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Differential Post-translational Amino Acid Isomerization Found Among Neuropeptides in *Aplysia californica*

David H. Mast^{1,§}, James W. Checco^{1,§,‡}, Jonathan V. Sweedler^{1,*}

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

Abstract

D-amino acid-containing peptides (DAACPs) are a class of post-translationally modified peptides in animals that play important roles as cell-to-cell signaling molecules. Despite the functional importance of L- to D-residue isomerization, little is known about its prevalence, mostly due to difficulties associated with detecting differences in peptide stereochemistry. Prior efforts to discover DAACPs have been largely focused on pursuing peptides based on homology to known DAACPs or DAACP-encoding precursors. Here, we used a combination of enzymatic screening, mass spectrometry, and chromatographic analysis to identify novel DAACPs in the central nervous system (CNS) of *Aplysia californica*. We identified five new DAACPs from the pleurin precursor, and three DAACPs from previously uncharacterized proteins. Further, two peptides from the pleurin precursor, Plrn2 and Plrn3, exist as DAACPs with the D-residue found at either position 2 or position 3. These differentially modified forms of Plrn2 and Plrn3 are located in specific regions of the animal's CNS. Plrn2 and Plrn3 appear to be the first animal DAACPs where the D-residue is found at more than one position and suggests that L- to D-residue isomerization may be a more variable / dynamic modification than previously thought. Overall, this study demonstrates the utility of non-targeted DAACP discovery approaches to identify new DAACPs and demonstrates that isomerization is prevalent throughout the CNS of *A. californica*.

Graphical Abstract

*Corresponding Author jsweedle@illinois.edu.

‡Present Address: Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588, United States

§Denotes equal contribution.

Supporting Information

Supporting Materials and Methods, Tables S1-S4, and Figures S1-S14 (as noted in the text); %D in buccal vs cerebral ganglion for peptide stereoisomers (Table S5); additional figures showing the amino acid sequence of the pleurin precursor (Figure S15), EICs, MS1, and MS2 spectra of identified DAACPs (Figures S16-S21), and MS/MS fragment ion identifications of endogenous peptides (Figures S22-S27); and supporting references.

The authors declare no competing financial interest.

INTRODUCTION

D-amino acid-containing peptides (DAACPs) can be formed by an age-related isomerization of amino acid residues, usually aspartate, and are most commonly found in long-lived peptides and proteins such as lens crystallin proteins and amyloid-beta peptide.¹⁻⁴ In the metazoan, DAACPs may also be the products of an enzyme-catalyzed post-translational modification (PTM), which isomerizes an L-amino acid residue in a peptide into a D-amino acid residue.⁵⁻⁶ The functions of DAACPs in animals range from defense (i.e., venoms and toxins) to cell-cell signaling (i.e., hormones and neuropeptides).⁵⁻⁶ For DAACPs with identified bioactivity, the D-residue can be critical for physiological functions or behaviors, with the all-L-stereoisomer displaying weaker potency or being completely inactive.⁵⁻¹¹ In fact, most DAACPs were discovered only after the biological activity of a purified endogenous peptide was not reproduced by the synthetic all-L-residue stereoisomer. However, several cases do exist in which a DAACP and the all-L-residue stereoisomer share similar receptor potency or physiological activity, but may differ in other functional properties, such as biological half-life.¹²⁻¹⁵

The biological relevance of animal cell-cell signaling DAACPs is not well known, likely because relatively few (~10–20) have been characterized and shown to be involved in this process.¹² In contrast to commonly identified neuropeptide PTMs, such as acetylation or amidation, peptide isomerization may be understudied because modern “peptidomics” characterization approaches do not routinely evaluate stereochemistry.¹⁶⁻²³ Unlike most PTMs, isomerization does not change the resulting peptide’s molecular mass or sequence, making this modification difficult to detect with commonly used liquid chromatography (LC)–tandem mass spectrometry (MS/MS) techniques.

We developed an approach to identify DAACPs in tissue extracts using a stereoselective enzymatic digestion.²⁴⁻²⁵ Peptides with a D-residue near the N-terminus can be identified and differentiated from peptides containing only L-residues in tissue extracts based on differences in the rate of degradation by the stereoselective exopeptidase, aminopeptidase M (APM). Variations of this approach have been previously used by our group and others to

either discover or confirm DAACPs in animals.^{12,24-26} When APM is added to a peptide mixture, the undigested peptides remaining after an extended incubation are putative DAACPs. However, choosing which peptides to pursue for DAACP confirmation studies based solely on a list of APM-resistant sequences is challenging because there are other peptide sequences and modifications that resist APM digestion, independent of the presence of a D-residue (see Supporting Information, Materials and Methods section).

In our previous work with *Aplysia californica*, **GdYFD** (**dX**, in bold indicates the D-residue) and **SdYADSKDEESNAALSDFA** from the *A. californica* achatin-like neuropeptide precursor,²⁴ and **GdFRLNSASRVAHGY-NH₂** from allatotropin-related peptide precursor,¹² were found to be DAACPs based on their resistance to APM digestion. However, these previous studies relied on homology-guided discovery. As an example, we targeted **GdYFD** and **SdYADSKDEESNAALSDFA** because they are derived from the same prohormone as a well-studied DAACP, **GdFFD**.⁷ We expected all peptides from the same precursor to be exposed to the same processing enzymes in the secretory pathway, including a putative isomerase. Similarly, we investigated the peptide **GdFRLNSASRVAHGY-NH₂** because of its sequence similarities to the previously characterized DAACP from cone snail venom, conomap-Vt.^{12,27}

Here for the first time, we applied our DAACP discovery funnel²⁴ in a completely non-targeted manner, with the goal of identifying novel DAACPs from precursors other than those already known to encode isomerized peptides in the central nervous system (CNS) of *A. californica*. We initially screened for DAACPs based on enzymatic stability, followed by evaluation of the chromatographic and mass spectrometric properties of the DAACP candidates. Using this non-targeted approach, we were able to identify eight novel DAACPs. Additionally, two peptide sequences from the pleurin precursor²⁸ were found to be isomerized into multiple epimer forms, with a D-residue at either position 2 or position 3 (from the N-termini), and the relative abundance of these epimers differed across brain regions of the *A. californica* CNS. Finally, three of the DAACPs we discovered are encoded by previously uncharacterized proteins that likely represent novel neuropeptide prohormones. These results suggest that DAACPs should be accounted for in future peptidomics studies, and that the discovery of novel DAACPs leads to uncovering novel prohormones.

RESULTS AND DISCUSSION

APM screening.

The *A. californica* CNS is comprised of five primary ganglia: buccal, cerebral, pedal, pleural, and abdominal (Figure S1). Expression of most prohormones is known to be ganglia specific and certain peptides can be present at significant quantities only in specific ganglia.²⁸ We performed LC-MS/MS peptide identification on APM-treated extracts from pleural, cerebral, abdominal, and buccal ganglia from the *A. californica* CNS to construct a list of peptide sequences that were resistant to APM degradation. Pedal ganglia were omitted from this study because they were the focus of our previous work.²⁴ The list of APM-resistant peptides was compared to a list of sequences present in extracts not treated with APM (no-APM) to ensure the peptides in the APM-treated list were present in the extract before

enzyme treatment. Peptides observed in both lists were considered DAACP candidates. Ideally, the all-L residue peptides would be degraded by APM, leaving only peptides with a D-residue near the N-terminus in the extract. However, not all peptides resistant to APM digestion have a D-residue because APM tends not to cleave certain residues, such as Asp, Glu, Pro or modified N-termini (see Supporting Information, Materials and Methods section).²⁹⁻³¹

DAACPs are often detected alongside the all-L residue peptides in biological extracts, and the two species are typically separable by reversed-phase LC.^{5,32-33} Therefore, we examined whether any of the candidates possibly existed as both the all-L and D-amino acid-containing forms in the extract without APM treatment by checking the LC-MS/MS chromatograms for multiple eluting peaks corresponding to the same peptide sequence. DAACP candidate sequences were selected for follow-up analysis if they were observed to elute at two distinct retention times in the no-APM extracts and the same sequence was only detected at one retention time after APM digestion. The APM-resistant peak likely corresponded to a DAACP, while the peak degraded by APM likely corresponded to the all-L peptide. Any peptides that had N-terminal Asp, Glu, Pro, Xaa-Pro, or modified N-termini were excluded from our analysis because resistance to APM by these peptides may not be the result of a D-residue.

Following our analysis, we found Plrn1, Plrn2, and Plrn3 from the pleurin prohormone, Ip1 and Ip2 from an uncharacterized protein (NCBI accession: XP_012946424.1), and FMRGF-NH₂ from one of three possible uncharacterized proteins (NCBI accessions: XP_005094941.1, XP_005094942.1, XP_005094943.1), eluted at two (or more) retention times in the no-APM extracts, and had only one peak resistant to degradation in the APM-treated extracts (Figure 1). Amino acid sequences of the identified DAACP candidates are reported in Table 1. The six APM-resistant peptides mentioned above were detected in the cerebral ganglia extracts, but some of these DAACP candidates were not observed in the extracts from other ganglia (Table S1). Extracted ion chromatograms (EICs) of putative DAACPs obtained from the buccal, pleural, and abdominal ganglia extracts are shown in Figure S2.

We expected that the resistant peptides mentioned above would likely have a D-residue near the N-terminus based on the stereoselectivity of APM, and more specifically, have a D-residue at the second position from the N-terminus because the D-residue for all previously reported *A. californica* DAACPs has been at position 2.^{7,9,12,24}

The stereochemistry of Plrn1, Plrn2, Plrn3, Ip1, Ip2, and FMRGF-NH₂ was tested by comparing the chromatographic retention of synthetic standards and endogenous peptides using reversed-phase LC with MS detection. Peptide standards were synthesized with isotopically labeled ¹³C-Gly; the sequences are reported in Table S2. Figure 2 shows the EICs of endogenous Plrn1, Plrn2, Plrn3, Ip1, Ip2, and FMRGF-NH₂, as well as the synthetic ¹³C-labeled D₂-peptide standards spiked into the cerebral ganglia extracts. The synthetic D₂-peptide standards co-eluted with the peaks corresponding to the endogenous APM-resistant Plrn1, Plrn2, Plrn3, Ip1, Ip2, and FMRGF-NH₂ (Figure 2). Co-elution of the synthetic D₂-standards with the endogenous APM-resistant peptides supports the idea that the

endogenous peptides are DAACPs with a D-residue in position 2. Further, the all-L-residue synthetic standards did not co-elute with the suspected DAACPs, but did co-elute with peaks that were susceptible to APM digestion (Figure S3).

Identification of [D-Phe³]-Plrn3.

As seen in Figure 2C, the EIC of Plrn3 shows three major peaks sharing the same parent mass and MS/MS fragmentation ions: the first peak (30.5 min) was identified as L-Plrn3 (Figure S3C) and the second peak (33.5 min) was identified as [D-alle²]-Plrn3, as described above. The third peak (34.5 min) was not fully resistant to APM (Figure 1C) and did not co-elute with either of the ¹³C-[D-alle²]-Plrn3 or ¹³C-L-Plrn3 synthetic standards (Figure 2C and Figure S3C, respectively). We considered that the third peak might be a stereoisomer of Plrn3 with a D-residue at an amino acid position further from the N-terminus than position 2 because it did not co-elute with the synthetic all-L or D2-peptides, but was still degraded by APM. Analysis of the APM digestion experiments showed an increase in partially digested Plrn3(2-14) lacking the Gly residue in position 1 after a 15 h digestion with APM that was not present in the controls (Figure S4). Because Plrn3(2-14) remained resistant to APM for the duration of the APM digestion, we expected that the identity of the third eluting Plrn3 peak (34.5 min) in Figure 2C corresponded to a stereoisomer bearing a D-Phe at position 3. Consistent with this supposition, when the synthetic ¹³C-[D-Phe³]-Plrn3 was spiked into the cerebral ganglia extracts, it co-eluted with the peak at 34.5 min (Figure 3C).

To ensure the origin of the endogenous Plrn3(2-14) observed in the APM-treated ganglia extracts is from [D-Phe³]-Plrn3 and not from endogenous L- or D2-peptide, an APM digestion was performed on synthetic standards (Figure S5). ¹³C-L-Plrn3 and ¹³C-[D-alle²]-Plrn3 were both treated with APM, but neither ¹³C-L-Plrn3(2-14) or ¹³C-[D-alle²]-Plrn3(2-14) were detected in the digests of these peptides. The synthetic ¹³C-[D-Phe³]-Plrn3 was also treated with APM and it was degraded into ¹³C-[D-Phe³]-Plrn3(2-14), which remained stable for as long as 43 h. The latter result indicates the Plrn3(2-14) observed in the APM-treated ganglia extracts likely originated from [D-Phe³]-Plrn3.

Identification of [D-Tyr³]-Plrn2.

Because endogenous Plrn3 was found to have a D-residue in position 3, we tested whether the other two peptides from the pleurin prohormone also existed with a D-residue at position 3. As seen in Figure 2A, B, endogenous Plrn1 and Plrn2 eluted at only two retention times, indicating either a third stereoisomer was not present, or that a third stereoisomer co-eluted with one of the two peaks. We tested this by comparing the retention times of synthetic ¹³C-[D-Tyr³]-Plrn1 and ¹³C-[D-Tyr³]-Plrn2 to those of endogenous peptides by spiking. As shown in Figure 3A, ¹³C-[D-Tyr³]-Plrn1 did not co-elute with either of the endogenous Plrn1 peaks. Only the L and D2 forms of Plrn1 could be identified in this study.

We found that ¹³C-[D-Tyr³]-Plrn2 and ¹³C-[D-Phe²]-Plrn2 both co-eluted with the endogenous APM-resistant Plrn2 peptide on a C18 stationary phase (Figure 3B). In a control experiment, we found that APM degraded the synthetic ¹³C-[D-Tyr³]-Plrn2 into ¹³C-[D-Tyr³]-Plrn2(2-14) (Figure S6). Thus, if [D-Tyr³]-Plrn2 was present in the cerebral ganglia extract, we would have expected it to be partially degraded by APM into [D-Tyr³]-

Plrn2(2-14). However, we did not detect the m/z corresponding to Plrn2(2-14) in APM-treated cerebral ganglia extracts (Figure S7A). In contrast, the second eluting Plrn2 peak in the APM-treated buccal extract was mostly degraded, and this degradation corresponded to an increase in the presence of Plrn2(2-14) (Figure S7B). These results suggest that [D-Tyr³]-Plrn2 may be present and localized to the buccal ganglion. The detection of Plrn2(2-14) in APM-treated ganglia extracts does not unambiguously demonstrate that [D-Tyr³]-Plrn2 is present because we did find that some peptides with a D-residue in position 2 can still be partially degraded by APM. For example, we observed partial degradation of [D-Phe²]-Plrn1 into [D-Phe²]-Plrn1(2-14) in the presence of APM (Figures S8 and S9).

To obtain further evidence for the presence of [D-Tyr³]-Plrn2, the endogenous Plrn2 stereoisomers were separated by LC using a chiral stationary phase, and their retention times were compared to the retention times of the synthetic standards. LC with multiple reaction monitoring (MRM) MS was used for detection of endogenous peptides. Extracts from both buccal and cerebral ganglia were tested for [D-Tyr³]-Plrn2 because APM screening had indicated [D-Tyr³]-Plrn2 would be detected in the buccal ganglia extracts, but not detected in the cerebral ganglia extracts. Three Plrn2 peaks were observed in both the cerebral and buccal ganglia extracts that eluted at the same retention times as the Plrn2 synthetic standards (Figure 4). The elution order of the synthetic Plrn2 standards on the chiral column was determined in a separate experiment (Figure S10). Consistent with the results obtained by APM screening, the LC-MRM results showed [D-Tyr³]-Plrn2 is present in the buccal ganglia extracts. Importantly, the relative abundance of [D-Phe²]-Plrn2 was found to be substantially reduced in the buccal ganglion compared to the cerebral ganglion. Additionally, the relative abundance of [D-Tyr³]-Plrn2 was increased in the buccal ganglion compared to the cerebral ganglion. This further confirms that [D-Tyr³]-Plrn2 is localized to the buccal ganglion.

Evaluation of [D-Tyr³]-Plrn2 by Trapped Ion Mobility Spectrometry (TIMS).

DAACPs can adopt different gas phase structures than their all-L-residue counterparts, allowing them to be separated via TIMS.³⁴⁻³⁷ As a complementary method to the LC-MS experiments described above, we next combined LC-MS/MS TIMS to evaluate whether [D-Tyr³]-Plrn2 was present endogenously. Initially a TIMS separation was performed on synthetic Plrn2 standards. The ¹³C-[D-Phe²]-Plrn2 and ¹³C-[D-Tyr³]-Plrn2 [$m+2H$]²⁺ ions were found to have slightly different inverse reduced mobilities ($1/k_0$) during TIMS. As shown in Figure 5A, the D2 and D3 stereoisomers of Plrn2 each adopted two gas phase conformations. The two ¹³C-[D-Tyr³]-Plrn2 peaks were only partially resolved but two distinct peaks at 1.010 V·s·cm⁻² and 1.020 V·s·cm⁻² were observed (Figure 5A). ¹³C-[D-Phe²]-Plrn2 had a major peak observed at 1.027 V·s·cm⁻² and a minor peak at 1.048 V·s·cm⁻². The extracted ion mobilogram (EIM) obtained for ¹³C-L-Plrn2 is provided in Figure S11A.

After optimizing the TIMS separation on synthetic standards, we measured the $1/k_0$ of the endogenous peptides. As mentioned above, APM screening indicated [D-Tyr³]-Plrn2 was detected in the buccal ganglia, but not the cerebral ganglia. Therefore, we measured the stereochemistry of Plrn2 from both the buccal and cerebral ganglia by TIMS. The EIM

obtained from the cerebral ganglia extracts showed peaks at $1.030 \text{ V}\cdot\text{s}\cdot\text{cm}^{-2}$ and $1.053 \text{ V}\cdot\text{s}\cdot\text{cm}^{-2}$, indicating $[\text{D-Phe}^2]\text{-Plrn2}$ was present. However, peaks at $1.010 \text{ V}\cdot\text{s}\cdot\text{cm}^{-2}$ or $1.020 \text{ V}\cdot\text{s}\cdot\text{cm}^{-2}$ were not observed in the EIM, indicating $[\text{D-Tyr}^3]\text{-Plrn2}$ was not detected in the cerebral ganglia extracts. In contrast, the EIM obtained from buccal ganglia extracts showed partially resolved peaks at $1.010 \text{ V}\cdot\text{s}\cdot\text{cm}^{-2}$ and $1.020 \text{ V}\cdot\text{s}\cdot\text{cm}^{-2}$, consistent with $1/k_0$ values measured for the synthetic $^{13}\text{C}\text{-}[\text{D-Tyr}^3]\text{-Plrn2}$ (Figure 5B). Additionally, a third, partially resolved peak was observed at $1.029 \text{ V}\cdot\text{s}\cdot\text{cm}^{-2}$ in the EIM from buccal ganglia extracts, indicating $[\text{D-Phe}^2]\text{-Plrn2}$ and $[\text{D-Tyr}^3]\text{-Plrn2}$ coexist in the buccal ganglion. Note, the $1/k_0$ for $^{13}\text{C}\text{-}[\text{D-Phe}^2]\text{-Plrn2}$ standard was shifted by roughly $0.003 \text{ V}\cdot\text{s}\cdot\text{cm}^{-2}$ compared to the $1/k_0$ measured for the endogenous $[\text{D-Phe}^2]\text{-Plrn2}$ from cerebral ganglia extracts. We expect this shift was the result of drift in the TIMS calibration over time. Importantly, the $1/k_0$ values reported here were not intended to be used to calculate the collisional cross sections of the Plrn2 stereoisomers.

Relative Abundance of D2 and D3 Stereoisomers Between Regions of the CNS.

The TIMS results described above were consistent with the results obtained from APM screening and LC–MRM, indicating $[\text{D-Tyr}^3]\text{-Plrn2}$ is present in the buccal ganglion. Furthermore, both TIMS and LC–MRM showed a reduced relative abundance of $[\text{D-Phe}^2]\text{-Plrn2}$ in buccal ganglia extracts compared to cerebral ganglia extracts.

The observed differences in the relative abundance of D2 and D3 stereoisomers between regions of the CNS were not unique to Plrn2. We also observed distinct differences in the relative abundance of Plrn3 stereoisomers between the buccal and cerebral ganglia extracts by LC–MS. Specifically, $[\text{D-alle}^2]\text{-Plrn3}$ was not detected in buccal ganglia extracts whereas both $[\text{D-alle}^2]\text{-Plrn3}$ and $[\text{D-Phe}^3]\text{-Plrn3}$ were detected in cerebral ganglia extracts. Because the three Plrn3 stereoisomers were well resolved by reversed-phase LC, we were able to quantify their relative abundances. Figure 6A shows EICs of Plrn3 obtained from the buccal or cerebral ganglia extracts, and Figure 6B shows the averaged relative abundance of Plrn3 stereoisomers from four biological sets. In four replicates, $[\text{D-alle}^2]\text{-Plrn3}$ was not detected in buccal ganglia extracts, demonstrating that the absence of $[\text{D-alle}^2]\text{-Plrn3}$ was not the result of biological variability.

Though we did not quantify the relative abundance of Plrn2 stereoisomers, both LC–MRM and TIMS showed a decreased relative abundance of $[\text{D-Phe}^2]\text{-Plrn2}$ in buccal ganglia extracts compared to cerebral ganglia extracts. Additionally, we were able to show the $[\text{D-Tyr}^3]\text{-Plrn2}$ stereoisomer comprises a greater relative abundance in the buccal ganglia extracts than in cerebral ganglia extracts by comparing the ion ratios obtained from collision induced dissociation (Figure S12). The region-specific distribution of Plrn2 and Plrn3 we observed may indicate that the PTM processing of these peptides is different in the buccal ganglion than in the cerebral ganglion.

Differential Isomerization of Plrn2 and Plrn3.

Plrn2 and Plrn3 are the first examples of isomerization occurring at the third position of a peptide in *A. californica*. To our knowledge, Plrn2 and Plrn3 also represent the first reported examples (in animals) of L- to D-residue isomerization occurring at different positions within

the same peptide sequence. The closest known example of this observed differential isomerization is found among hormones from crustaceans. It was previously found that the crustacean hyperglycemic hormone (CHH) is isomerized at [Phe³] and the vitellogenesis-inhibiting hormone (VIH) is isomerized at [Trp⁴].^{14,38} Studies on the cellular distribution of VIH and CHH found that isomerization of these peptides occurs in unique cell populations, indicating differential cellular expression of one or more isomerases.⁶ However, unlike VIH and CHH from crustaceans, Plrn2 and Plrn3 from *A. californica* are differentially isomerized, with the D-residue occurring at either of two positions within the same peptide sequence.

The unexpected discovery of [D-Tyr³]-Plrn2 and [D-Phe³]-Plrn3 might indicate the L/D-isomerase responsible for the biogenesis of these DAACPs has somewhat loose specificity for isomerization of positions 2 versus 3. The five previously identified DAACPs in *A. californica* are isomerized at aromatic amino acids in position 2. Isomerization of Plrn2 and Plrn3 at positions 2 and 3 may result because the isomerase favors aromatic amino acid residues. The lack of [D-Tyr³]-Plrn1 in biological extracts was somewhat surprising, given that Plrn1 also derives from the pleurin prohormone and shares a similar N-terminal sequence to Plrn2 (Table 1). This difference in the specificity of the L/D-isomerase towards residue positions may arise from subtle differences in the peptide sequences. Alternatively, it is possible that there exist two distinct L/D-isomerases responsible for isomerization at position 2 vs. position 3.

Identification of *Aplysia* Isomerized Peptides Precursor and FMRGFamide Precursors.

As mentioned above, the two newly identified DAACPs, Ip1 and Ip2, are encoded on an uncharacterized predicted protein (NCBI accession: XP_012946424.1) supported by transcript and expressed sequence tag evidence in *A. californica*. In prior experiments our lab identified the Ip1 sequence by *de novo* sequencing of whole CNS lysates.³⁹ In the current study, a total of 11 peptides, including Ip1 and Ip2, were detected in screening experiments that derived from the same uncharacterized protein. Consistent with the proteolytic processing expected of peptide prohormones, all detected peptides were flanked by dibasic cleavage sites on the precursor protein. According to SignalP,⁴⁰ there is a 50% probability of signal peptide cleavage between residues 31 and 32 of the precursor, suggesting the protein can be targeted to the secretory pathway. A BLAST search of this protein showed it has 32% sequence similarities to *A. californica* pedal peptides 2 precursor (NP_001191623.1), and 38% similarity to feeding circuit activating peptides precursor (FCAP, NP_001191518.1). We refer to this uncharacterized protein as the *Aplysia* isomerized peptides precursor (AIPP) to describe the PTM observed on two of the encoded peptides. The sequence coverage of AIPP by the detected peptides is reported in the Supporting Information (Figure S13, Table S3); and the complete annotation of the processing of AIPP will be the subject of a follow-up study.

Another novel DAACP identified in this work, FMRGF-NH₂, is one of 40 peptides matching sequences encoded in three uncharacterized protein isoforms (XP_005094941.1, XP_005094942.1, XP_005094943.1) predicted from the *A. californica* transcripts (XM_005094884.2, XM_005094885.2, XM_005094886.2). The three proteoforms have

95% signal peptide cleavage probability between residues 29 and 30 of the precursor, and numerous conventional proteolytic cleavage sites characteristic of neuropeptide precursors. A BLAST search showed the proteins have >90% sequence similarity between themselves and ~40% similarity to the *A. californica* FMRGFamide precursor (AAA27752.1, P08021.3) and mytilus inhibitory peptide-related peptides precursor (NP_001191614.1). We refer to these proteins as the *A. californica* FMRGFamide precursor(s). The novel prohormone sequence(s) is supported by LC–MS/MS detection of numerous predicted peptides (Figure S14, Table S4). Peptides detected by LC–MS/MS matched sequences common in all three predicted proteoforms and could have originated from any of the three proteins. It was beyond the scope of this work to determine the exact transcript variant that leads to the observed FMRGF-NH₂ peptides. An interesting question is whether only one of the three FMRGFamide proteins produces the [D-Met²]-FMRGF-NH₂, and this will be a subject for future studies.

The evidence presented above indicates that the AIPP and FMRGFamide-related peptides detected during this screening were likely the result of post-translational proteolytic processing of the precursor by prohormone convertases.

Prior studies have indicated that multiple peptides from the same prohormone are often isomerized.^{24,41–42} Consistent with previously characterized prohormones encoding DAACPs, we identified three peptides from the pleurin precursor and two peptides from AIPP (Ip1 and Ip2) as DAACPs. This is consistent with a mechanism by which peptides are first cleaved from their precursor by prohormone convertases, and then isomerized within the dense core vesicles. However, in APM screening and peptide identification experiments, nine peptides were detected from AIPP that all shared similar sequences (Table S3), but only Ip1 and Ip2 were found to be DAACPs. This was surprising given the sequence similarities between these peptides. Similarly, among numerous similar peptides predicted and detected from FMRGFamide precursor(s), [D-Met²]-FMRGF-NH₂ was the only DAACP identified from the prohormone (Table S4).

While the selective isomerization may be caused by enzyme processing, it may be technical in nature. Although FMRGFamide and AIPP encode numerous similar peptide sequences, Ip1, Ip2, and FMRGF-NH₂ have multiple repeats of the same sequence compared to the other peptides encoded on their precursors. Following proteolytic processing, Ip1, Ip2, and FMRGF-NH₂ exist at much higher concentrations within the dense core vesicles relative to the other possible isomerase substrates encoded on the prohormones. Therefore, although the other possible isomerase substrates may be present from the same prohormone, the isomerase is more likely to encounter Ip1 and Ip2 or FMRGF-NH₂, or perhaps the isomerized peptides present at lower levels are not detectable.

CONCLUSIONS

Using a combination of enzymatic screening, analysis of LC–MS/MS properties, and synthetic peptide standards, we identified eight novel DAACPs from three different prohormone precursors—two of which were previously uncharacterized and are described for the first time in this study. By systematically evaluating potential DAACPs based on both

their APM resistance and chromatographic properties, we were able to identify DAACPs purely based on their chemical properties without regard to homology to other known DAACPs. Importantly, our approach also allowed us to identify the first animal peptides to undergo differential post-translational isomerization within the same sequence, and we found that this processing differed across different regions of the CNS. This study represents the most comprehensive discovery effort for endogenous DAACPs in a single organism to date. However, we recognize that our DAACP discovery method is restricted to finding D-residues near the N-terminus and for sequences having N-terminal Asp, Glu, Pro, Xaa-Pro or modified N-termini. Thus, a fraction of the DAACPs may have been overlooked and future work will require new methods to address these sequence constraints. Despite the latter limitations, our findings indicate that DAACPs are more common than previously thought in the *A. californica* CNS. They also suggest that bioactivity studies on all-L-peptides in *A. californica* may have missed activity because of the possible presence of unrecognized D-residues. Isomerization of amino acid residues in peptides appears to be an underreported PTM and may have a broader scope than *A. californica*. Future studies should be carried out to determine the prevalence of peptide isomerization in a range of animals. An important next step is the identification and characterization of the isomerase enzyme responsible for this modification.

METHODS

Detailed procedures can be found in the Supporting Information.

Chemicals.

Solvents and reagents used, unless specified otherwise, were purchased from Thermo Fisher Scientific or MilliporeSigma.

Peptide Extraction.

A. californica weighing 70–160 g were purchased from the NIH/University of Miami National Resource for *Aplysia*, and maintained at 14 °C in an aquarium filled with Instant Ocean (Aquarium Systems Inc.). Before dissection, animals were injected (66% volume/body weight) with a solution of 74 g/L MgCl₂·6H₂O to anesthetize, after which the buccal, cerebral, left pleural, right pleural, and abdominal ganglia from 3–5 animals were dissected and separated into 50 mL beakers filled with artificial sea water (ASW) cooled on ice. Following dissection, individual ganglia were removed from the ASW and placed directly into a 500 µL solution of 70/30 acetone/water (v/v) with HCl at a final concentration of 0.2 N (acidified acetone). Ganglia were homogenized manually using mechanical homogenization, and sonicated for 1 min in an ice bath before centrifugation at 15,000 × g, 10 min, 4 °C. The supernatant was transferred to a clean tube and the remaining pellet was washed once with 200 µL of LC–MS-grade water and centrifuged at 15,000 × g, 5 min at 4 °C. The resulting supernatant was combined with the acidified acetone extract and dried. Samples were desalted using C18 solid-phase extraction (SPE). The SPE samples were dried and stored at –80 °C until analysis.

APM Digestion.

APM from porcine kidney was purchased from EMD Millipore. APM screening was performed as previously described, with minor alterations.¹² The approximate peptide concentration of ganglia extracts from five animals was estimated using bicinchoninic acid. Following quantitation, each ganglia extract was dried and reconstituted in a solution of 50 mM Tris, 500 mM NaCl, pH 7.5, to a final peptide concentration of 25 µg/mL. Each ganglia extract was then divided into two separate tubes: APM was added to one tube to a final concentration of 0.3 U/mL (APM-treated); an equal volume of water was added to another tube (no-APM). The tubes were then incubated for 15 h at 37 °C on a shaker. Following incubation, the reaction was quenched by bringing each tube to 5% CH₃CN and 0.1% formic acid (FA) and placing the reaction on ice, and the quenched reactions were desalted by SPE. After desalting, the samples were dried, and each reconstituted in 40 µL of 5% CH₃CN, 0.1 % FA. For each ganglia extract, 3–6 µL of extract was used for LC–MS/MS analysis.

LC–MS/MS Analysis.

LC was carried out on an Ultimate 3000 Nano-RSLC system (Thermo Fisher Scientific) coupled to a Bruker Impact quadrupole time-of-flight mass spectrometer with a CaptiveSpray ionization source, operated in positive ionization mode. MS/MS peptide sequencing was performed by collision induced dissociation fragmentation. Separation was performed using an Acclaim™ Pepmap™ 100 C18 nano-LC column, 75 µm i.d. × 15 cm, 2 µm particle size, 100 Å pore size (P/N 164534, Thermo Fisher Scientific) at 35 °C with a flow rate of 300 nL/min. Mobile phase compositions were as follows: 95% H₂O, 5% CH₃CN, 0.1% FA (solvent A) and 95% CH₃CN, 5% H₂O, 0.1% FA (solvent B). The gradient used was 4–50% B over 90 min, 50–90% B over 5 min, 90% B for 10 min, 90–4% B over 5 min, 4% B for 10 min.

Peptide Identification.

Peptides present in the no-APM control and the APM-treated extracts of buccal, cerebral, abdominal, pleural left, and pleural right ganglia were identified using PEAKS Studio 8.0 (Bioinformatics Solutions Inc.), searching against the NCBI *A. californica* protein database. EICs, MS1, and MS2 spectra of identified DAACPs are shown in Figures S16–S21. MS/MS fragment ion identifications of endogenous peptides are provided in Figures S22–S27.

Relative Quantitation of Plrn3 Stereoisomers.

Cerebral and buccal ganglia extracts were prepared as above and run under the same LC–MS/MS analysis conditions described above. Relative abundances of Plrn3 were calculated using the peak areas from an EIC of m/z 772.9 ± 0.1. Significance was tested using a two-sample t-test with a significance of $\alpha < 0.05$. Relative abundances were an average of N=4 biological sets of buccal and cerebral ganglia, where each biological set contained ganglia pooled from 3–5 animals.

LC Separations of Plrn2 Stereoisomers with MRM.

Cerebral and buccal ganglia extracts were prepared as above, reconstituted in 50 μ L of 0.1% FA in water and sonicated for 5 min; 30 μ L of the resulting solution was injected onto an Agilent 1200 series HPLC system (Agilent Technologies). The LC separation of Plrn2 stereoisomers was performed on a Supelco Aztec Chirobiotic T2 column, 4.6 mm i.d. $\times$ 25 cm, 5 μ m particle size, 200 $\text{\AA}$ pore size (P/N 16024AST, MilliporeSigma). Mobile phase compositions were as follows: 0.1% FA in water (solvent A) and 0.1% FA in acetonitrile (solvent B). The separation was performed at 35 $^{\circ}$ C using a flow rate of 1 mL/min and running a linear gradient as follows: 15% B over 2 min, 15–25% B over 15 min, 70% B over 2 min, and 15% B over 3 min. MRM MS analysis was performed using a benchtop hybrid triple quadrupole-linear accelerator trap LC–MS/MS system (5500 QTRAP; Sciex) located in the Metabolomics Lab of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Mass spectra were acquired under positive electrospray ionization. The parent ion m/z monitored was m/z 814.4 for ^{13}C -labeled synthetic peptide standards, and 813.4 for the endogenous peptide. Fragment ions monitored were m/z 547.1, 384.1, and 207.0 for both synthetic and endogenous Plrn2.

LC–MS/MS with TIMS.

For the TIMS measurements of Plrn2 stereoisomers, LC was carried out on a nanoElute system (Bruker) using a nanoElute FIFTEEN C18 ReproSil AQ reversed-phase analytical column, 75 μ m i.d. $\times$ 15 cm, 1.9 μ m particle size, 120 $\text{\AA}$ pore size (P/N 1842621, Bruker) coupled to a timsTOF Pro mass spectrometer with a CaptiveSpray ionization source (Bruker). Separation was carried out at 40 $^{\circ}$ C at a flowrate of 30 nL/min. Mobile phase compositions were as follows: H_2O , 0.1% FA (solvent A) and CH_3N , 0.1% FA (solvent B). The LC gradient conditions used were 4–50% B in 50 min, 50–95% B in 2 min, 95% B for 10 min. MS parameters used for TIMS: MS was collected in positive mode, with TIMS enabled and the ion charge control target set to 2.00 Mio. The imex setting was set to custom resolution. The reduced mobility ($1/k_0$) range was set to 0.9–1.2 $\text{V}\cdot\text{s}\cdot\text{cm}^{-2}$, ramp time 350 ms, spectra rate 2.82 Hz, and the lock accumulation to mobility range was selected. The TIMS tuning delta values were as follows: $\delta_1 = -20.0$ V, $\delta_2 = -80.0$ V, $\delta_3 = 110.0$ V, $\delta_4 = 110.0$ V, $\delta_5 = 0.0$ V, $\delta_6 = 120.0$ V. The tunnel out pressure was 8.06×10^{-1} mBar and the tunnel in pressure was 2.57 mBar. For TIMS MS measurements, the TOF and TIMS were calibrated using Agilent low concentration tune mix introduced through the CaptiveSpray source. Additional details can be found in the Supporting Information, Materials and Methods section.

Peptide Synthesis.

Synthetic Plrn1, Plrn2, Plrn3, FMRGF-NH₂, Ip1, and Ip2 peptides, labeled with ^{13}C -Gly, were synthesized using Fmoc solid-phase peptide synthesis, as described previously.¹² ^{13}C -Fmoc-Gly-OH was purchased from Anaspec Inc. and labeled with one ^{13}C at the carbonyl carbon. MS/MS spectra of synthetic peptides with labeled fragment ion identifications are shown in Figures S22–S27.

Spiking Synthetic Standards.

Peptide standards were diluted to 1 μ M in water with 0.1% FA. For spiking, each standard was then combined with cerebral ganglia extracts (prepared as described above) to a final concentration between 0.01–0.5 μ M, and 3–6 μ L of extract was used for LC–MS/MS analysis using the method described above

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

APM screening results from cerebral ganglia extracts showing the APM-resistant peptides. Plots show LC-MS EICs of (A) Plrn1 (m/z 560.6, $z = 3+$), (B) Plrn2 (m/z 542.6, $z = 3+$), (C) Plrn3 (m/z 772.9, $z = 2+$), (D) Ip1 (m/z 552.3, $z = 2+$), (E) Ip2 (m/z 1051.6, $z = 1+$), and (F) FMRGF-NH₂ (m/z 328.3, $z = 2+$) incubated for 15 h without APM (black trace) and incubated with APM for 15 h (dashed red trace). The red arrows indicate the APM-resistant peak. The peak labeled with an X in (E) was identified as an unrelated peptide sequence.

Figure 2.

Confirmation of endogenous peptide stereochemistry identifications by LC-MS using ^{13}C -labeled synthetic D2 peptides spiked into *A. californica* cerebral ganglia extracts. For each plot, the black trace shows EICs for the monoisotopic m/z predicted for the endogenous peptide, while the dashed red trace shows the EIC for $(m+1)/z$, corresponding to the m/z of the ^{13}C -labeled synthetic standard. For each plot, the upper trace shows the EICs of the endogenous peptides in cerebral ganglia extracts spiked with water, and the lower trace shows the synthetic D2 standard spiked into cerebral ganglia extracts. (A) Plrn1, m/z 560.3 ± 0.1 , $z = 3+$ (black trace), $(m+1)/z$ 560.6 ± 0.1 , $z = 3+$ (red trace), (B) Plrn2, m/z 542.6 ± 0.1 , $z = 3+$ (black trace), $(m+1)/z$ 542.9 ± 0.1 , $z = 3+$ (red trace), (C) Plrn3, m/z 772.9 ± 0.1 , $z = 2+$ (black trace), $(m+1)/z$ 773.4 ± 0.1 , $z = 2+$ (red trace), (D) Ip1, m/z 552.3 ± 0.1 , $z = 2$ (black trace), $(m+1)/z$ 552.8 ± 0.1 , $z = 2+$ (red trace), (E) Ip2, m/z 1051.6 ± 0.1 , $z = 1+$ (black trace), $(m+1)/z$ 1052.6 ± 0.1 , $z = 1+$ (red trace), (F) FMRGF-NH₂, m/z 328.7 ± 0.1 , $z = 2+$ (black trace), $(m+1)/z$ 329.2 ± 0.1 , $z = 2+$ (red trace). For each peptide, the $(m+1)/z$ isotope is present in the endogenous sample due to the natural isotopic pattern of peptides in this mass range. However, when the ^{13}C -labeled synthetic standard is spiked into the extract, the increase in the $(m+1)/z$ is due to the presence of the isotope-labeled peptide co-eluting with the endogenous peptide. The peak labeled with an X in (F) was identified as an unrelated endogenous peptide sharing the same m/z as the synthetic standard.

Figure 3.

Testing for the presence of endogenous Plrn1, Plrn2, and Plrn3 D3-stereoisomers by spiking of ^{13}C -labeled synthetic D3 forms of Plrn1, Plrn2, and Plrn3 peptides into *A. californica* cerebral ganglia extracts. Plots show LC-MS EICs of (A) endogenous Plrn1, m/z 560.3, $z = 3+$ (black trace) and synthetic ^{13}C -[D-Tyr³]-Plrn1, $(m+1)/z$ 560.6, $z = 3+$ (dashed red trace), (B) endogenous Plrn2 m/z 542.6, $z = 3+$ (black trace) and ^{13}C -[D-Tyr³]-Plrn2, $(m+2)/z$ 543.3, $z = 3+$ (dashed red trace), and (C) endogenous Plrn3, m/z 772.9 $\pm$ 0.1, $z = 2+$ (black trace), and ^{13}C -[D-Phe³]-Plrn3, $(m+2)/z$ 773.9 $\pm$ 0.1, $z = 2+$ (dashed red trace). Shown in (B), ^{13}C -[D-Tyr³]-Plrn2 co-eluted with the peak that was earlier identified as [D-Phe²]-Plrn2 based on spiking and APM resistance. The peak labeled with an “X” in (B) indicates an unrelated endogenous peptide that has the same m/z as the synthetic standard.

Figure 4. Separation of endogenous Plrn2 peptides using chiral LC-MRM MS. Chromatograms show (A) ^{13}C -labeled synthetic standards, ^{13}C -L-Plrn2 (10.7 min), ^{13}C -[D-Phe²]-Plrn2 (12.3 min), and ^{13}C -[D-Tyr³]-Plrn2 (14.4 min), (B) endogenous Plrn2 stereoisomers in cerebral ganglia extracts, and (C) buccal ganglia extracts. For ^{13}C synthetic Plrn2 stereoisomers, the channel monitored parent m/z 814.4 and fragment m/z 547.1. For the endogenous Plrn2, the channel monitored parent m/z 813.4 and fragment m/z 547.1.

Figure 5.

Evidence for [D-Tyr³]-Plrn2 in buccal ganglia extracts using TIMS. (A) EIMs of synthetic Plrn2 standards, ¹³C-[D-Phe²]-Plrn2 (*m/z* 813.9, *z* = 2+, solid black trace) and ¹³C-[D-Tyr³]-Plrn2 (*m/z* 814.4, *z* = 2+, dashed black trace). (B) EIMs of endogenous Plrn2 (*m/z* 813.4, *z* = 2+) from cerebral ganglia extracts (solid black trace) and buccal ganglia extracts (dashed red trace). EIMs of the L-peptides are reported in Figure S11.

Figure 6.

Relative abundance comparison of Pln3 between buccal and cerebral ganglia. (A) LC-MS EICs of Pln3 (m/z 772.9 $\pm$ 0.1, z = 2+) from cerebral ganglia extracts (upper) and buccal ganglia extracts (lower). The chromatogram from the cerebral ganglia is from Figure 2C for comparison purposes. (B) Quantitative comparison of the relative abundance of Pln3 stereoisomers in the buccal and cerebral ganglia measured by LC-MS. The bars represent averaged relative abundances $\pm$ standard deviation from N=4 biological sets. *** p = 3.8×10^{-7} , ** p = 3.6×10^{-5} , * p = 0.01. Significance was tested using a two-sample t-test.

Table 1.

Peptide stereoisomers identified by APM screening.

Peptide name	Peptide Sequence	Prohormone
L-Plrn1	MFYTKGSDSDYPRI-NH ₂	Pleurin
L-Plrn2	SFYTTGNGNHYPRI-NH ₂	Pleurin
L-Plrn3	GIFTQSAYGSYPRV-NH ₂	Pleurin
L-IP1	YLDHLGSSLV	AIPP ^a
L-IP2	YLDGIASSLI	AIPP
L-FMRGF-NH ₂	FMRGF-NH ₂	FMRGFamide ^b
DAACPs		
[D-Phe ²]-Plrn1	MdFYTKGSDSDYPRI-NH ₂	Pleurin
[D-Phe ²]-Plrn2	SdFYTTGNGNHYPRI-NH ₂	Pleurin
[D-allo ²]-Plrn3 ^c	GdIFTQSAYGSYPRV-NH ₂	Pleurin
[D-Leu ²]-IP1	YdLDHLGSSLV	AIPP
[D-Leu ²]-IP2	YdLDGIASSLI	AIPP
[D-Met ²]-FMRGF-NH ₂	FdMRGF-NH ₂	FMRGFamide
[D-Tyr ³]-Plrn2	SFdYTTGNGNHYPRI-NH ₂	Pleurin
[D-Phe ³]-Plrn3	GldFTQSAYGSYPRV-NH ₂	Pleurin

^a *Aplysia* isomerized peptides precursor.^b Three possible proteins (XP_005094941.1, XP_005094942.1, XP_005094943.1).^c D-allo refers to (2R,3S)-2-amino-3-methylpentanoic acid.The predicted D-residue in each peptide is indicated in the single amino acid code preceded by a lowercase “d” (**dX**, bold font).