Comparative Analysis of Neuropeptides in Homologous Interneurons and Prohormone Annotation in Nudipleuran Sea Slugs

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Despite substantial research on neuronal circuits in nudipleuran gastropods, few peptides have been implicated in nudipleuran behavior. In this study, we expanded the understanding of peptides in this clade, using three species with well-studied nervous systems, Hermissenda crassicornis, Melibe leonina, and Pleurobranchaea californica. For each species, we performed sequence homology analysis of de novo transcriptome predictions to identify homologs to 34 of 36 prohormones previously characterized in the gastropods Aplysia californica and Lymnaea stagnalis. We then used single-cell mass spectrometry to characterize peptide profiles in homologous feeding interneurons: the multifunctional ventral white cell (VWC) in P. californica and the small cardioactive peptide B large buccal (SLB) cells in H. crassicornis and M. leonina. The neurons produced overlapping, but not identical, peptide profiles. The H. crassicornis SLB cells expressed peptides from homologs to the FMRFamide (FMRFa), small cardioactive peptide (SCP), LFRFamide (LFRFa), and feeding circuit activating peptides prohormones. The M. leonina SLB cells expressed peptides from homologs to the FMRFa, SCP, LFRFa, and MIP-related peptides prohormones. The VWC, previously shown to express peptides from the FMRFamide (FMRFa), small cardioactive peptide (SCP), LFRFamide (LFRFa), and feeding circuit activating peptides prohormones, was shown to also contain SCP peptides. Thus, each neuron expressed peptides from homologs to the FMRFa, SCP, LFRFa, and MIP-related peptides prohormones. The VWC, previously shown to express peptides from the FMRFa and QNFLa (a homolog of A. californica pedal peptide 4) prohormones, was shown to also contain SCP peptides. Thus, each neuron expressed peptides from the FMRFa and SCP families, the H. crassicornis and M. leonina SLB cells expressed peptides from the LFRFa family, and each neuron contained peptides from a prohormone not found in the others. These data suggest each neuron performs complex co-transmission, which potentially facilitates a multifunctional role in feeding. Additionally, the unique feeding characteristics of each species may relate, in part, to differences in the peptide profiles of these neurons. These data add chemical insight to enhance our understanding of the neuronal basis of behavior in nudipleurans and other gastropods.

Keywords: mass spectrometry, bioinformatics, peptidomics, neuroethology, mollusk, invertebrate, evolution
INTRODUCTION

Due to their relatively simple nervous systems and individually identifiable neurons, nudipleuran gastropods have yielded considerable insight into the neuronal basis of behavior (Katz and Quinlan, 2019). The neuronal circuits governing certain behaviors are well described (Elliott and Susswein, 2002; Crow, 2004; Gillette and Brown, 2015; Sakurai and Katz, 2015), yet there is relatively little information on the peptides and hormones regulating these circuits. Only one nudipleuran, Tritonia diomedeae, has been the subject of a broad scale peptidomic study (Senatore et al., 2015) and physiological studies have largely focused on only three peptides: pedal peptide (Beck et al., 2000; Baltzley et al., 2011), FMRFamide (FMRFa; Lillvis et al., 2012; Webber et al., 2017), and one of the small cardioactive peptides (SCP; Watson and Willows, 1992; Lillvis et al., 2012; Watson et al., 2020). Because 100s of peptides and hormones are present in many species (Ma et al., 2009; Cafe-Mendes et al., 2014; Gan et al., 2015; Christie and Pascual, 2016; Van Camp et al., 2017), and peptidergic signaling is diverse and essential to animal behavior (Liu et al., 2008), the limited peptidomic insights constitute a considerable gap in our understanding of nudipleuran physiology. Research on other gastropods further highlights this gap. Numerous peptides and peptide prohormones have been identified in the gastropods Aplysia californica and Lymnaea stagnalis (Hummon et al., 2003b; Di Cosmo and Di Cristo, 2006; Feng et al., 2009; Cropper et al., 2018; Wood et al., 2021), and recent large-scale transcriptomic studies have examined peptides in the snails Theba pisana (Adamson et al., 2015), Deroceras reticulatum (Ahn et al., 2017), and Charonia tritonis (Bose et al., 2017). Identifying more peptides in nudipleurans will help to rectify this deficit.

Nudipleurans also permit analysis of individual, homologous neurons, that is, neurons with conserved neuroanatomy and function (Bulloch and Ridgway, 1995; Sakurai and Katz, 2019). By comparing homologous neurons one can examine the evolution of behavior at the level of a single neuron (Croll, 1987), and moreover, enables examination of traits at the level of a clade rather than a single species, revealing trends in specific traits (Jourrine and Hoekstra, 2021). In most species, it is difficult to reproducibly identify individual neurons, but nudipleurans, with their large neuronal cell bodies, simple behaviors, and relatively few neurons (less than 10,0000; Boyle et al., 1983), allow for such identification (Katz and Quinlan, 2019). Additionally, the large neuronal sizes are well suited for single-cell peptidomic analysis, performed using matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) mass spectrometry (MS; Garden et al., 1996; Li et al., 2000a). Several homologous neurons have been characterized across nudipleurans (Baltzley et al., 2011; Lillvis et al., 2012; Newcomb et al., 2012), and detailed analyses have uncovered the subtle differences in their circuit-level roles (Katz, 2016; Sakurai and Katz, 2019). Characterizing the peptide profiles of homologous neurons is a logical next step to this research.

This study characterized peptides in both homologous neurons and central nervous system (CNS) transcriptomes for three nudipleuran sea slugs, Hermissenda crassicornis, Melibe leonina, and Pleurobranchaea californica. Each species has been the subject of extensive neurophysiological research (Crow and Tian, 2006; Sakurai et al., 2014; Gillette and Brown, 2015), has a publicly deposited CNS transcriptome, and an easily identified, homologous feeding interneuron in its buccal ganglion. In each species, this neuron drives fictive feeding rhythms and extends axons to extensively innervate the esophagus. In P. californica, this cell, the ventral white cell (VWC), has been shown to be multifunctional (Gillette et al., 1980; Gillette and Gillette, 1983), playing both a command and a motor role in feeding behavior. In H. crassicornis and M. leonina, these are the SCPs, large buccal (SLB) cells, which stain for SCPb (Watson and Willows, 1992). Furthermore, each species has unique feeding characteristics that can be correlated with peptide identities. M. leonina lacks a buccal mass or radula for food breakdown, is a filter feeder, engages in hours-long feeding bouts, and has only approximately 40 neurons in its buccal ganglion (Watson and Trimarchi, 1992; Lee and Watson, 2016). H. crassicornis feeds primarily on polyps and jellyfish (Hoover et al., 2012), and P. californica is an active, generalist predator that indulges in cannibalism (Noboa and Gillette, 2013). These species thus allow for a meaningful comparison of homologous interneurons, using existing resources for rapid annotation of their transcriptomes.

For prohormone annotation, we obtained the CNS de novo transcriptome assemblies because no genomic assemblies for H. crassicornis, M. leonina, and P. californica are available. Using 36 known A. californica and L. stagnalis prohormones, we identified homologous prohormones within each species, and from these compiled a library of putative encoded peptides for each species. We then performed single-cell MS on individual VWCs and SLB cells and used the peptide library to perform peptide mass fingerprinting (Thiede et al., 2005) on the resulting spectra. In H. crassicornis we detected peptides from homologs to the FMRFa, SCP, LFRFamide (LFRFa), and feeding circuit activating peptides (FCAP) prohormones, and in M. leonina, peptides from homologs to the FMRFa, SCP, LFRFa, and myoinhibitory peptide (MIP)-related prohormones. The P. californica VWC has previously been shown to have peptides from the FMRFa and QNFLa [a homolog of the A. californica pedal peptide 4; (Green et al., 2018)] prohormones, and we found that it also contains peptides from the SCP prohormone. Thus, each species’ neuron expressed peptides from both the FMRFa and SCP prohormones, and both H. crassicornis and M. leonina expressed peptides from the LFRFa prohormone; however, each neuron also expressed peptides not seen in the others. This work provides an untargeted peptidomic characterization of single homologous neurons and a large-scale prohormone annotation of multiple nudipleuran sea slugs.
MATERIALS AND METHODS

Animal Care

P. californica and H. crassicornis were trapped by the Monterey Abalone Company (Monterey Bay, CA) and shipped overnight to the University of Illinois Urbana-Champaign. There they were housed individually in artificial seawater at 12°C. M. leonina were collected at the San Juan Islands, either off the docks of Friday Harbor Labs (San Juan, WA), or via snorkel/scuba diving at Park's Bay (Shaw Island, WA), and maintained at Friday Harbor Labs in sea tables with flow-through sea water.

In silico Prohormone Annotation and Peptide Library Establishment

For each species, prohormone annotations were performed on publicly available de novo RNA transcriptome assemblies from the NCBI Sequence Read Archive (Kodama et al., 2012; Christie, 2017; Southey et al., 2020). Species-specific information is as follows: H. crassicornis – SRR1719366 (Goodheart et al., 2017), M. leonina – SRR1950947 and SRR3738852 (Goodheart et al., 2017), and P. californica – SRR026692, SRR026693, SRR026694, SRR026695, SRR1505130, and SRR3928990 (Zapata et al., 2014). For each experimental data set, de novo assemblies were created without any preprocessing of reads using MEGAHIT (Li et al., 2015), SOAPdenovo (Luo et al., 2012), and Trinity (Grabherr et al., 2011; Haas et al., 2013) with default settings. De novo assemblies from the same species were combined into a single BLAST (Altschul et al., 1997) database.

For each species, A TBLASTN search was performed on a database of de novo assemblies for 34 A. californica and two L. stagnalis neuropeptide prohormones obtained from the UniProt database (Apweiler et al., 2004), yielding both RNA and protein matches. RNA matches were translated using the ExPaSy “Translate” tool (Gasteiger et al., 2003), and the longest predicted protein sequence from the matched region was selected for further analysis. SignalP 5.0 (Armenteros et al., 2019) and Phobius (Kall et al., 2007) were then used to analyze both translated proteins and direct protein sequences for the presence of a signal sequence, which is required for targeting into the secretory pathway (Rusch and Kendall, 1995). Finally, potential neuropeptides from each matching protein, whether complete (i.e., possessing a signal sequence) or not, were predicted using NeuroPred (Southey et al., 2006a,b, 2008) with the Mollusc model (Hummon et al., 2003a) and common PTMs selected. The resulting predicted peptides were compiled to form a putative peptide library for each species.

Single-Cell Isolation and MALDI-TOF MS Analysis

We followed prior approaches (Li et al., 2000a) for single-cell isolation and MALDI MS characterization. Subjects were pinned out in dissecting trays, and buccal ganglia were surgically removed. Ganglia were then incubated for 6 min in 1% type 14 protease prepared in saline (460 mm NaCl, 10 mm KCl, 10 mm CaCl2, 25 mm MgCl2, 25 mm MgSO4, 10 mm HEPES, pH = 7.6), which loosened the surrounding connective sheath. Moria scissors were then used to cut through the connective sheath (one layer in M. leonina and H. crassicornis, two layers in P. californica) to expose the neurons. Neurons were identified visually by their distinct morphology, color, and landmark location within the ganglion (Gillette et al., 1980; Watson and Willows, 1992), and then carefully teased away from the rest of the ganglion using either pulled glass capillaries or tungsten needles. Isolated neurons were then aspirated into a custom-made transfer pipette and spotted onto a ground steel MALDI plate sample (Bruker Corp., Billerica, MA), and 0.5 µl of matrix solution (dihydroxybenzoic acid, 20 mg/ml in deionized water) was applied to each neuron. Following drying and matrix crystallization, samples were analyzed by MALDI-TOF MS using an ultrafleXtreme mass spectrometer (Bruker Corp.) in positive reflectron mode, with a surveyed mass range of 530–5,000 m/z and external calibration. Once the spectra had been collected, detected masses were matched to those in the peptide libraries by peptide mass fingerprinting (Thiede et al., 2005) with an allowed mass match error of 200 ppm.

RESULTS

In silico Transcriptomic Annotation of Putative Neuropeptide Prohormones

De novo transcriptomes of the three nudipleurans were queried against 34 A. californica and two L. stagnalis neuropeptide prohormones to identify 35 transcripts in H. crassicornis and M. leonina, and 34 P. californica (Table 1; Supplementary Tables S1–S3). This included two SCP prohormones in H. crassicornis and M. leonina, and two M. leonina temptin proteins. Two proteins, A. californica attractin and egg-laying hormone, were searched but did not yield matches in any of the three species. The majority (27 in H. crassicornis, 27 in M. leonina, and 26 in P. californica) contained a predicted signal sequence. However, only 19, 17, and 12 transcripts of H. crassicornis, M. leonina, and P. californica, respectively, had sequence lengths of at least 95% of A. californica and L. stagnalis neuropeptide prohormone sequence lengths. Each prohormone encoded peptides homologous to those found in the A. californica/L. stagnalis versions of the prohormone.

Identification of Novel Small Cardioactive Peptide C

Further analysis of the transcriptomes found two protein isoforms for the SCP prohormone in H. crassicornis and four in M. leonina (Figure 1). The M. leonina isoforms all expressed identical signal sequences and both SCPα and SCPβ neuropeptides, and three of the four shared the same 94 C-terminal amino acids. Two of the isoforms also expressed a novel peptide, SCPc, which differed from the other SCPs in that it has a serine at the C-terminus, lacked amidation, and is 10 amino acids long rather than nine. However, it retains the YXXFPRM motif seen in all other SCPs, including...
| Name | Query | Accn | Organism | H. crassicornis | M. leonina | P. californica |
|------|-------|------|----------|----------------|-----------|--------------|
|      |       |      | nR | % Id | Evalue | Signal length | nR | % Id | Evalue | Signal length | nR | % Id | Evalue | Signal length | nR |
| Abdominal ganglion neuropeptide L11 | P06518 | Aplysia californica | 151 | 8.1 | 0.756 | 25 | 161 | 14.3 | 3.00E-04 | 27 | 175 |
| Abdominal ganglion neuropeptide L5-L67 | P07712 | A. californica | 112 | 33.9 | 3.35E-20 | 22 | 124 | 37.9 | 1.42E-18 | 23 | 103 |
| Abdominal ganglion neuropeptide R3-14 | P01364 | A. californica | 108 | 21.9 | 5.18E-06 | 23 | 114 | 23.4 | 5.7 | 25 | 77 |
| Achatin | Q5MAR6 | A. californica | 158 | 31.3 | 5.14E-05 | 24 | 131 | 25.4 | 1.87 | 22 | 134 |
| Adipokinetic hormone (AKH) | I6YDN8 | A. californica | 80 | 29.7 | 8.17E-09 | 31 | 91 | 30.9 | 2.42E-07 | 22 | 81 |
| Atrial gland and calcin peptides | P01360 | A. californica | 173 | 21.3 | 0.0521 | 94 | 8.8 | 0.0132 | 29 | 306 | 14.4 | 1.22 | 153 |
| Buccalin | P20481 | A. californica | 505 | 35.4 | 3.63E-60 | 24 | 328 | 48.6 | 4.75E-96 | 368 |
| Cerebral Peptide Cerebrin | Q10998 | A. californica | 209 | 54.1 | 1.53E-52 | 19 | 194 | 28.2 | 5.45E-09 | 30 | 142 |
| Enterin | Q95P23 | A. californica | 837 | 44.7 | 1.02E-43 | 215 | 46.4 | 1.5 | 25 | 414 | 48 | 9.59E-57 | 323 |
| Enticin | Q8I817 | A. californica | 88 | 33.3 | 5.48 | 33 | 31.6 | 3.37 | 57 |
| Feeding circuit activating peptides (FCAP) | Q8ISH7 | A. californica | 504 | 31.5 | 6.23E-92 | 22 | 743 | 48.8 | 4.70E-86 | 26 | 404 |
| FMRF-amide neuropeptide | P08021 | A. californica | 597 | 27.7 | 2.46E-23 | 23 | 386 | 22.6 | 5.00E-20 | 25 | 285 | 15.1 | 5.34E-19 | 23 | 885 |
| FMRFa-related neuropeptides | P42565 | L. stagnalis | 360 | 49.4 | 9.15E-21 | 154 | 21.9 | 7.89E-31 | 25 | 430 | 40.2 | 3.32E-70 | 24 | 338 |
| Gonadotropin-releasing hormone (GNRH) | A8WA77 | A. californica | 147 | 36.3 | 1.13E-11 | 23 | 124 | 27.9 | 1.56E-05 | 34 | 104 |
| Insulin | Q9NDE7 | A. californica | 156 | 33.3 | 2.69E-21 | 29 | 168 | 46.1 | 2.35E-22 | 31 | 141 |

(Continued)
A. californica or L. stagnalis versions of each prohormone were searched against de novo transcriptome assemblies for each species' CNS. Accn – accession number. nR – number of amino acid residues in protein. % Id – degree to which the two prohormones have the same residue at the same point in the alignment. Evalue – likelihood of achieving a comparable match by chance. Signal length – length of signal peptide. nR – number of amino acid residues in returned prohormone.

| Name                                | Query     | H. crassicornis | M. leonina | P. californica |
|--------------------------------------|-----------|-----------------|------------|---------------|
|                                      | Accn      | Organism        | nR | % Id | Evalue | Signal length | nR | % Id | Evalue | Signal length | nR | % Id | Evalue | Signal length | nR |
| LFRF                                 | Q5U900    | L. stagnalis    | 194 | 38  | 1.62E-15 | 22 | 208 | 37.5 | 1.00E-16 | 22 | 176 | 41.2 | 2.11E-19 | 22 | 215 |
| MIP-related peptides                 | Q9NDE8    | A. californica  | 735 | 35.3| 6.05E-26 | 22 | 272 | 31.9 | 4.00E-12 | 22 | 668 | 9.7  | 6.97E-12 | 22 | 444 |
| Myomodulin 1                         | P15513    | A. californica  | 370 | 32.4| 4.30E-42 | 21 | 426 | 58.1 | 0.53  | 16 | 277 | 37.5 | 2.06E-41 | 21 | 344 |
| Myomodulin 2                         | Q2VF17    | A. californica  | 240 | 28.8| 1.01E-14 | 25 | 187 | 41.4 | 6.00E-14 | 22 | 215 | 33.2 | 3.06E-20 | 25 | 244 |
| NdWFamide                            | A0A161R9R0| A. californica  | 90  | 45.9| 6.77E-11 | 25 | 83  | 45.8 | 9.75E-11 | 25 | 83  | 40.5 | 6.22E-10 | 25 | 84  |
| Neuroactive Polyprotein R15          | P12285    | A. californica  | 156 | 21.2| 1.01E-08 | 28 | 151 | 33.5 | 0.95  | 28 | 170 | 34.5 | 1.50E-05 | 25 | 84  |
| Neuropeptides CP2 NPY                | Q8TDY7    | A. californica  | 141 | 14  | 0.00217 | 23 | 129 | 9.5  | 2.412 | 23 | 126 | 18   | 2.59   | 24 | 128 |
| Pedal Peptide 1                      | Q27441    | A. californica  | 92  | 45.7| 3.14E-22 | 21 | 92  | 52.7 | 9.00E-18 | 21 | 91  | 35.6 | 3.88E-13 | 21 | 90  |
| Pedal Peptide 2                      | Q5PSU2    | A. californica  | 385 | 38.8| 2.77E-113| 18 | 418 | 11   | 3.25E-13 | 18 | 281 | 51.7 | 4.49E-47 | 20 | 174 |
| Pedal Peptide 3                      | A1XP49    | A. californica  | 628 | 52.4| 2.47E-47 | 21 | 203 | 55.2 | 0     | 645 | 27.7 | 9.74E-43 | 34 | 476 |
| Pedal Peptide 4                      | A1XP50    | A. californica  | 307 | 34.1| 1.59E-56 | 21 | 323 | 35.7 | 3.61E-45 | 23 | 249 | 43.8 | 4.72E-39 | 20 | 256 |
| Pleurin                              | Q5PSJ5    | A. californica  | 188 | 35.1| 1.41E-24 | 19 | 205 | 35.7 | 2.00E-26 | 19 | 196 | 44.9 | 2.36E-32 | 18 | 176 |
| PROFV                                | Q9E6MA7   | A. californica  | 862 | 32.6| 2.26E-56 | 763 | 23  | 28.8 | 4.00E-22 | 23 | 319 | 53.9 | 3.59E-95 | 495 | 782 |
| Small cardioactive peptides 1        | P09892    | A. californica  | 136 | 43.9| 1.09E-35 | 24 | 132 | 49.6 | 2.00E-27 | 24 | 141 | 57   | 3.82E-31 | 25 | 135 |
| Small cardioactive peptide 2         | P09892    | A. californica  | 136 | 58.3| 1.19E-34 | 24 | 132 | 62   | 3.23E-35 | 24 | 122 |       |        |     |      |
| Sensorin A                           | P29233    | A. californica  | 113 | 38.5| 2.40E-16 | 31 | 117 | 34.4 | 3.00E-16 | 30 | 160 | 30.4 | 7.04E-16 | 29 | 115 |
| Temptin 1                            | Q7Z0T3    | A. californica  | 125 | 27.3| 4.76E-20 | 25 | 128 | 44.6 | 9.00E-33 | 40 | 157 | 29.5 | 1.63E-33 | 22 | 190 |
| Temptin 2                            | Q7Z0T3    | A. californica  | 125 | 52.3| 3.00E-31 | 16 | 100 |       |        |     |      |       |        |     |      |
| Whitnin                              | Q5PSJ3    | A. californica  | 116 | 54.7| 1.96E-38 | 23 | 117 | 54.7 | 9.01E-40 | 23 | 117 | 0.5  | 6.49E-36 | 23 | 116 |
FIGURE 2 | Representative spectrum from a *H. crassicornis* SLB cell. Six neurons were analyzed, and peptides from the FMRFa (black), SCP (blue), LFRFa (purple), and FCAP (red) prohormones were present. The LFRF prohormone is predicted to produce five peptides with a C-terminal LFRFa motif, the FCAP prohormone, three versions of the FCAP peptide, and the SCP prohormone, both SCPa and SCPb; all of these were present in the cell. The FMRFa prohormone is predicted to produce multiple tetrapeptides, but only FMRFa was present in the cell.

FIGURE 1 | Multiple sequence alignment of SCP prohormones across species and predicted protein isoforms. *Melibe leonina* yielded four SCP prohormone isoforms, and *Hermissenda crassicornis* and *Tritonia diomedea* each yielded two. Each prohormone encoded SCPb (orange highlight) and SCPa (green highlight), and one *T. diomedea* and two *M. leonina* isoforms encoded SCPc (blue highlight). Conserved cysteine (red font) and predicted or known cleavage sites (purple font) are also indicated.
those found in *A. californica* (P09892), *L. stagnalis* (O97374), the snail *T. pisana* (A0A0S1RSH0), and the snail *D. reticulatum* (A0A1X9WEF6; Figure 1). Although SCP C has not been observed in any of the above species, it was identified in one of two SCP protein isoforms in *T. diomedea*.

**Unique Neuropeptide Profiles of SLB Cells and VWC Among Nudipleuran Species**

Mass spectrometric analysis of individual SLB cells found that the peptides SCP A and SCP B, encoded by species-specific homologues of the *A. californica* SCP prohormone (UniProt accession number: P09892), were present (Figures 2, 3). The *M. leonina* SLB cells contained two other peptides encoded by the SCP prohormone (GGCA01092244.1), including SCP C. In addition, peptides encoded by several other neuropeptide genes co-localized in the SLB cells, although combinations differed by species (Table 2). The *H. crassicornis* SLB cells contained FMRFa and peptides from homologs to the *L. stagnalis* LFRFa prohormone (Q5U900) (Supplementary Figure S1) and *A. californica* FCAP prohormone (Q81SH7) (Supplementary Figure S2). The *H. crassicornis* LFRFa prohormone encodes five different peptides with a conserved LFRFa motif and amidated C-terminus (Supplementary Figure S1), and each was present within the *H. crassicornis* SLB cells. Finally, the *H. crassicornis* FCAP prohormone encoded three structurally similar peptides, and each was confirmed by MS in the neuron.

In addition to peptides from the SCP prohormone, the *M. leonina* SLB cells contained peptides matching three other prohormones (Figure 3): a homolog to the *A. californica* MIP-related prohormone (Q9NDE8) (Supplementary Figure S3), a homolog to the *A. californica* FMRFa prohormone (P08021) (Supplementary Figure S4), and a homolog to the *L. stagnalis* LFRFa prohormone (Supplementary Figure S1). Eleven peptides derived from the MIP-related prohormone, including eight with a C-terminal amidation and a PRFV or PTFV motif, similar to the PRFX motif found in the *A. californica* MIP-related prohormone. Six peptides came from the FMRFa prohormone, including FMRFa and three other -RFa tetrapeptides, and four peptides from the LFRFa prohormone (Table 2). Two of these four had the same amino acid sequence (GGTLFRF), differing only in the post-translational addition of an acetyl group, and a third peptide also shared the LFRFa motif. Interestingly, two other peptides with an LFRFa motif were putatively encoded on the *M. leonina* LFRFa prohormone, yet were not detected in the SLB cells by MS. Finally, the *P. californica* VWC also contained both SCP A and SCP B (Figure 4; Table 2).
DISCUSSION

**De novo Assembly of CNS Transcriptomes**

Peptides in nudipleurans remain understudied and yet have been extensively studied in the gastropods *A. californica* and *L. stagnalis*. Exploring peptides in nudipleurans can enhance the existing understanding of their neuronal circuits while also allowing for comparison with other species, an essential task for the study of brain evolution (Webber et al., 2017; Moroz, 2018). Using de novo transcriptome assemblies, we predicted putative peptide prohormones for *H. crassicornis*, *M. leonina*, and *P. californica*, and identified several homologs to prohormones previously characterized in *A. californica* and *L. stagnalis*. Additionally, in *M. leonina*

| Species       | Prohormone family homology | Peptide sequence | Peptide name | Mean M + H | Theoretical M + H | Mass error ppm |
|---------------|----------------------------|-----------------|---------------|-------------|------------------|----------------|
| *H. crassicornis* | FCAP                        | GLDSLGGFNVHGQGW | FCAP<sub>1</sub> | 1415.684   | 1415.668        | 11.3           |
|                | FCAP                        | GLDSLGGFQVHGQGW | FCAP<sub>1</sub> | 1429.71    | 1429.684        | 18.2           |
|                | FCAP                        | GLDSLGGFHVHGQGW | FCAP<sub>2</sub> | 1495.7     | 1495.706        | -4             |
|                | FMRFa                       | FMRFamide       | FMRFa         | 599.279    | 599.312         | -55.1          |
|                | LFRFa                       | TLFRFamide      | TLFRFa        | 682.393    | 682.403         | -14.7          |
|                | LFRFa                       | GSIFRFamide     | GSIFRFa       | 725.404    | 725.409         | -6.9           |
|                | LFRFa                       | ASLRFamide      | ASLRFa        | 739.423    | 739.425         | -2.7           |
|                | LFRFa                       | GOSLRFamide     | GOSLRFa       | 782.464    | 782.431         | 42.2           |
|                | LFRFa                       | ARGSLRFamide    | ARGSLRFa      | 952.594    | 952.547         | 49.3           |
|                | SCP                         | SGYLAFFPRMamid  | SCP<sub>1</sub> | 1014.588 | 1014.535      | 50.9           |
|                | SCP                         | VNYLAPRFMamid   | SCP<sub>2</sub> | 1109.638 | 1109.592      | 41.5           |
| *M. leonina*   | FMRFa                       | FVRFamide       | FVRFa         | 567.313    | 567.34          | -47.6          |
|                | FMRFa                       | FLRFamide       | FLRFa         | 581.338    | 581.356        | -31            |
|                | FMRFa                       | YLRFamide       | YLRFa         | 597.335    | 597.35          | -25.1          |
|                | FMRFa                       | FMRFamide       | FMRFa         | 599.303    | 599.312        | -15            |
|                | FMRFa                       | RSVDOMSTRSGDVD | FMRFa peptides-2 | 1882.809 | 1882.806      | 1.6            |
|                | FMRFa                       | SQCPNVDDIYNKALLEEPS | FMRFa peptides-1 | 2564.237 | 2564.249     | -4.7           |
|                | LFRFa                       | SSLRFamide      | SSLRFa        | 755.442    | 755.42         | 28.1           |
|                | LFRFa                       | GOTTLRFamide    | GOTTLRFa      | 796.477    | 796.448        | 38.9           |
|                | LFRFa                       | acSSLRFamide    | SSLRFa        | 797.432    | 797.43         | 2.5            |
|                | LFRFa                       | acSGQPSNEGGM    | LFRF peptides-2 | 948.51    | 948.371       | 146.6          |
|                | MIP-related                 | GPPRFVamide     | GPPRFVa       | 671.414    | 671.398        | 23.8           |
|                | MIP-related                 | pQAPRFVamide    | QAPRFVa       | 699.411    | 699.393        | 25.7           |
|                | MIP-related                 | QAPRFVamide     | QAPRFVa       | 716.442    | 716.42         | 30.7           |
|                | MIP-related                 | YPPRFVamide     | YPPRFVa       | 779.493    | 779.456        | 47.5           |
|                | MIP-related                 | YPPRFVamide     | YPPRFVa       | 793.511    | 793.472        | 49.2           |
|                | MIP-related                 | AQOPRFVamide    | AQOPRFa       | 829.546    | 829.504        | 50.6           |
|                | MIP-related                 | YDPPRFVamide    | YDPPRFa       | 892.514    | 892.467        | 52.3           |
|                | MIP-related                 | ARSPPRFVamide   | ARSPPRFa      | 928.594    | 928.5472       | 50.3           |
|                | MIP-related                 | acGPSLQASEE     | MIP-related peptides 1 | 959.519 | 959.43         | 92.8           |
|                | MIP-related                 | YGRPPIPGQuamide | YGRPPIPGQa | 1112.707 | 1112.6572 | 44.8           |
|                | MIP-related                 | DYDTIFDLLHNSA   | MIP-related peptides 2 | 1523.721 | 1523.699 | 14.4           |
|                | SCP                         | acSFSVSEDamid  | SCP peptides-1 | 940.5413 | 940.389 | 162           |
|                | SCP                         | SGYAGPFRMS     | SCP<sub>1</sub> | 1072.541 | 1072.486 | 51.3           |
|                | SCP                         | SNYLAPRFMamid  | SCP<sub>1</sub> | 1097.605 | 1097.556 | 44.6           |
|                | SCP                         | MNYLAPRFMamid  | SCP<sub>1</sub> | 1141.616 | 1141.564 | 45.6           |
|                | SCP                         | SGYLAFFPRMamid | SCP<sub>1</sub> | 1041.6403 | 1041.535 | 101.1          |
|                | SCP                         | MNYLAPRFMamid  | SCP<sub>1</sub> | 1141.7383 | 1141.564 | 152.7          |
| *P. californica*| FMRFa                       | ASAGQRSEESLREALMQAEEPLY | AEEPLY | Previously characterized |
|                | FMRFa                       | SEEESLREALMQAEEPLY | AEEPLY' |                    |
|                | FMRFa                       | FLRFamide      | FLRFa         |            |                  |
|                | FMRFa                       | FMRFamide      | FMRFa         |            |                  |
|                | FMRFa                       | DVQVQSAAGDAEDEEDISRQILGGGQGVSQDGVDGF | FMRFa peptide 3 |            |                  |
|                | FMRFa                       | PSNAALGEOGE    | FMRFa peptide 5 |            |                  |
|                | QNFLa                       | (p-OLDISGAGM/VSLHOKNFL(Amide) | QNFLa-peptide 5 |            |                  |
|                | QNFLa                       | FDSISSGRLGGNFANFL(Amide) | QNFLa-peptide 6 |            |                  |

Six SLB cells analyzed from *H. crassicornis*, four SLB cells from *M. leonina*, and five VWCs from *P. californica*. PPM – parts per million. Both mean and theoretical M + H values are the average molecular weight.
we identified alternatively spliced SCP transcripts that encoded a novel peptide. Although we recovered homologs to almost every searched transcript, in some instances we could not recover the full protein sequence, and thus it is possible that we missed certain peptides. It is also possible that the transcripts not found here are indeed present in these species, but simply not expressed in the tissues used to generate the transcriptome assemblies.

Peptide Profile Diversity of the VWC and SLB Cells in Nudibranchs, and Functional Implications

We found that the neuropeptide complements of the VWC and SLB cells overlapped but were not identical (Figure 5), which may reflect adaptation to the species’ different feeding habits. SCP peptides were present in every cell, consistent with earlier immunological work (Watson and Willows, 1992) and suggesting a conserved role for these peptides in feeding. In P. californica, VWC firing drives esophageal dilation (Gillette and Gillette, 1983), and in M. leonina, SCP\textsubscript{B} application causes esophageal contractions (Watson et al., 2020), so the data suggest that these neurons use SCP\textsubscript{B} to regulate esophageal movement.

Meanwhile, SCPs are extensively involved in the control of feeding motor programs in A. californica (Lloyd, 1986; Lloyd et al., 1987) and L. stagnalis (Santama et al., 1994; Perry et al., 1999). The A. californica B1 and B2 and the L. stagnalis B2 neurons contain SCP\textsubscript{B} and SCP\textsubscript{A} and project axons to the esophageal nerve (Lloyd et al., 1988; Santama et al., 1994; Perry et al., 1998, 1999), and additional A. californica SCP-immunoreactive neurons innervate buccal musculature (Lloyd, 1988; Church et al., 1991). In both species, the SCPs co-localize with each other (Perry et al., 1998; Perry et al., 1999; Li et al., 2000b), and act as co-transmitters with both classical neurotransmitters (Weiss et al., 1992; Perry et al., 1999) and other neuropeptides (Santama et al., 1994). SCP also drives rhythmic bursting in the buccal ganglion of the snail Helisoma trivolvis (Murphy et al., 1985) and has even been implicated in feeding in Octopus vulgaris, as it drives contraction of the radula protractor muscle, and is transcribed in the buccal ganglion (Kanda and Minakata, 2006). The studies
discussed here are insufficient to determine if *A. californica* and *L. stagnalis* have homologs to the VWC/SLB cells, but a clear conserved role for SCP can be seen in feeding-related movements.

FMRFAs was previously found in the VWCs (Green et al., 2018) and was also found here within the SLB cells, which was surprising given that it inhibits feeding in other gastropods. In *A. californica*, the FMRFa peptide partially shifts feeding rhythms from ingestive to egestive and is released from sensory neurons to reduce accessory radula closer (ARC) muscle contractions (Vilim et al., 2010). Meanwhile, in *L. stagnalis* (Kyriakides and McCrohan, 1989) and *H. trivolvis* (Murphy et al., 1985), FMRFa perfusion inhibits the feeding rhythm, although in *L. stagnalis* it appears to be released from a pleural interneuron involved in defensive responses (Alania et al., 2004), rather than from an element of the feeding neural network. The *L. stagnalis* buccal mass is immunopositive for FMRFa and the buccal ganglion contains a single, bilateral neuron pair with immuneactivity to the related peptide SEQPDYDDYLDDLQSEEPLY (Santama et al., 1994), but FMRFa itself has not been detected in the *L. stagnalis* buccal ganglion by MS. Meanwhile, numerous sensory and motor neurons express FMRFa in the *A. californica* buccal ganglion (Vilim et al., 2010). Does the FMRFa released from the VWC/SLB cells in some way attenuate feeding, or does the presence of FMRFa in these cells reflect divergence from its role in *A. californica* and *L. stagnalis*? Our finding opens the door for future functional studies to address this question.

LFRFa peptides were observed in the *H. crassicornis* and *M. leonina* SLB cells but not the *P. californica* VWC, a pattern that perhaps reflects the three species’ phylogeny. The nudipleuran clade separates into nudibranchia and pleurobranchomorpha; *H. crassicornis* and *M. leonina* are nudibranchs whereas *P. californica* is a pleurobranch. In *A. californica*, LFRFa peptides have a similar effect as FMRFa, modulating contraction of the ARC muscle and weakening ingestive feeding rhythms (Cropper et al., 1994; Vilim et al., 2010). In *L. stagnalis*, MS analysis of the buccal ganglion found the presence of the six peptides encoded by the LFRFa prohormone, which inhibit neurons that regulate metabolism (Hoek et al., 2005). However, immunostaining and single-cell analysis have not been carried out thus far, nor is it known if these peptides have a role in *L. stagnalis* feeding circuitry. Thus, it will be of interest to determine the roles of LFRFa peptides in feeding in other species. Similarly, it will be of interest will be to determine if other cells within the *P. californica* buccal ganglion express LFRFa.

Finally, each species’ neuron expressed peptides from a prohormone not detected by MS in the others. First, the *H. crassicornis* SLB cells contained three peptides from the homolog to the *A. californica* FCAP prohormone and may contain more, as our annotation of the *H. crassicornis* FCAP prohormone returned an incomplete protein. FCAP drives feeding rhythms in *A. californica*, and interestingly is co-expressed with SCPi in a mechanosensory neuron (Sweedler et al., 2002), but its effects on feeding appear to come via the cerebral ganglion neuron CBI-2 (Friedman et al., 2015). FCAP has not been implicated in feeding in any other species. The *M. leonina* SLB cells express peptides from the MIP-related prohormone, which is found in one bilaterally paired set of buccal neurons in *A. californica* (Fujisawa et al., 1999), and many small buccal neurons in *L. stagnalis* and *Helix pomatia* (Elekes et al., 2000). In each species, application of MIP-related peptides drives contractions of the gut. Finally, the *P. californica* VWC contains peptides from the QNFLa prohormone that is a homolog to the *A. californica* pedal peptide 4 prohormone (Green et al., 2018). Pedal peptide 4 has not been investigated physiologically, but in *Biomphalaria glabrata* was observed to be less abundant 12 days post-infection with the parasite *Schistosoma* (Wang et al., 2017).

What are the implications of peptide co-localization in these neurons? Co-localization suggests co-transmission, which can increase the flexibility of post-synaptic control. Co-transmitters, particularly those released from different prohormones, can confer numerous possible abilities onto a single neuron, notably, the modulation of a different neurotransmitter’s effects (Kiss, 2011), more refined control of a single target (Breza et al., 1995; Vilim et al., 2010), or the differential control of multiple targets (Svensson et al., 2019). This final mechanism seems especially possible in the VWC/SLB cells, which affect both feeding circuitry and the gut. Interestingly, SCP and FMRF co-localize in a cerebral interneuron in five different nudipleurans, including *H. crassicornis*, *M. leonina*, and *P. californica* (Lillvis et al., 2012). We cannot say definitively what each peptide does in these three cells, but it seems possible that SCP is released to drive esophageal contractions, and the other peptides to regulate feeding circuits.

Additionally, what are the functional consequences of the unique aspects of each neurons’ peptide profiles? Differences in the intrinsic properties and synaptic wiring of homologous neurons can lead to subtle differences in behavior (Newcomb et al., 2012; Ding et al., 2019), and it may be that these chemical differences are another mechanism of this change. *M. leonina* differs markedly from the other species in this study in the lack of a buccal mass, and differs further in its prey capture apparatus, feeding mechanics, feeding bout duration, and prey. *P. californica* and *H. crassicornis* differ from each other in prey choice and the relative size of their feeding apparatuses. Additionally, the *M. leonina* buccal ganglion is considerably smaller than that of the others, consisting of only 30 to 40 neurons (Trimarchi and Watson, 1992). The differences in peptide profiles may relate in part to these anatomical and behavioral differences.

Finally, in prior studies, neuron homology has been inferred based on synaptic wiring, neuroanatomical position, function, and overlap in immunohistochemical staining (Faulkes, 2008; Lillvis et al., 2012; Sakurai and Katz, 2019). The data in this study do not address the first three characteristics, but do suggest that limits should be placed on interpretations made based on immunohistochemical staining. Our data suggest that at least some of the peptides expressed in homologous neurons will not overlap, and thus if staining is performed for a peptide found in only some of the neurons, it may lead to incorrect conclusions regarding homology.
CONCLUSION

Characterization of the neuropeptides present in a variety of animals is essential to our understanding of neurotransmission. Combining de novo transcriptomics and peptidomics allows us to examine the functional consequences of different peptide profiles without requiring a genomic assembly. The usage of different species in this work helped reveal what is “typical” of neuropeptide signaling, which is essential to the translatability of comparative research. Examining neuropeptides in nudipleuran sea slugs furthers this goal, and moreover, does so in a clade that has provided great insight into neuronal circuits.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CL – conceptualization, methodology, data collection, and writing. ER – conceptualization, methodology, and writing. BS – methodology, data collection, and writing. RG and JS – conceptualization and writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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