acetylation (K, N-term, and protein N-term), amidation, oxidation (M), pyro-Glu from E, pyro-Glu from Q, phosphorylation (STY), and half-disulfide bond (C). Identified peptides in the database search results were filtered by -10logP value cut-offs, corresponding to a 1% false discovery rate (FDR) or lower.

The raw data files acquired on the LC–FT-ICR MS system (LTQ-FT Ultra) were processed with the cRAWler algorithm in ProsightPC (Version 2.0, Thermo Fisher Scientific), which outputs .puf files containing the deconvoluted monoisotopic masses of precursor and fragment ions. The .puf files were then loaded into ProsightPC and searched against a lab-built rat proteome database based on information downloaded from UniProt.⁴¹ The database search was conducted in Biomarker search mode with the precursor and fragment mass error tolerances set at 81 Da and 10 ppm, respectively. In biomarker search mode, the detected precursor mass was compared with the calculated masses of all of the possible peptide subsequences present in the database. For subsequences with masses within an 81 Da tolerance of a precursor mass, detected fragment masses were matched with theoretical fragment masses from these candidate peptide subsequences. Identified peptides with p values lower than 1e-4 were retained.

Peptide Identification: Targeted Database Search

Data in the .raw format from the Orbitrap MS analysis were also searched against a lab-built rat neuropeptide prohormone library that contains prohormone sequences in FASTA format using the PEAKS software. The same precursor and fragment ion mass error tolerance value were used as described above. Up to 7 variable PTMs, including acetylation (K, N-term, and protein N-term), amidation, oxidation (M), pyro-glu from E, pyro-glu from Q, phosphorylation (STY), and half-disulfide bond (C) were allowed for each peptide, so that the neuropeptides with multiple PTMs not detected in the initial round of the untargeted database search described above could be discovered and identified in the second round of targeted search. The FDR value was set at a stricter level of 0.1%.

Sulfated Peptide Identification

The .raw files from the HCD + EThcD method for the sulfated peptide analysis were processed with Byonic software (version 2.0, Protein Metrics, San Carlos, CA). Data were searched against a rat neuropeptide database (<http://isyslab.info/NeuroPep/>). A precursor

tolerance of 10 ppm and a fragment mass tolerance of 20 ppm were allowed. Acetylation (N-term), oxidation (M), pyro-glu from E, and pyro-glu from Q were set as rare dynamic modifications. Common dynamic modifications consisted of phosphorylation (STY) and amidation (C-term). The mass spectra of the peptides identified to be sulfated were manually analyzed for the presence of a neutral loss of SO_3 that corresponds to a mass difference of 80 Da.^{42,43}

LC–MALDI MS Analysis

Peptide extracts were injected into a Dionex capLC system equipped with a capillary C18 column (300 μm ID $\times$ 150 mm, 100 Å, Thermo Fisher Scientific). Solvents A and B were H_2O with 0.1% FA and ACN with 0.1% FA, respectively. Flow rate was set at 4 $\mu\text{L}/\text{min}$. Peptides were separated over 45 min with gradient solvent B rising from 1% to 50%. Fractions were collected every 3 min. Collected fractions were dried with a vacuum pre-concentrator and reconstituted in 5 μL of H_2O . 1 μL of sample from each fraction was spotted on a MALDI sample plate, mixed with 50 mg/mL 2, 5-dihydroxybenzoic acid matrix, and dried at room temperature ($\sim 24^\circ\text{C}$). The dried samples were analyzed with a MALDI FT-ICR mass spectrometer (solariX XR, Bruker Daltonics, Billerica, MA) over a mass range of 200–4000 m/z . The putative identities of peaks in the MALDI mass spectra were assigned by matching the m/z of detected analytes to the m/z of known neuropeptides contained in the SwePep rat database (<http://www.swepep.org/>), with a 0.1 Da allowable mass error.

RESULTS AND DISCUSSION

The habenular nuclei encompass two small regions symmetrically located in the brain hemispheres; these nuclei have important roles in a range of behavioral phenomena, including drug addiction and depression.^{1,9,44} Neuropeptides are important cell-to-cell signaling molecules involved in these phenomena.^{45–47} While immunohistochemical methods and *in situ* hybridization have been used to study the distribution of selected neuropeptides or their mRNAs within the habenular nuclei,^{48–51} it appears surprising that an inventory of the endogenous neuropeptide molecules present in the structure is not available. Here, we employed a combination of high resolution MS platforms to explore the neuropeptidome of the lateral and medial habenula in rat.

Evaluation of the morphology, connectivity to other brain regions, and biochemistry of the habenula nuclei suggests that the habenula is comprised of distinct lateral and medial subnuclei, each of which can be further divided into several smaller subnuclear regions.⁵² Difficulties in the observation, isolation, and LC–MS analysis of these smaller subnuclei restricted our investigation of the lateral and medial habenula nuclei.

Figure 1 shows the workflow of the multifaceted mass spectrometric approach used in this study. Acquired data from analysis 1 using the LC–Orbitrap system were searched against a rat proteome database for peptide identification. A targeted database search was also performed on the raw Orbitrap MS data using a prohormone library instead of a proteome database. For the targeted search, up to seven PTMs on individual peptides were allowed, facilitating the discovery of neuropeptides possessing multiple PTMs. For example, orexin-

A has pyroglutamination at its N-terminus, two disulfide bonds, and amidation at its C-termini. In our analysis, six neuropeptides from the lateral habenula region, which were missed in the initial untargeted search, were found by the targeted database search. Using LC-FT-ICR MS/MS and LC-Orbitrap MS/MS, 262 known and putative neuropeptides derived from 27 prohormones were identified from the medial habenula (Table S1); in the lateral habenula, 177 known and putative neuropeptides derived from 20 prohormones were found (Table S2). These small brain nuclei display a high molecular heterogeneity, which is in agreement with the complex spatial expression of genes and high heterogeneity of cell types.⁵³ Among the identified neuropeptides, 126 were detected and identified in both the medial and lateral habenula (Figure 2). Table 1 summarizes the prohormones detected in the medial and lateral habenula. Peptides from seven prohormones were exclusively detected in the medial habenula, including pro-opiomelanocortin, gastrin-releasing peptide, insulin-like growth factor-binding protein 5, neuropeptide S, neuroendocrine protein 7B2, corticotiberin, and tachykinin-3. These observations demonstrate that the medial and lateral habenula contain distinct sets of cell-cell signaling peptides. Mass spectra illustrating the results of the MS/MS characterization of selected physiologically important neuropeptides are shown in Figure S1.

We also investigated the habenula using MALDI-FT-ICR MS. Mass-to-charge ratio (m/z) of around 3% of all the well-resolved peaks (~800 peaks were considered from seven different mass spectra) matched to their expected m/z of the known neuropeptides compiled in the SwePep database (Table S3), as described previously.⁷⁸ Several of the unassigned peaks may correspond to neuropeptides possessing endogenous cleavage sites at both termini but not included in the database. Other unassigned signals could represent peptides originating from the processing of non-prohormone proteins. Twenty-two signals from the 24 signals detected by MALDI-FT-ICR MS were also found in the ESI MS data sets containing MS/MS-supported analyte characterization, thereby improving our confidence in the assignments of neuropeptide identities. Two signals were detected only with MALDI-FT-ICR MS, and likely represent osteostatin and pro-FMRamide-related neuropeptides. However, confirmation of the presence of these peptides needs further validation since no MS/MS spectra were acquired in the MALDI MS analysis. Nevertheless, the Allen Brain Atlas⁷⁹ *in situ* hybridization data demonstrate that mRNA of the parathyroid hormone-related protein containing the osteostatin sequence is present in the medial habenula region (Figure S2). Also, pro-FMRamide-related neuropeptide FF receptors have been reported in the habenula region previously,⁸⁰ but the presence of parathyroid hormone-related protein mRNA and pro-FMRamide-related neuropeptide FF receptors do not confirm their protein expression.

Although a comprehensive study of the neuropeptide complement of this physiologically important region has not been performed previously, a large body of information on the identity and function of several neuropeptides found in the habenular nuclei does exist. Our results align well with the results from previous studies, some of which are discussed below.

The prohormone protachykinin-1, which belongs to the tachykinin protein family, has been reported in the medial habenula region.⁸¹ We identified multiple neuropeptides cleaved at mono/di-basic sites from tachykinin-1, including substance P, neurokinin A, short neuropeptide K, C-terminal flanking peptide, and protachykinin [111–118]. Prior

immunocytochemistry and in situ hybridization investigations demonstrated the presence of cells expressing substance P and neurokinin B in the medial habenula, with their axons projecting to the interpeduncular nucleus and ventral tegmental area.⁸² The neurokinin signaling pathway involving substance P, neurokinin B, and their receptors facilitates enhancement of nicotine-induced excitability in medial habenula neurons.⁸³ However, the biological functions of other protachykinin-1-derived peptides identified in the habenula are not clear. Further research on these neurokinin peptides can lead to a better understanding of the mechanism of nicotine addiction and perhaps neurokinin signaling-based therapies for smoking cessation and other disorders for which the actions of this neuropeptide system in the medial habenula may be relevant. Work done by Pertizzello et al.⁸⁴ on the effect of chronic nicotine treatment in the regulation of opioid and non-opioid peptides has revealed a significant difference in peptide levels derived from prohormones such as cerebellin-1, cholecystokinin, neurotensin, proenkephalin-A/B, proNPY, and proSAAS in the dorsal striatum of model rats compared to the controls. In the current study, several endogenously cleaved full-length peptides from these prohormones were also found, further reinforcing the involvement of the habenular nuclei in nicotine addiction.

NPY [30–65] is a 36-amino acid cell-to-cell signaling molecule found in the nervous system that is involved in regulating different physiological and pathological events, such as food intake, stress, and depression.^{85,86} NPY-immunoreactive fibers were detected in both lateral and medial habenula regions.⁸⁷ We detected and identified the full-length form of NPY and its C-terminal flanking peptide. A novel shortened NPY [30–47] peptide (YPSKPDNPGEDAPAEDMA.R) was also characterized with high confidence (34.0 for -10logP score). This shortened NPY has an arginine at its C-terminal, a common cleavage site in prohormone processing, supporting its endogenous nature, in contrast to the possibility that it was generated by degradation in post-mortem tissue. McBride and coworkers⁸⁸ have reported that mRNA transcripts encoding NPY are significantly reduced in the medial habenula of alcohol-preferring rats, suggesting a role for NPY in ethanol intake control.⁸⁸ This raises the interesting possibility that NPY, and novel modified forms of the peptide, expressed in the habenula may contribute to alcohol dependence vulnerability.

While NPY is an appetite stimulator, cholecystokinin (CCK)-derived peptides have been associated with satiety and shown to decrease food intake.⁸⁹ Even in invertebrates, sulfakinins, the peptides with high sequence similarity to vertebrate CCK peptides, were shown to reduce food intake.⁹⁰ In this work, we detected a full-length mature peptide of the CCK prohormone (CCK[21–45]) with high confidence in both lateral and medial habenula regions, suggesting a role for the habenula in the regulation of food intake. Additionally, a recent study by Ye et al.⁷² has shown a direct correlation between food intake and brain levels of the proSAAS-derived peptides, big LEN (proSAAS[245–260]) and PEN (proSAAS[221–242]). Both of these peptides have been identified with high confidence scores in the current study.

The habenular nuclei are anatomically connected to the pineal gland, which together comprise the epithalamus.⁸ The pineal gland is known to regulate sleep. Also, the epithalamus receives extrinsic circadian signals sent from the main circadian pacemaker, the suprachiasmatic nucleus (SCN).⁹¹ The SCN is a well-investigated brain structure containing

hundreds of peptides.^{15,92} For example, SCN-originating orexin-A and -B modulate sleep by regulating melatonin synthesis and secretion through the orexinergic neuron-pineal gland circuit in zebrafish and rat, as well as influence neuronal activities in the lateral habenula.^{93,94} We detected and identified full-length orexin-B and other pro-orexin-derived peptides in both the lateral and medial habenula. Though the sequence identity of orexin-A was not confirmed, low intensity peaks of *m/z* corresponding to +4, +5, and +6 charged orexin-A molecules were detected (Figure S3). The relatively low levels of this molecule present in the studied structures, and/or biological matrix interferences, likely led to the low quality MS/MS spectra. In addition, the PTMs of orexin-A⁹⁵ (N-terminal pyroglutamyl cyclization, C-terminal amidation and two disulfide bonds between residues 38↔44 and 39↔46) complicate confident identification of the molecules with a targeted search. The identification of orexin-B in the habenular nuclei suggests that pro-orexin likely undergoes local translation and processing in the habenula, which may in turn contribute to sleep regulation by the epithalamus. In addition to sleep modulation, orexin is also thought to play a role in regulating the motivational properties of addictive drugs, including opioids, psychomotor stimulants, nicotine, and ethanol.^{96–98} Most conceptualizations of how orexin modulates drug seeking behaviors have focused on its actions in the ventral midbrain to regulate dopamine neurons.^{99,100} The present findings raise the intriguing possibility that orexin also acts in the habenula, which is known to exert an inhibitory action on midbrain dopamine neurons, to regulate the motivational properties of addictive drugs.⁵

We also identified two new pituitary adenylate cyclase-activating polypeptide-derived neuropeptides, PACAP [111-128] and PACAP [130-143], in the habenular nuclei. Classic cleavage sites flank the N- and C-termini of both peptides. Immunohistochemistry and *in situ* hybridization studies have shown that these peptides and their receptors are present in the medial and lateral habenula.^{66,101} Recently, the medial habenula was shown to play a critical role in regulating the extinction of fear memories in rodents.¹⁰² PACAP is known to regulate the strength of fear memories and has been implicated in post-traumatic stress disorder (PTSD).¹⁰³ It is therefore possible that PACAP acts in the habenula to regulate the strength and persistence of fear memories and thereby influence vulnerability or resilience to PTSD. Habenular nuclei neurons are also activated by stress-inducing stimuli, resulting in a significant decrease in c-fos expression.¹⁰⁴ PACAP is associated with stress and anxiety-like behaviors.^{8,44}

Another cell-to-cell signaling peptide observed in this study is somatostatin, which is related to chronic stress. It has been reported that somatostatin receptors were significantly upregulated in the medial habenula in stressed rats, together with an increase of somatostatin in the plasma.¹⁰⁵ Multiple pain-related peptides were also detected in this study, including those derived from prohormones such as nociceptin, pro-enkephalin-A, and pro-dynorphin-related, which are known to produce endogenous opiate peptides.^{106–108} These peptides bind to various classes of opioid and nociception receptors, and are involved in mechanisms of pain signaling.¹⁰⁹ Our finding of pain-related peptides in the habenular nuclei is consistent with results from a preclinical study demonstrating an important role for the habenula in pain and analgesia.¹¹⁰ In fact, because opioid peptides are so enriched in the habenula, it raises the possibility that this structure is involved in the pain relieving or addiction-relevant actions of opioid drugs. Understanding the contribution of the habenular nuclei to the actions

of opioid drugs is particularly timely, considering the dramatic increase in opioid abuse over recent years.

An additional experiment to identify the sites of sulfation revealed the location of five Tyr residues with O-sulfation. All of the five locations correspond to the prohormone SCG1. One of them (SCG1 [339]) has been previously reported in rats,¹¹¹ whereas the other four are novel PTM sites of sulfation (SCG1 [330], [466], [469] and [501]). SCG1 [469] is predicted to be a sulfotyrosine by homology with a previously reported sulfation site in a bovine model.¹¹² While secretogranins are a class of secretory proteins that have been previously shown to be sulfated,¹¹³ only one site of sulfation had been previously confirmed in the rat SCG1 prohormone.¹¹¹ Moreover, all of the identified peptides (Table S4) are either full-length forms or smaller fragments of two parent peptides (SCG1 [324–343] and SCG1 [454–508]), both with mono/di-basic amino acid cleavage sites. Sulfation is associated with the secretory pathway and the modification is known to occur in the *trans*-Golgi network. Modified Tyr residues have been shown to modulate ligand-receptor interaction in several previous studies.^{35–37,114} The important role of sulfotyrosine in the regulation of ligand-receptor interactions, combined with the mono/di-basic cleavage sites of the corresponding peptides containing this modification, make them interesting candidates for further evaluation of biological activity. Secondly, although the sulfation experiments were performed on an MS platform with a different collision energy, ion detection, and search engine parameters in a separate laboratory, we did not observe significant differences in the total number of precursor proteins identified (data available on request). This consistency in results reflects the efficient sample stabilization and peptide extraction protocols used in this study.

Finally, we also detected and identified several novel neuropeptides, which have not been reported previously or have only corresponding mRNA transcripts detected in the habenular nuclei. These include peptides derived from CART, neurosecretory protein VGF, and several secretogranins.¹¹⁵ However, their role in the habenula is unknown and further investigation will be required to understand their functions.

CONCLUSIONS

Habenular nuclei are morphologically, chemically, and functionally complex brain structures. Our peptidomic analyses utilized high-mass resolution MS-based and bioinformatics approaches to detect and identify 262 and 177 neuropeptides in the rat medial and lateral habenula regions, respectively. We determined the spatial specificity of the prohormone expressions and revealed the endogenous forms of neuropeptides in these habenular structures. Recently, the Hu group¹¹⁶ reported that enhancement in the neuron bursting activity in the lateral habenula mediated by an astroglial potassium channel directly impacts depression. Although the impact of neuropeptides have yet to be reported for this ion channel, neuron burst firing can facilitate synaptic plasticity and neuropeptide release.¹¹⁷

The new compendium of habenular-expressed peptides reveals the striking chemical complexity of the habenular nuclei and adds critical details to the overall picture of this region's functional and structural biochemistry. The habenular nuclei receive inputs from

multiple brain regions and act as a bridge between the forebrain and midbrain. The neuropeptides identified in our study may originate from within the habenula or they may be from cells outside of the habenula and transported into the habenula.

Regardless, the richness of our peptide results corroborate prior habenular nuclei neuropeptide data and support that idea that many normal and pathological conditions, as well as behaviors, depend on the activity of cells in this brain region. Due to the subtle and at times redundant effects of individual peptides and the high plasticity of the evaluated systems, most of the peptides detected here have yet to be linked to function; more studies are needed. We expect that our results will be useful in identifying endogenous ligands for the habenular nuclei orphan GPCRs,^{11,12} which may be targets for the development of novel therapeutics for neuropsychiatric disorders in which the habenula has been implicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Workflow of the MS-based neuropeptidomic analyses of the rat habenular nuclei.

Symmetrical habenular nuclei were isolated from the left and right brain hemispheres and characterized in three ways: (1) The medial or lateral habenula was analyzed with LC–ESI–Orbitrap MS and a PEAKS search against a rat proteome database (untargeted analysis), and a rat prohormone database (targeted analysis). (2) The medial or lateral habenula analyte extracts were first fractionated during C18 solid phase extraction, then analyzed with ESI–FT–ICR MS. (3) Analytes extracted from the entire habenula were fractionated with capLC and analyzed by MALDI–FT–ICR MS. (4) The entire habenula extracts were analyzed using specific settings for characterization of sulfated peptides on the LC–ESI–Orbitrap mass spectrometer. The resultant raw spectra were searched against a rat prohormone database using Byonic 2.0.

Figure 2.

Venn diagram summarizing the results of the peptidomic analyses of the medial and lateral habenula. A total of 262 and 177 neuropeptides were detected and identified in the medial and lateral habenula, respectively, with 126 neuropeptides detected in both regions.

Table 1

Prohormones expressed in the medial and lateral habenula and detected using MS-based peptidomic analyses.

Prohormone	Localization in mammalian central nervous system	Medial habenula	Lateral habenula
Cerebellin-1	Burnet et al. ^{54,a}	X	X
Cholecystokinin	Schiffmann et al. ^{55,a}	X	X
Chromogranin A	Hawkins Et al. ^{56,a}	X	X
Cocaine- and amphetamine-regulated transcript protein	Koylu et al. ⁵⁷	X	X
Corticoliberin	Wong et al. ^{58,a,b}	X	
Galanin peptides	Skofitch et al. ^{59,a}	X	X
Gastrin-releasing peptide	Kamichi et al. ^{60,a}	X	
Insulin-like growth factor-binding protein 5	Stenvers et al. ^{61,a}	X	
Neuroendocrine protein 7B2	Mbikay et al. ^{62,a}	X	
Neuropeptide S	Leonard et al. ^{63,b}	X	
Neurosecretory protein VGF	Snyder et al. ⁶⁴	X	X
Neurotensin	Moyse et al. ⁶⁵	X	X
Orexin	Marcus et al. ²⁹	X	X
Pituitary adenylate cyclase-activating polypeptide	Hannibal et al. ⁶⁶	X	X
Prepronociceptin	Schulz et al. ⁶⁷	X	X
Proenkephalin-A	Merchenthaler et al. ⁶⁸	X	X
Proenkephalin-B	Merchenthaler et al. ⁶⁸	X	X
Pro-MCH	Hervieu et al. ⁶⁹	X	X
Pro-neuropeptide Y	Allen et al., ⁷⁰ Kopp et al., ³¹ Lynch et al. ³⁰	X	X
Pro-opiomelanocortin	Kineman et al. ⁷¹	X	
ProSAAS	Ye et al. ⁷²	X	X
Protachykinin-1	Lucas et al. ⁷³	X	X
Protachykinin-3	Lucas et al. ⁷³	X	
Prothyroliberin	Winokur et al., ⁷⁴ Calza et al. ⁷⁵	X	X
Secretogranin-1	-	X	X
Secretogranin-2	Marksteiner et al. ⁷⁶	X	X
Secretogranin-3	-	X	X
Somatostatin	Finley et al. ⁷⁷	X	X

^aNo reported significant presence in the habenular nuclei;

^bDetection of the protein/peptide receptor