A D-amino acid-containing neuropeptide discovery funnel

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Additional Experimental Details

Isolation of GdFFD neurons

The pedal ganglia were dissected and incubated in 1% (w/v) protease (type IX, Bacterial; Sigma P8811) (Sigma-Aldrich, St. Louis, MO, USA) in artificial sea water (ASW: 460 mM NaCl, 10 mM KCl, 10 mM CaCl₂, 22 mM MgCl₂, 26 mM MgSO₄, 2.5 mM NaHCO₃, and 10 mM HEPES, pH 7.7) supplemented with antibiotics (penicillin G, gentamycin, and streptomycin) and maintained at 34 °C for 30 min to loosen the connective tissue sheath. Following a 1 h rinse in ASW with antibiotics to remove the bulk of the protease, the ganglia were stretched onto a silicone elastomer (Sylgard, Dow Corning, Midland, MI, USA) layer in a Petri dish containing 3–4 mL of the ASW/antibiotic medium using 0.15 mm diameter tungsten needles (WPI, Sarasota, FL, USA); the connective tissue was surgically removed to expose neurons.

Clusters of GFFD-expressing neurons were identified according to their relative position to prominent morphological landmarks in the ganglion and excised with tungsten needles. The clusters were quickly transferred via a plastic micropipette filled with Milli-Q water to remove excess salts and deposited onto the MALDI target. Excess liquid was aspirated from the target, and spot-to-spot cell transfers were performed for sampling of individual neurons as described previously. Matrix consisting of 0.5 μL of 2,5-dihydroxybenzoic acid (Sigma-Aldrich), 50 mg/mL in 50% acetone (Thermo Fisher Scientific, Waltham, MA, USA) was added to each spot. An equal volume of matrix and 1 mg/mL synthetic peptides dissolved in water were mixed and deposited on the target.

LC isolation of potential DAACPs

Separation methods were optimized based on the characteristics of a particular peptide and its co-eluents. All LC-grade solvents were purchased from Thermo Fisher Scientific.

For endogenous GYFD, two stages of analytic scale LC were employed using a Breeze 2 HPLC instrument (Waters, Milford, MA, USA). In the first stage, a reversed-phase Waters XSELECT CSH C18 column (3.5 μm particle size, 4.6 mm inner diameter (ID) × 150 mm) was used with the following: Buffer A: H₂O, 0.1% formic acid (FA). Buffer B: methanol, 0.1% FA. Flow rate: 500 μL/min. The gradient started with 5% B for 3 min, rising to 15% B in 2 min, then 50% B in 50 min, then 80% B in 1 min and held for 5 min. The gradient was dropped from 80% B to 5% B at the end of the 60-min run. In the second stage, Buffer A: H₂O, 0.1% FA. Buffer B: acetonitrile (ACN), 0.1% FA. Flow rate 500 μL/min. The gradient started with 5% B for 1 min, rising to 30% B in 5 min, then 50% B in 40 min, then 80% B in 4 min and held for 2 min. The gradient was dropped from 80% B to 5% B at the end of the 55-min run.

For endogenous SYADSKDEESNAALSDFA, two stages of analytic scale LC using the Breeze 2 were employed. In the first stage, a reversed-phase Waters XSELECT CSH C18 column (3.5 μm particle size, 4.6 mm ID × 150 mm) was used with the following: Buffer A: H₂O, 0.1% FA. Buffer B: methanol, 0.1% FA. Flow rate: 500 μL/min. The gradient started with 5% B for 5 min, rising to 10% B in 5 min, then 70% B in 80 min, then 80% B in 10 min and held for 8 min. The gradient was dropped from 80% B to 5% B at the end of the 110-min run. In the second stage, a reversed-phase Waters Atlantis T3 column (5 μm particle size, 2.1 mm ID × 150 mm) was used with the following: Buffer A: H₂O.
Buffer B: ACN. Flow rate 400 µL/min. The gradient started with 2% B for 5 min, rising to 5% B in 5 min, then 70% B in 60 min, then 90% B in 15 min and held for 5 min. The gradient was dropped from 90% B to 2% B at the end of the 100-min run.

**MS and MS/MS parameters for LC–tandem mass spectrometry (MS/MS)**

The mass spectrometer used was an amaZon speed ETD (Bruker Daltonics, Billerica, MA). The MS data was acquired in positive ion/enhanced resolution mode. The mass range scanned was 300 m/z to 2800 m/z. The MS/MS data was acquired in a data-dependent manner, where the three most intense precursor ions were selected for MS/MS fragmentation followed by their active exclusion from MS/MS for 0.50 min after 2 spectra each.
The use of DCl/D₂O for acid hydrolysis and its effect on the detection of labeled amino acids

Many peptides are present at low levels in biological samples, making MRM useful for detecting the peptide’s constituent D-amino acids after acid hydrolysis because of MRM’s excellent signal-to-noise ratio. However, during acid hydrolysis a low rate of amino acid racemization occurs. This produces D-amino acids,³ which may lead to the incorrect conclusion of D-amino acids being present in the original sample. As a solution to this problem, the reactions were carried out in 6 M DCl in D₂O with 0.1% phenol (added to prevent destruction of tryptophan and other amino acids).⁴,⁵ Under these conditions, digestion-induced racemization extracts a deuteron from the solution, causing a shift in mass of 1 Dalton compared to an amino acid initially in the D-form. Here, we use MRM to monitor parent ions based on their molecular weight within an m/z ± 0.35 window. As a result, a shift of m/z +1 causes acid-hydrolysis-induced racemization to be undetected in the same channel as the endogenously present D-amino amino acids. In addition, MRM measures the labeled L- and D-amino acid enantiomers in different channels in a single analysis step (Figure 1, Stage 2).⁶⁻¹⁴

An interesting observation was found for Tyrosine (Tyr), which was seen to have a different molecular weight than expected by only Tyr + label. This observation did not affect the retention time of the labeled L- or D-Tyr in the MRM that did not undergo hydrolysis (Figure S2). Tyr can be substituted with chloride during acid hydrolysis; rapid vapor phase hydrolysis is meant to help prevent this phenomenon. A shift of +1 Dalton may be expected due to random racemization during deuterium-assisted acid hydrolysis. However, no racemized residue was detected in the +2 Dalton channel, so it is unlikely that the deuteron is on the alpha carbon of the amino acid (which would result in racemization). In addition, these amino acids were in solutions that were not deuterated following deuterium hydrolysis, so the deuteron is not the result of readily exchangeable hydrogens. Rather, the deuteron may result from a substitution reaction in the ortho position on both sides, as has been noted with chloride substitutions on Tyr while using acid chlorides. No shift was observed in Tyr’s molecular weight following HCl-based hydrolysis (Figure S2).

For Marfey’s Reagent-based labeling, the L-form was observed to always elute before the D-form, presumably due to the fixed structure of the tag where the L-amino acid’s side chain will be in a trans formation with the –CH₃ of L-alanine amide (already on the tag) compared to the plane of the benzene ring on the derivatization reagent, whereas the D-amino acid’s side chain will be in a cis formation with the –CH₃ of L-alanine amide compared to the plane of the benzene ring on the derivatization reagent.¹⁵,¹⁶
In the first set of experiments with cerebral and buccal ganglia, we examined if the peptides could elicit feeding motor programs. We found that under resting conditions (Figure 5A1), there are few spontaneous motor programs (note the absence of bursts in I2). However, upon perfusion of both $10^{-6}$ M and $10^{-5}$ M GdYFD, spontaneous motor programs occurred at an increased rate in a concentration-dependent manner (Figure 5A2, A4, $F_{3,21} = 32.81$, $p < 0.0001$, $n = 8$). Interestingly, the motor programs are mostly egestive as radula closer B8 fired primarily during protraction (open bar) (Figure 5A2). In contrast, GYFD had no obvious effects ($n = 3$). In addition, neither SdYADSKDEESNAALSDFA ($n = 3$), nor its all L-amino acid epimer ($n = 3$), had any observable effects on the activity of the cerebral and buccal ganglia.

Previously, we have shown that GdFFD also promotes egestive programs. Consistently, B65, an interneuron that promotes the generation of egestive programs, was strongly active during the spontaneously generated programs in the presence of both GdFFD (see Figure 8B of Bai et al. 2013) and GdYFD (Figure 5A2). To determine if these two peptides might act directly on B65 to promote the generation of egestive programs, we examined if both peptides affected B65 excitability. For this, we applied 3-s current pulses every 30 s in B65 in high-divalent saline, which was used to curtail polysynaptic connections. We then perfused $10^{-6}$ M and $10^{-5}$ M peptides, and found that both GdFFD (Figure 5B, $F_{3,12} = 21$, $p < 0.001$, $n = 5$) and GdYFD (Figure 5C, $F_{3,9} = 17.63$, $p < 0.001$, $n = 4$) significantly enhanced B65 excitability in a concentration-dependent manner.

Given that GdYFD promoted egestive programs, we tested if GdYFD can also influence motor programs evoked by a command-like interneuron, CBI-2. We elicited feeding motor programs by stimulating CBI-2 at 8–10 Hz, with inter-stimulation intervals of 1.5 min. Under control conditions, the programs were ingestive, as radula closer B8 fired mostly during retraction (filled bar) rather than during protraction (open bar) (Figure 5D1, E1). Perfusion of GYFD at a concentration of up to $10^{-5}$ M had no obvious effects on the programs (Figure 5D2, $n = 4$). In contrast, perfusion of $10^{-6}$ M GdYFD (Figure 5E2) shortened protraction duration (Figure 5E4, $F_{2,6} = 19.94$, $p < 0.01$, $n = 4$; protraction value at 100%: 22.34 ± 4.92 s). Perfusion of $10^{-6}$ M GdYFD also slightly shortened retraction duration, but the effects were not statistically significant (Figure 5E5, $F_{2,6} = 2.35$, $p > 0.05$; retraction value at 100%: 7.38 ± 1.06 s). In addition, the programs appeared to be less ingestive because B8 frequency during protraction increased somewhat (although not statistically significant, Figure 5E6, $F_{2,6} = 1.97$, $p > 0.05$; protraction frequency value at 100%: 3.88 ± 0.70 Hz), and B8 frequency during retraction decreased (Figure 5E7, $F_{2,6} = 7.89$, $p < 0.05$; retraction frequency value at 100%: 10.39 ± 0.51 Hz).

We also tested the effects of GdYFD on the locomotor network. First, we examined if the peptides could elicit activity in the parapedal commissural nerve (PPCN) of the isolated nervous systems of cerebral and pedal ganglia. We found that perfusion of $10^{-6}$ M and $10^{-5}$ M GYFD did not change PPCN activity in the pedal ganglion (Figure S7A, $F_{3,9} = 1.85$, $p > 0.05$, $n = 4$), whereas perfusion of $10^{-6}$ M and $10^{-5}$ M GdYFD (Figure S7B4) significantly increased activity (Figure S7B5, $F_{3,15} = 17.77$, $p < 0.0001$, $n = 6$). We also found that perfusion of $10^{-6}$ M and $10^{-5}$ M GdYFD significantly increased frequency within bursts (Figure S7B6, $F_{3,15} = 12.38$, $p < 0.001$, $n = 6$), and frequency between bursts (Figure S7B7, $F_{3,15} = 10.1$, $p < 0.001$, $n = 6$). These effects are similar to the effects of GdFFD described earlier. In addition,
neither SdYADSKDEESNAALSDFA (n = 3) nor its all L-amino acid epimer (n = 3) had any observable effects on the activity of cerebral and pedal ganglia.

Because we showed that the increase in the frequency between bursts in GdFFD appeared to be detrimental to locomotor behavior, which acted to reduce locomotion in intact animals,\textsuperscript{19} we also tested the effects of GdYFD on the locomotor behavior. Initially we used 10\textsuperscript{-7} M GdYFD and found that it reduced locomotor velocity but the effects were not statistically significant. We then increased the concentration to 10\textsuperscript{-6} M GdYFD, and found that it reduced locomotor velocity significantly (Figure S7). Using normalized data (natural logarithm of velocity) as described in the previous study,\textsuperscript{19} we found that whereas injection of ASW had no effects on locomotion (natural logarithm of velocity, before: 8.53 ±0.26; after: 8.28 ±0.47; two tailed t-test, p > 0.05, n = 4), injection of GdYFD significantly reduced locomotion (natural logarithm of velocity, before: 8.52 ±0.42; after: 5.95 ±0.21; two tailed t-test, p < 0.05, n = 4).
Table S1. Triple quadrupole MS conditions for labeled amino acids.

| Amino acid      | Parent Ion | Parent Resolution | Collision Energy (V) | Fragment Ion | Fragment Resolution | Time (ms) | Mode |
|-----------------|------------|-------------------|----------------------|--------------|---------------------|-----------|------|
| Alanine         | 340        | Unit (0.7)        | 14                   | 278.1        | Standard (2.0)      | 50        | -    |
| Arginine        | 426.9      | Unit (0.7)        | 10                   | 70.1         | Standard (2.0)      | 50        | +    |
| Asparagine      | 382.9      | Unit (0.7)        | 33                   | 175.9        | Standard (2.0)      | 50        | -    |
| Aspartatic Acid | 384        | Unit (0.7)        | 24                   | 267.9        | Standard (2.0)      | 50        | -    |
| Cysteine        | 372.1      | Unit (0.7)        | 21                   | 284.9        | Standard (2.0)      | 50        | -    |
| Glutamic Acid   | 398        | Unit (0.7)        | 24                   | 201.98       | Standard (2.0)      | 50        | -    |
| Glutamine       | 396.9      | Unit (0.7)        | 20                   | 353          | Standard (2.0)      | 50        | -    |
| Glycine         | 326.1      | Unit (0.7)        | 32                   | 162          | Standard (2.0)      | 50        | -    |
| Histidine       | 658.2      | Unit (0.7)        | 30                   | 549.1        | Standard (2.0)      | 50        | -    |
| Isoleucine/Leucine | 382   | Unit (0.7)        | 18                   | 319.8        | Standard (2.0)      | 50        | -    |
| Lysine          | 649.1      | Unit (0.7)        | 36                   | 479.1        | Standard (2.0)      | 50        | -    |
| Methionine      | 400        | Unit (0.7)        | 17                   | 337.9        | Standard (2.0)      | 50        | -    |
| Phenylalanine   | 416        | Unit (0.7)        | 20                   | 337.1        | Standard (2.0)      | 50        | -    |
| Proline         | 365.9      | Unit (0.7)        | 15                   | 321.9        | Standard (2.0)      | 50        | -    |
| Serine          | 355.9      | Unit (0.7)        | 16                   | 263.9        | Standard (2.0)      | 50        | -    |
| Threonine       | 370        | Unit (0.7)        | 17                   | 263.9        | Standard (2.0)      | 50        | -    |
| Tryptophan      | 457.1      | Unit (0.7)        | 10                   | 188          | Standard (2.0)      | 50        | +    |
| Tyrosine        | 684.1      | Unit (0.7)        | 28                   | 351.95       | Standard (2.0)      | 50        | -    |
| Tyrosine(+2)    | 686.1      | Unit (0.7)        | 27                   | 353.9        | Standard (2.0)      | 50        | -    |
| Valine          | 367.9      | Unit (0.7)        | 15                   | 306          | Standard (2.0)      | 50        | -    |
Figure S1. Labeled amino acid standard separation using LC–MS with a triple quadrupole mass spectrometer. Labeling with Marfey’s Reagent leads to separation of all the common chiral amino acids. Their separation allows for identification of D-amino acids in a peptide, either through retention-time matching or through spiking.
Figure S2. Tyrosine (Tyr) had an unexpected molecular weight following DCI-assisted acid hydrolysis. DCI-assisted acid hydrolysis of Tyr (red trace) versus HCl-assisted hydrolysis (black trace). Tyr reveals that DCI hydrolysis leads to an unracemized (but different molecular weight) species. The amino acid used was L-Tyr and the peaks match the retention time of L-Tyr + label with no acid hydrolysis (see Figure S1). (A) The expected molecular weight for Tyr + label yields a large peak in HCl-assisted hydrolysis, but almost no peak in DCI-assisted hydrolysis. (B) In the channel for Tyr + label + 1 Da, there is still almost no molecular species seen in DCI-assisted hydrolysis, even though a single deuteron would lead to Tyr being detected in this channel. There is still a substantial peak for HCl-assisted hydrolysis of Tyr, but this can result from naturally occurring carbon and hydrogen isotopes. (C) In the channel for Tyr + label + 2Da, there is a large peak in the DCI-assisted hydrolysis, which accounts for the majority of this species. This is an unracemized (L-Tyr) species. Meanwhile, little (< 5% of intensity of original species on the left) is detected for Tyr in HCl-assisted hydrolysis.
Figure S3. *A. californica* endogenous peptide digestions lead to the identification of SYADSKDEESNAALSDFA as a potential DAACP. Top: LC–MS trace of an *A. californica* peptide fraction after 48 hours of APM digestion. *Black trace*: base peak chromatogram of the peptide fraction. *Red trace*: extracted ion chromatogram of m/z 960.4, which with z = +2 matches SYADSKDEESNAALSDFA. Bottom: MS/MS spectrum of SYADSKDEESNAALSDFA, with fragment peaks assigned to confirm the identity of this peptide.
Figure S4. Direct cell MALDI-TOF MS/MS of neurons expressing the apALNP prohormone reveals the presence of GYFD. (A) apALNP from *A. californica*, with the sequences of interest in bold for this study, and others in italic. GFFG was detected as GFFamide in Figure S5. GDAS has not been detected. GFFD and YYGS have been detected previously.\(^\text{17}\) (B) MALDI-MS spectrum of neurons that express apALNP, with some peptides assigned based on mass matching. (C) MALDI-MS/MS spectrum of GYFD in neurons that express apALNP. GYFD is assigned based on the fragment spectra. Parent ion 501.2 m/z. (D) MALDI-MS/MS spectrum of SYADSKDEESNAALSDFA in neurons that express apALNP. SYADSKDEESNAALSDFA is assigned based on the fragment spectra. Parent ion 1919.8 m/z. (E) MALDI-MS/MS spectrum of SYADSKDEESNAALSDFAED in neurons that express apALNP. SYADSKDEESNAALSDFAED is assigned based on the fragment spectra. Parent ion 2163.9 m/z.
Figure S5. GFFamide, another peptide of the apALNP, is not present as a DAACP in the *A. californica* nervous system. (A) The top chromatogram is of an LC–MS (base peak chromatogram) trace of endogenous GFFamide. GFFamide was identified in *A. californica* pedal ganglia peptide extracts, but did not resist digestion by APM (data not shown). It had a retention time of 23.6 min. The middle LC–MS trace is of GdFFamide synthetic standard, with a retention time of 25.9 min. The bottom LC–MS trace is of GFFamide, with a retention time of 23.6 min. The endogenous peptide matches that of an all-L-amino acid peptide standard and did not resist digestion by APM, establishing that it is not a DAACP. (B) The MS/MS spectrum of GFFamide with assigned fragment peaks.
Figure S6. MALDI-TOF MS spectrum of a peptide fraction collected from multi-stage LC containing endogenous SYADSKDEESNAALSDF. (A) Results shown demonstrate the relative purity of this fraction. (B) The MALDI-MS/MS fragmentation.
Figure S7. GdYFD was bioactive, whereas GYFD was not, in the *A. californica* locomotor circuit (with cerebral and pedal ganglia). (A) GYFD did not change activity in the PPCN of the pedal ganglion significantly. Motor neuron: a presumed motor neuron on the ventral surface of the pedal ganglion. A5: group data. (B) GdYFD increased activity in the PPCN of the pedal ganglion, i.e., the average frequency (B5), frequency within bursts (B6), and frequency between bursts (B7). Open bars: Bursting activity in PPCN. Bonferroni post hoc test is as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure S8. GdYFD reduced locomotor activity in intact animals. (A) Locomotor path within 25 min. Left: before injection of GdYFD; right: after injection of GdYFD. (B) Group data. Left two bars: before and after injection of ASW; right two bars: before and after injection of GdYFD.

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