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# A D-Amino Acid-Containing Neuropeptide Discovery Funnel

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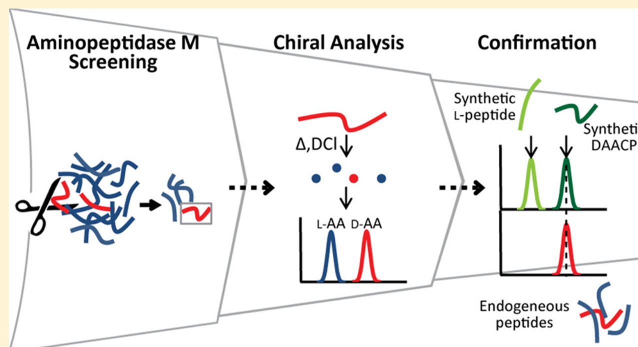
*Supporting Information*

**ABSTRACT:** A receptor binding class of D-amino acid-containing peptides (DAACPs) is formed in animals from an enzymatically mediated post-translational modification of ribosomally translated all-L-amino acid peptides. Although this modification can be required for biological actions, detecting it is challenging because DAACPs have the same mass as their all-L-amino acid counterparts. We developed a suite of mass spectrometry (MS) protocols for the nontargeted discovery of DAACPs and validated their effectiveness using neurons from *Aplysia californica*. The approach involves the following three steps, with each confirming and refining the hits found in the prior step. The first step is screening for peptides resistant to digestion by aminopeptidase M. The second verifies the presence of a chiral amino acid via acid hydrolysis in deuterium chloride, labeling with Marfey's reagent, and liquid chromatography−mass spectrometry to determine the chirality of each amino acid. The third involves synthesizing the putative DAACPs and comparing them to the endogenous standards. Advantages of the method, the D-amino acid-containing neuropeptide discovery funnel, are that it is capable of detecting the D-form of any common chiral amino acid, and the first two steps do not require peptide standards. Using these protocols, we report that two peptides from the *Aplysia* achatin-like neuropeptide precursor exist as GdYFD and SdYADSKDEESNAALSDFA. Interestingly, GdYFD was bioactive in the *Aplysia* feeding and locomotor circuits but SdYADSKDEESNAALSDFA was not. The discovery funnel provides an effective means to characterize DAACPs in the nervous systems of animals in a nontargeted manner.




Neuropeptides are a class of cell−cell signaling molecules processed from protein prohormones. They are produced in the nervous system and exert effects on virtually all organs, being implicated in processes like reproduction,[1] food intake,[2] and circadian rhythms.[3] The proteins used to generate neuropeptides are ribosomally translated using only L-amino acids. During this process, they undergo enzymatic post-translational modifications (PTMs), which include cleavages by prohormone convertases and additional modifications such as amidation.[4] These PTMs are required to create the bioactive form of the peptide. One understudied PTM of neuropeptides is peptide isomerization, where roughly 40 DAACPs have been found as toxins, neurohormones, and neuropeptides.[5,6] In neuropeptides, this involves the enzymatic conversion of one residue near the N-terminus from an L-amino acid to a D-amino acid.[5,7−13]

However, unlike most other PTMs, isomerization is a zero-Dalton shift in molecular weight and so it is not easily detectable by mass spectrometry (MS); several strategies have thus emerged to enable isomerization detection.[6] One way to overcome this difficulty is via the use of MS/MS-based techniques and comparing peptides with only L-amino acids to their DAACP epimers.[7,14−18] Recently, MALDI-TOF/TOF was used for the discovery of D-Leu in the peptide GH-2.[19] This study also established a stereosensitive fragmentation effect of D-amino acids from various amino acid classes on different peptide sequences, which can aid in the discovery of DAACPs through MALDI-TOF/TOF by observing the fragmentation ratios of specific peptides. Another way to facilitate the

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discovery is to use bioinformatics to find neuropeptides that are homologous to known DAACPs in related species.[7] What is needed is a set of measurement techniques capable of identifying DAACPs in a nontargeted manner, rather than targeting a suspected DAACP based on homology.

Other methods, such as those using chemical tags or enzymatic approaches, have been developed to detect DAACPs that are formed spontaneously, where racemization of a residue is a pathological or aging-related process.[18,20−22] Using photolabile tags combined with electron capture dissociation MS, D-Ala, D-Ser, and D-Asp were identified in peptides of lens proteins.[18] Isomers of L-Asp, including D-Asp, L-iso-Asp, and D-iso-Asp, were distinguished in lens protein peptides using differential enzyme digestion with endoprotease Asp-N, protein-L-isoaspartyl methyltransferase, and paenidase (D-aspartic acid endopeptidase).[21] Deuterium chloride-assisted acid hydrolysis was also used in conjunction with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) labeling to identify D-amino acid enantiomers of Ala, Asp, Glu, Pro, and Ser in peptides of ovalbumin.[22]

While these methods have increased the rate and sensitivity of identifying spontaneously forming DAACPs, the ability to identify endogenous DAACPs in a complex biological system remains elusive. Moreover, these approaches have been directed to specific proteins and also have involved the use of standards for initial inquiry, an undesirable option if hundreds of peptides are potential DAACPs. If the incorrect L-form of the peptide is tested, this potentially creates a gap in peptidomic studies, thereby underestimating the number of biologically active peptides in an organism. Since MS has been an enabling tool in modern peptidomic studies, there could be DAACPs hiding among the peptides already discovered.

We report a toolset, or discovery funnel, for the nontargeted detection of DAACPs, particularly neuropeptides, that takes the following issues into consideration. First, for neuropeptides (unlike spontaneously formed DAACPs and some toxins), isomerization occurs at the second position from the N-termini, except for the crustacean neurohormones.[23,24] Second, the variety of amino acid residues that are isomerized may include any of the chiral amino acids. When including toxin peptides, endogenous DAACPs have included D-amino acid enantiomers of aromatic amino acids, polar amino acids, and aliphatic amino acids.[5,6] Thus, a method to uncover DAACPs should take advantage of isomerization near the termini and be able to simultaneously assay the enantiomers of the 19 common chiral amino acids. Third, there are literally hundreds to thousands of brain peptides in most animal models. Thus, requiring peptide standards for the initial screening steps for DAACP discovery appears impractical and economically infeasible.

Specifically, the first stage of discovery takes advantage of the fact that DAACPs are resistant to peptidases such as aminopeptidase M (APM) (Figure 1, stage 1).[25] Next, candidate DAACPs are isolated and subjected to a chiral amino acid analysis, where deuterium chloride-assisted acid hydrolysis and labeling with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA) are used to assay the chirality of amino acid residues in peptides (Figure 1, stage 2).[26,27] On the basis of information from the chiral analysis, a short list of candidate peptides is determined. The peptides of interest are synthesized and their retention times compared to the native forms with liquid chromatography (LC) coupled to MS (Figure 1, stage 3). Some MS-based approaches for DAACP discovery[7,14−18] require a peptide standard for initial inquiry or targeting specific sequences, a limitation that is reduced in this method through the use of the enzyme APM. Synthetic peptides were also used in the current study for characterization of the bioactivity of DAACPs in a system-specific manner.

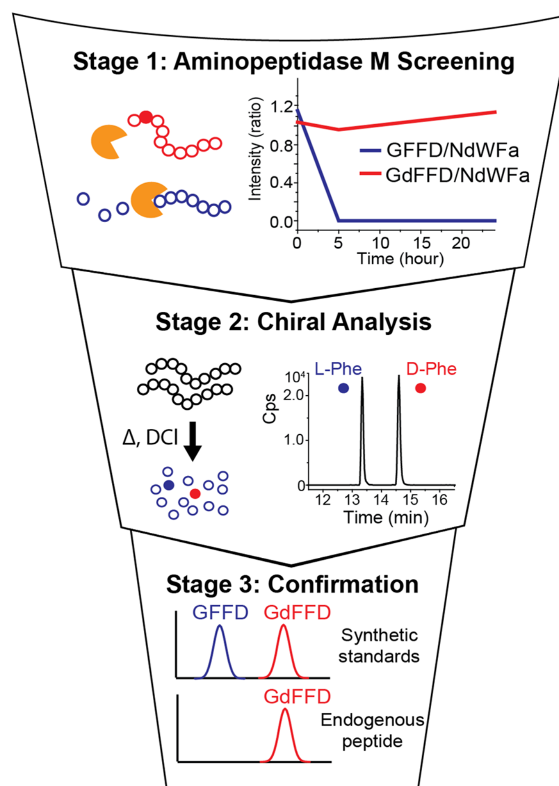


**Figure 1.** DAACP discovery funnel is capable of identifying DAACPs in three stages, as illustrated with GdFFD. In stage 1, MS-based detection of APM digestion is capable of identifying potential DAACPs in the screening phase of the discovery funnel. Here, GFFD, used as an example, is rapidly degraded after 5 h, whereas its DAACP counterpart, GdFFD, is not degraded after 24 h. Both are shown as a ratio to NdWFamide, a peptide that is known to resist degradation by APM. In stage 2, chiral analysis utilizes the MRM mode of MS to detect L- and D-amino acids in a peptide following acid hydrolysis and labeling. First, microwave-assisted vapor phase hydrolysis is carried out in DCl to break down peptides into their component amino acids. Importantly, DCl-based acid hydrolysis reduces detection of racemized residues in peptides. The amino acids are then labeled with Marfey's reagent, separated, and detected using a triple quadrupole mass spectrometer. The result of this step is outlined using GdFFD, where a D-Phe is detected. In stage 3, confirmation of DAACPs, peptides are synthesized with the suspected chirality at each position and then compared to the endogenous peptides. Here, the retention time of the endogenous peptide matches that of the GdFFD synthetic standard, confirming its presence as a DAACP.

We validated the approach using *Aplysia californica* because two D-amino acid-containing neuropeptides have already been identified in its nervous system: NdWFamide (Asn-D-Trp-Phe-$NH_2$) and GdFFD (Gly-D-Phe-Phe-Asp-OH).[7,28] More importantly, GdFFD originates from the *Aplysia* achatin-like neuropeptide precursor (apALNP), which produces additional peptides.[7] However, whether these additional neuropeptides are DAACPs has not been explored because of the lack of a systematic method for their study. Interestingly, in most known cases, a single neuropeptide precursor appears to contain a single DAACP.[7,10,13] However, there is at least one example



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where a single precursor produced two DAACPs.[11,12] Thus, we both validate our discovery funnel and then use it to determine whether there may be additional DAACPs in the *Aplysia* apALNP precursor. The value of using *Aplysia* as our model is that its neuropeptidome has been the subject of intensive study, and it is an excellent physiological model organism.[29−35]

The discovery funnel can be readily applied to investigating biological model systems in which DAACPs have not been discovered, uncovering potential DAACPs from any precursor with N-terminally modified DAACPs. Indeed, this approach has allowed us to identify three DAACPs from a single precursor in *Aplysia*, suggesting that more DAACPs in *Aplysia* remain to be discovered.

## ■ EXPERIMENTAL SECTION

**Animals.** *A. californica* (120−1200 g) were purchased from Marinus Scientific (Long Beach, CA) and the *Aplysia* Research Facility (Miami, FL). Animals were kept in an aquarium containing aerated and filtered artificial seawater (Instant Ocean, Aquarium Systems Inc., Mentor, OH) at ∼14 °C until used. Prior to dissection, animals were anesthetized by injection of isotonic 333 mM $MgCl_2$ (about 50% of body weight) into the body cavity.

**Reagents and Peptides.** All reagents were purchased from Sigma-Aldrich (St. Louis, MO) except where otherwise indicated. NdWFamide was synthesized by the Protein Sciences Facility of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana−Champaign. Other peptides (GFFamide, GdFFamide, GYFD, GdYFD, SYADSKDEESNAALSDFA, and SdYADSKDEESNAALSDFA) were synthesized by CPC Scientific (Sunnyvale, CA). Human angiotensin I acetate salt hydrate was purchased from Sigma-Aldrich.

**Cell Sampling of GdFFD Neurons.** In situ hybridization data were used as guidance for the isolation of neurons expressing the GFFD prohormone (apALNP).[7] The isolation and sampling procedures are described in the Supporting Information, Additional Methods.

**Peptide Extraction.** For initial screening of DAACPs, neuropeptide extracts from pedal ganglia were pooled from 24 euthanized animals (including discovery of GdYFD); follow-up studies for SdYADSKDEESNAALSDFAED involved 10 animals. Peptide extraction was performed in acidified acetone (40:6:1 acetone: $H_2O$:12 N HCl, v/v/v, acetone and HCl from Thermo Fisher Scientific, Waltham, MA), followed by centrifugation at 14 000 rpm, at 4 °C for 20 min (Centrifuge 5804R, Eppendorf, Westbury, NY). The supernatant was collected, dried (Savant Instruments, Farmingdale, NY), and reconstituted in 5% acetonitrile (ACN) in $H_2O$ with formic acid (FA) (95% $H_2O$, 5% $CH_3CN$, 0.1% FA v/v/v).

**APM Screening.** Potential DAACPs in *A. californica* were selected from a complex peptide mixture by their resistance to digestion by APM.[5,6,25] APM (EC 3.4.11.2, Merck, Darmstadt, Germany) reaction conditions were 25 mM Tris-HCl with 0.5 M NaCl, pH 7.5, reacted at 37 °C for 24−48 h, depending on the application (48 h ensures a more thorough digestion for peptide isolation). APM (at 60.6 U/mL) was added to be 0.6% of the final reaction volume. Included in the digestion experiments were a positive control, human angiotensin I, which is digested by APM, and a negative control, NdWFamide, which is not digested by APM.[25] Peptide content was compared between 0 and 24 h of the enzymatic digestion.

**Separations and Mass Spectrometry.** Several different instruments and procedures were used for separating the peptides, surveying the peptide contents, characterization of the peptides, and quantitation of their constituent amino acids as described below and in the Supporting Information.

*Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight (MALDI-TOF) and MALDI-TOF/TOF Analysis.* For APM screening, MALDI-TOF and MALDI-TOF/TOF analyses were performed using an ultrafleXtreme mass spectrometer (Bruker Daltonics, Billerica, MA). Mass spectra were analyzed using flexAnalysis 3.4 (Bruker Daltonics). The mass was calibrated using a peptide mixture of bradykinin 1−7 (757.4 $[M + H]^+$), angiotensin II (1046.5 $[M + H]^+$), angiotensin I (1296.7 $[M + H]^+$), substance P (1347.7 $[M + H]^+$), bombesin (1619.8 $[M + H]^+$), ACTH clip 1−17 (2093.1 $[M + H]^+$), ACTH clip 18−39 (2465.2 $[M + H]^+$), and somatostatin 28 (3147.5 $[M + H]^+$). MS analysis of each neuron was performed in positive-ion reflectron mode. The laser size was set to "ultra", ∼85 μm. Each MALDI-TOF spectrum was summed from 5000 laser shots, 500 Hz. Selected masses matching to apALNP peptides were chosen for follow-up MALDI-TOF/TOF analysis via the "LIFT" mode with argon as the collision gas.

*Sequencing of Peptides.* Peptide sequences were confirmed using an LC−MS set up comprised of an UltiMate 3000 RSLC system (Thermo Scientific Dionex, Sunnyvale, CA) coupled to an amaZon speed ETD mass spectrometer (Bruker Daltonics), by comparing MS/MS fragmentation (CID mode) to predicted fragment ions from Protein Prospector (UCSF, http://prospector.ucsf.edu) or fragment ion matching using BioTools 3.2 (Bruker Daltonics), fragment mass tolerance 0.2 Da, minimal intensity threshold 800.

*LC−Tandem Mass Spectrometry (MS/MS) for Structure Confirmation.* Solvents for LC−MS/MS were purchased from Thermo Fisher Scientific. To confirm that a peptide was a DAACP, peptide standards were synthesized and compared to the endogenous peptides. For this purpose, a hyphenated LC−MS/MS platform was employed; the LC instrument was an UltiMate 3000 RSLC (Thermo Scientific Dionex) and the mass spectrometer was an amaZon speed ETD (Bruker Daltonics). The column was a reversed-phase Acclaim PepMap100 C18 (3 μm particle size, 100 Å pore size, 300 μm internal diameter (ID) × 15 cm (length) Thermo Scientific Dionex) with the following: Buffer A: $H_2O$, 0.1% FA. Buffer B: ACN, 0.1% FA. Loading solvent: $H_2O$, 0.1% FA. Flow rate: 4 μL/min. For the determination of GdYFD, the gradient started with 4% B for 5 min, rising to 15% B in 3 min, then 50% B in 35 min, then 90% B in 1 min and held for 4 min. The gradient was dropped from 90% B to 4% B and held for 15 min at the end of the 60 min run. For the determination of SdYADSKDEESNAALSDFA, the gradient started with 5% B for 5 min, rising to 10% B in 3 min, then 45% B in 32 min, then 90% B in 1 min and held for 4 min. The gradient was dropped from 90% B to 5% B and held for 15 min at the end of the 60 min run.

*Chiral Analysis—Acid Hydrolysis and Amino Acid Labeling.* Acid hydrolysis was performed on each semipurified peptide fraction in the vapor phase at 150 °C for 30 min in a CEM Discover microwave (CEM, Mathews, NC).[36] The reactions were carried out in 6 M DCl in $D_2O$ with 0.1% phenol (added to prevent destruction of tryptophan and other amino acids).[37,38] Next, to enhance separation, L- and D-amino acids were derivatized with FDAA.[26,27] The amino acids from the acid hydrolysates were dried and reconstituted in 25 μL of 0.5 M $NaHCO_3$ (Thermo Fisher Scientific). For derivatization, 20 μL of 1 mg/mL FDAA dissolved in ACN were added to



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these amino acids and reacted at 60 °C for 3 h. Further details of this process are included in the Supporting Information.

*LC–MS/MS Multiple Reaction Monitoring (MRM) Conditions for the Detection of Labeled Amino Acids.* Solvents and solutes for chiral analysis were purchased from Thermo Fisher Scientific. The LC–MS/MS-MRM setup consisted of an EVOQ Elite Triple Quadrupole Mass Spectrometer equipped with the Advance UHPLC module (Bruker Daltonics). The column was a Kinetex 2.6 μm particle Phenyl-Hexyl column, 100 Å pore size, 100 mm (length) × 2.1 mm ID (Phenomenex, Torrance, CA). A binary solvent system was used (A, 25 mM ammonium formate; B, methanol; flow rate, 300 μL/min). The gradient started with 5% B for 2 min, rising to 15% B in 5 min, then 60% B in 5 min and held for 3 min, then 100% B in 3 min and held for 3 min. The gradient was dropped from 100% B to 5% B toward the end of the 24 min run. The EVOQ source parameters were as follows: HESI, spray voltage (±) 3500 V; cone temperature, 250 °C; cone gas flow, 20; heated probe temperature, 400 °C; probe gas flow, 45; nebulizer gas flow, 50; exhaust gas, Off. EVOQ MRM chromatograms were analyzed using Data Review 8.2 (Bruker Daltonics). A standard mixture of the achiral amino acid glycine and the L- and D-forms of the 19 common chiral amino acids was derivatized and used in the MRM to establish a table of retention times.

**Electrophysiology.** Intracellular and extracellular recordings of the physiological activity from *Aplysia* central nervous system preparations (either the cerebral and buccal ganglia, or the cerebral and pedal ganglia) were performed as described previously.[7,35,39,40] The ganglia were desheathed, transferred to a recording chamber containing 1.5 mL of artificial seawater (ASW) (460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM HEPES, pH 7.6), continuously perfused at 0.3 mL/min, and maintained at 14–17 °C. Peptides were dissolved in ASW immediately before each physiological test, and the peptide/ASW solution was perfused into the recording chamber. As noted in the Results and Discussion section, some experiments were also performed in high divalent (HiDi) saline (368 mM NaCl, 8 mM KCl, 13.8 mM $CaCl_2$, 115 mM $MgCl_2$, and 10 mM HEPES, pH 7.6), which increases the spiking threshold of neurons and therefore curtails polysynaptic influences. Intracellular recordings were obtained using 5–10 MΩ sharp microelectrodes filled with 0.6 M $K_2SO_4$ plus 60 mM KCl.

To test the peptide effects on the feeding circuit, we included cerebral and buccal ganglia. The buccal ganglion innervates the feeding organ (radula). Feeding motor programs were monitored by cyclic bursts in the I2 nerve of the buccal ganglion.[41,42] To test the peptide effects on the locomotor circuit, we included cerebral and pedal ganglia. The pedal ganglion innervates the foot. Locomotor programs were monitored by cyclic bursts in the parapedal commissural nerve of the pedal ganglion.[43−45] Electrophysiological recordings were digitized on line using AxoScope software (version 9, Molecular Devices, LLC, Sunnyvale, CA) and plotted with CorelDRAW (version 11, Corel Corporation, Ottawa, ON, Canada). Bar graphs were plotted using SigmaPlot (version 10, Systat, San Jose, CA). Data are expressed as mean ± SE. All statistical tests (e.g., repeated measures one-way analysis of variance) were performed using Prism (version 5, GraphPad Software, La Jolla, CA). When the data showed significant effects in analysis of variance, further individual comparisons were performed with Bonferroni's correction.

**Behavioral Studies.** Peptide effects on locomotor behavior were examined with the method and software described recently.[40] Each video recording session used to calculate the locomotor path lasted for 25 min. One notable difference with the previous work was that we used a round behavior box (diameter, ~43 cm and height, ~18 cm) instead of the square behavioral box used previously (see Figure S8A). We have not observed obvious differences in locomotor behavior between the two boxes.

## RESULTS AND DISCUSSION

**Discovery Funnel.** The three stages of the discovery funnel involve screening, chiral analysis, and confirmation (Figure 1). The goal of the first step is to identify suspected DAACPs for follow-up studies, where it is acceptable for the first step to have false positives, but it cannot require peptide standards (as it is not practical to create standards for all possible DAACPs). Thus, we used enzymatic screening of peptides with APM (Figure 1, stage 1). DAACPs are expected to resist degradation by peptidases, and indeed APM has been previously shown to degrade peptides containing only L-amino acids at a higher rate than DAACPs.[25] We observed a distinct difference in the rates at which the all-L-peptides and their corresponding DAACPs were degraded, as demonstrated with GFFD and GdFFD. GFFD was rapidly degraded within 5 h, whereas GdFFD was virtually intact after 24 h (Figure 1, stage 1), verifying that APM is effective for the screening of potential DAACPs.

Identifying potential DAACPs by resistance to APM digestion generates false positives because in addition to isomerization, specific N-terminal residues and select PTMs increase the resistance to APM digestion. For example, peptides with proline at the N-terminus can resist APM digestion.[25] In addition, although every D-amino acid-containing neuropeptide discovered so far has had isomerization occur at the second residue from the N-terminus, this does not preclude the possibility of isomerization occurring at a different residue near the N-terminus.[5,6] Ultimately, the limitations of the first step make the subsequent steps in our method necessary.

The second step is designed to confirm the presence of D-amino acids in suspected peptides and so reduces the number of false positives. First, acid hydrolysis is performed in the vapor phase, which hydrolyzes the peptide into its component amino acids.[36] While there are several possible chromatographic methods, including chiral LC, we derivatized L- and D-amino acids with Marfey's reagent to ensure good separation of all chiral amino acids in one analysis (Figure S1 and Table S1),[26,27] and detected them with a triple quadrupole mass spectrometer.

Interestingly, following DCl-based acid hydrolysis and analysis, labeled tyrosine (Tyr) was seen to have a different molecular weight than expected: two more Daltons than the label plus Tyr (Figure S2).[37,46] For more details, see the Supporting Information (page S-4, the use of $DCl/D_2O$ for acid hydrolysis and its effect on the detection of labeled amino acids). This mass shift raises the possibility of other unknown changes during DCl hydrolysis that can be observed with MS, which may account for losses of intensity of certain species. DCl hydrolysis may not be able to analyze all sequences, as there are amino acids that are destroyed (or modified) by acid hydrolysis.[47] Short, high temperature vapor-phase hydrolysis mitigates this, but with low enough levels of neuropeptide, this may not always be sufficient. Also, certain sequences do not hydrolyze readily, such as chains of some aliphatic amino acids.



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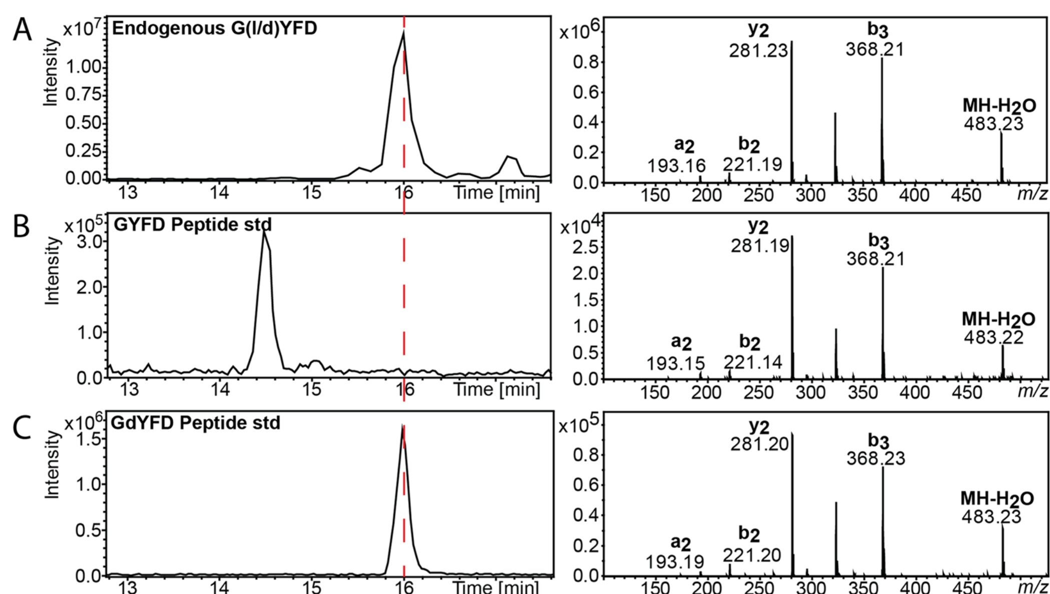


**Figure 2.** LC−MS/MS characterization of GdYFD, which is confirmed by comparing to the retention time of standards. (A) Left, LC−MS (base peak chromatogram) trace of endogenous GYFD after 48 h of APM digestion, with a retention time of 15.9 min. Right, the MS/MS fragmentation with fragment assignments is shown. (B) Left, an LC−MS trace of the all-L-amino acid synthetic GYFD, with a retention time of 14.5 min. Right, the MS/MS fragmentation with fragment assignments. (C) Left, an LC−MS trace of the synthetic DAACP GdYFD, with a retention time of 15.9 min. Right, MS/MS fragmentation with fragment assignments is shown. The matching retention time of the synthetic GdYFD standard with the endogenous GYFD demonstrates that the sequence for the endogenous peptide is in fact GdYFD.

The final step of the discovery funnel validates the DAACP and involves synthesizing the putative peptide in both the all L-form and the D-form. One way to verify structure is to compare LC retention times between endogenous and synthetic peptides. In combination with high quality MS/MS data,[15] a matching retention time from the LC separation for the native peptide and a synthetic DAACP is sufficient to confirm the identity of a DAACP (Figure 1, stage 3).[5,6]

**Screening: Peptides from apALNP Resist Digestion to Aminopeptidase M.** apALNP is predicted to produce several peptides and in situ hybridization has indicated that apALNP is localized in a cluster of neurons in the pedal ganglia.[7] This led us to consider that further DAACP discovery could be facilitated by targeting the pedal ganglia, as the isomerizing enzyme is inferred to be expressed by some neurons of this ganglia (the enzyme is currently unknown). Extracts from pedal ganglia were subjected to 48 h of APM digestion. After digestion, several peptides were seen to resist degradation, including GYFD (Figure 2) and SYADSKDEESNAALSDFA (Figure S3).

Both of these peptides are derived from apALNP. Considering that GdFFD is also present on this prohormone, these were promising hits that demonstrated the potential ability of this approach to discover novel DAACPs. GFFD was also detected after APM digestion, but was not explored further as it is a previously characterized DAACP (data not shown).[7,15] GYFD and GFFD are also homologous and differ only in the second residue. The isomerized residues Tyr and phenylalanine (Phe) are both aromatic amino acids. Thus, it would not be surprising that GYFD could also exist as GdYFD. Importantly, the peptide was not targeted for its homology, but was chosen here for further studies based on APM screening. While this peptide was not identified in MALDI-TOF profiling of the neurons expressing apALNP previously,[7] here we detected GYFD using MALDI-TOF/TOF from isolated neurons (Figure S4).

GFFD and GYFD are similar, but there are several longer peptides in apALNP.[7] The other observed peptide, SYADSKDEESNAALSDFAED, bears some sequence homology in its N-terminus to GYFD. Interestingly, a form of this peptide truncated by two C-terminal amino acids (SYADSKDEESNAALSDFA) was also seen to resist digestion by APM after 48 h (Figure S3) and was identified in previous work.[7] In addition, GFFamide was detected, which has not previously been reported as an observed peptide from apALNP. However, this peptide was digested by APM (Figure S5), suggesting it is not a DAACP.

**Chiral Analysis: Detection of D-Amino Acids in Peptides Resistant to Digestion.** Endogenous GYFD and SYADSKDEESNAALSDFA were purified to relative homogeneity (Figures 2 and S6), seen to contain a D-Tyr following chiral analysis (Figure 3), and confirmed with synthetic GdYFD and SdYADSKDEESNAALSDFA (Figures 2 and 4). Thus, we have added two new DAACPs to the products from apALNP. In Figure 3, the L-amino acids suggest the presence of additional peptides in the fraction containing L-amino acids. These may be low levels of the epimeric peptide, which can be somewhat difficult to separate through fraction collection. While the epimeric peptides were not detected with LC−MS, the TQ-MS channels are especially sensitive to several amino acids over others, based on fragmentation energies (see Figure S1; equimolar D- and L-tyrosine favors detection of L-tyrosine, with sometimes dramatic effects, as seen in detection of L- and D-threonine).

The discovery of GdYFD and SdYADSKDEESNAALSDFA as DAACPs has several interesting implications for DAACP formation. This is the first instance of an endogenous DAACP containing D-Tyr. In contrast, the peptide GFFamide is not isomerized; as both GFFamide and GFFD have Phe in the second position, and yet GdFFamide has not been detected, perhaps the ability of the enzyme to isomerize the peptide involves the flanking sequences, as has been noted in the frog


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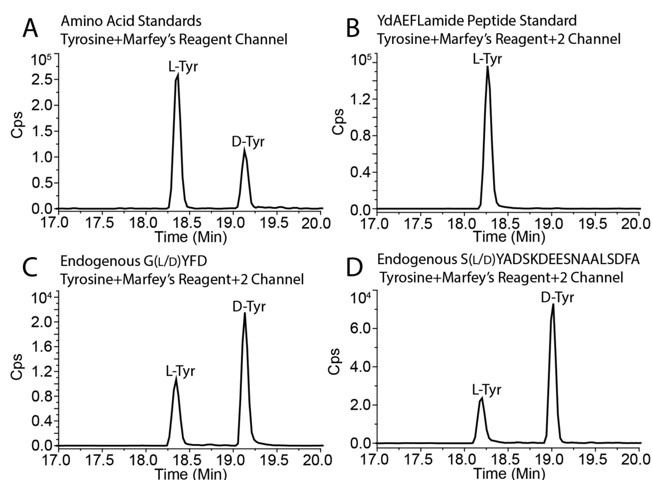


**Figure 3.** Chiral analysis and LC−electrospray ionization MRM detection of endogenous *A. californica* peptide fractions containing GYFD and SYADSKDEESNAALSDFA are seen to have a D-Tyr. (A) Retention times of labeled standard amino acids. (B) Chiral analysis of YdAEFLamide peptide standard, to demonstrate the retention time of L-Tyr only. (C) An endogenous peptide fraction containing GYFD is seen to contain a D-Tyr. This suggests that the endogenous peptide exists as GdYFD. (D) An endogenous peptide fraction containing SYADSKDEESNAALSDFA is seen to contain a D-Tyr. This suggests that the endogenous peptide exists as SdYADSKDEESNAALSDFA.

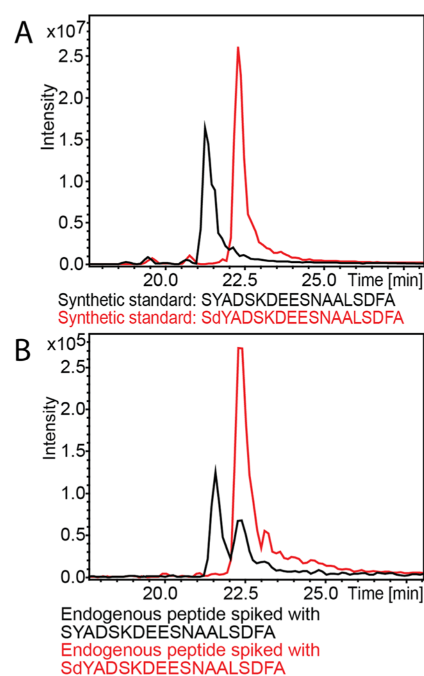


**Figure 4.** Through spiking of synthetic standards, endogenous SYADSKDEESNAALSDFA is seen to exist as a DAACP, SdYADSKDEESNAALSDFA, in the *A. californica* nervous system. (A) LC−MS (base peak chromatogram) of synthetic peptides of SYADSKDEESNAALSDFA (21.6 min) and SdYADSKDEESNAALSDFA (22.4 min) demonstrates that both peptides separate. (B) LC−MS (base peak chromatogram) of the endogenous SYADSKDEESNAALSDFA spiked with either synthetic SYADSKDEESNAALSDFA (black trace) or SdYADSKDEESNAALSDFA (red trace). Black trace: Spiking SYADSKDEESNAALSDFA results in two peaks corresponding to 21.6 and 22.4 min; the endogenous peptide, the second peak, matches the retention time of the DAACP. This is confirmed in the red trace where spiking SdYADSKDEESNAALSDFA results in one larger peak at 22.4 min, the additive intensity of the endogenous peptide and the standard. This confirms that the endogenous peptide is in fact SdYADSKDEESNAALSDFA.

enzyme.[48] However, GYFD, GFFD, and NWFamide are sequences that are recognized by the isomerase, while GFFamide is not. Perhaps GdFFamide does not extract under the protocols used, or perhaps it was simply below the limit of detection; therefore, the presence of GdFFamide cannot be precluded.

Our results are defining the endogenous substrates used by a yet unknown isomerizing enzyme in *Aplysia*. It appears to be flexible in regards to sequence and also length. After all, GdFFD is only 4 amino acids versus the 20 amino acids of SdYADSKDEESNAALSDFAED. This is consistent with studies of the frog enzyme: substrate specificity studies showed the frog isomerase was able to act upon sequences of only the N-terminus of the peptide.[48]

**GdYFD is Bioactive in the Feeding and Locomotor Circuits of *A. californica,* whereas SdYADSKDEESNAALSDFA is Not.** Previous work has shown that GdFFD is bioactive in both *Aplysia* feeding[7] and locomotor[40] circuits, whereas the all L-amino acid epimer, GFFD is not. We therefore tested whether GdYFD and SdYADSKDEESNAALSDFA may also be bioactive in the feeding and locomotor circuits. Only GdYFD was found to be bioactive in the feeding (Figure 5) and locomotor networks (Figures S7 and S8). (For further description of these results, see the page S-5 of the Supporting Information).

The physiological data showed that of the two new DAACPs identified from apALNP, only GdYFD is bioactive, whereas the other is not. In addition, for GdYFD, similar to our previous study with GdFFD,[7] only the DAACP, that is, GdYFD, is active, whereas GYFD is not. Finally, our data also suggest that the overall effects of GdYFD and GdFFD in both the feeding and locomotor circuits are similar, but the effects of GdYFD may be weaker than GdFFD, particularly the effects on CBI-2 programs (Figure 5F) and on fictive locomotor programs (Figure S7). Given that each apALNP produces three copies of GdFFD, whereas it only produces one copy of GdYFD, the main bioactivity of the peptides from apALNP likely originates from GdFFD. Nonetheless, our discovery funnel does provide an effective means to identify functionally important DAACPs from multiple peptides originating from a single precursor.

## ■ CONCLUSIONS

We developed and validated a series of protocols we term a DAACP discovery funnel, and used it to identify two novel D-amino acid-containing neuropeptides in the nervous system of *A. californica*: GdYFD and SdYADSKDEESNAALSDFA. This method is nontargeted and does not require the use of bioactivity to identify novel DAACPs. APM digestion takes advantage of a common property of neuropeptide DAACPs: that the modification is found near the N-terminus.[25] Although the individual methods have been used in various contexts, this is the first time that these methods have been combined into a cohesive workflow and optimized for the purpose of endogenous peptide discovery.

We improved every aspect of these methods, most notably, using triple quadrupole MS for the sensitive detection of low levels of D-amino acids, as is necessary for low levels of neuropeptides. Because we separate the L- and D-forms into different fractions, the ratio of the L-D forms is less important, but the absolute amount of the DAACP must be high enough


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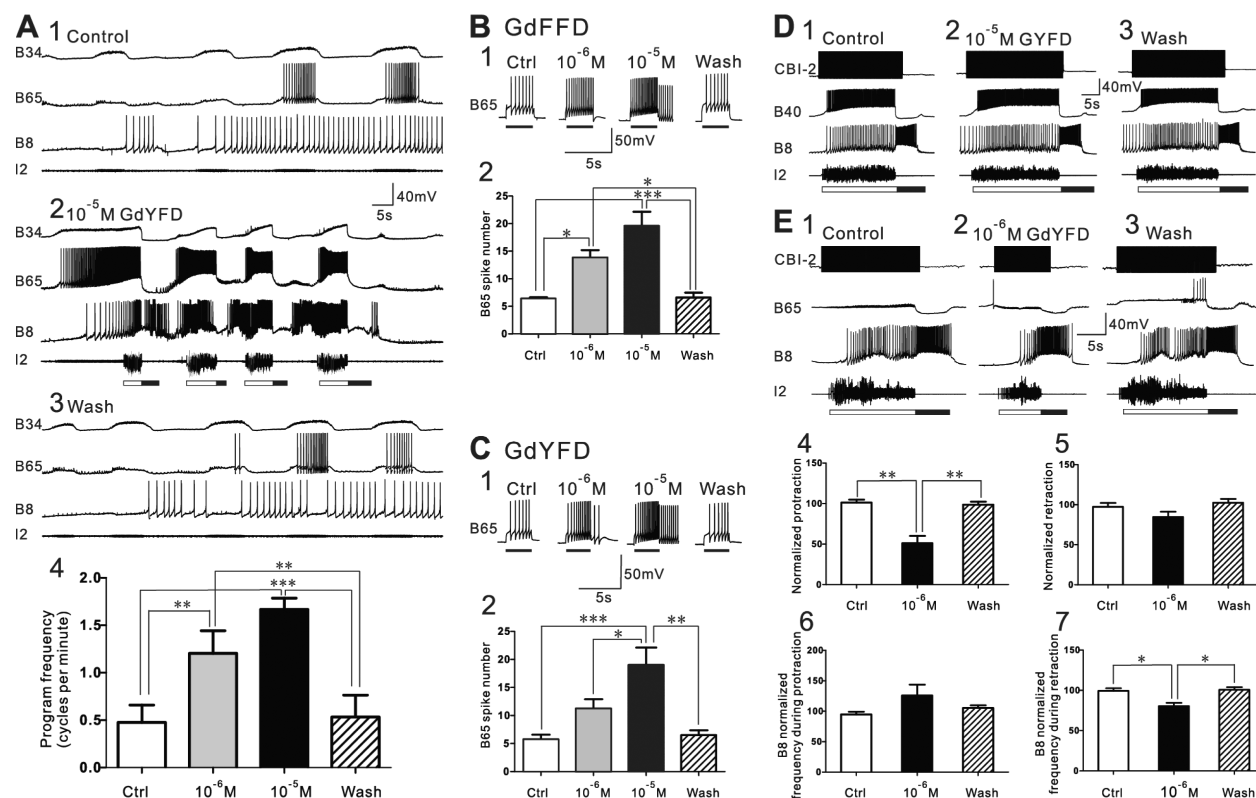


**Figure 5.** GdYFD was bioactive whereas GYFD was not in the *A. californica* feeding circuit (with cerebral and buccal ganglia). (A) Perfusion of GdYFD increased activity in the cerebral and buccal ganglia (A2) in a concentration-dependent manner (A4, group data). Protraction, open bar; retraction, filled bar. Radula closer B8 was mostly active during protraction, so the programs were egestive. Note that B65 is strongly active in A2. (B, C) Both GdFFD (B) and GdYFD (C) increased B65 excitability in a concentration-dependent manner. Bars in B1 and C1 indicate current injections in B65. (B2 and C2) Group data. (D) GYFD at $10^{-5}$ M had no effects on motor programs elicited by stimulation of CBI-2 (9 Hz) (D2). (E) GdYFD at $10^{-6}$ M made CBI-2-elicited motor programs less ingestive (E2): that is, it reduced protraction duration (E4) and reduced B8 activity during retraction (E7). Experiments were performed in ASW, except B and C, which were performed in HiDi saline. Bonferroni post hoc test is as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

to characterize with MS. We do note a limitation of this approach: it assumes that the modification occurs near the N-terminus; if the modification occurs near the C-terminus, our method could miss it. Carboxypeptidases may be studied in the future to see if they can achieve the kind of selectivity that is seen with APM. Perhaps APM may yet be shown to have the capability to degrade certain DAACP sequences more rapidly than anticipated, leading to false negatives.

Regardless, using this approach, further DAACP discovery efforts are underway in other models, including vertebrates. We are actively determining the extent to which post-translational amino acid isomerization is found in the nervous systems of animals. In *Aplysia*, novel peptide precursors have recently been identified.[49−52] Our approach can determine if there are multiple DAACPs from a single precursor, especially those peptides with little or no bioactivity. In mammals, although most bioactive DAACPs are exogenous, several are reported to have effects; for example, dermorphin acts upon opioid receptors and has a higher potency than morphine.[53,54] Discovery of new DAACPs may provide novel biologics for therapeutic use, and should help in the hunt for the enzymes responsible for this modification.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03658.

Additional experimental details, the use of $DCl/D_2O$ for acid hydrolysis and its effect on the detection of labeled amino acids, analysis of GdYFD and SdYADSKDEE-SNAALSDFA activity, Table S1, and Figures S1−S8 (PDF)

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### Notes

The authors declare no competing financial interest.

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