Molecular and Physiological Characterization of a Receptor for \(d\)-Amino Acid-Containing Neuropeptides

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Supporting Information

ABSTRACT: Neuropeptides in several animals undergo an unusual post-translational modification, the isomerization of an amino acid residue from the \(L\)-stereoisomer to the \(D\)-stereoisomer. The resulting \(d\)-amino acid-containing peptide (DAACP) often displays biological activity higher than that of its all-\(L\)-residue analogue, with the \(D\)-residue being critical for function in many cases. However, little is known about the full physiological roles played by DAACPs, and few studies have examined the interaction of DAACPs with their cognate receptors. Here, we characterized the signaling of several DAACPs derived from a single neuropeptide prohormone, the Aplysia californica achatin-like neuropeptide precursor (apALNP), at their putative receptor, the achatin-like neuropeptide receptor (apALNR). We first used quantitative polymerase chain reaction and \textit{in situ} hybridization experiments to demonstrate receptor (apALNR) expression throughout the central nervous system; on the basis of the expression pattern, we identified novel physiological functions that may be mediated by apALNR. To gain insight into ligand signaling through apALNR, we created a library of native and non-native neuropeptide analogues derived from apALNP (the neuropeptide prohormone) and evaluated them for activity in cells co-transfected with apALNR and the promiscuous \(G\) subunit \(G\)-16. Several of these neuropeptide analogues were also evaluated for their ability to induce circuit activity in a well-defined neural network associated with feeding behavior in intact ganglia from \textit{Aplysia}. Our results reveal the specificity of apALNR and provide strong evidence that this receptor mediates diverse physiological functions throughout the central nervous system. Finally, we show that some native apALNP-derived DAACPs exhibit enhanced stability toward endogenous proteases, suggesting that the \(D\)-residues in these DAACPs may increase the peptide lifetime, in addition to influencing receptor specificity, in the nervous system. Ultimately, these studies provide insight into signaling at one of the few known DAACP-specific receptors and advance our understanding of the roles that \(L\)- to \(D\)-residue isomerization play in neuropeptide signaling.

Polypeptide, including neuropeptides, often undergo post-translational modifications (PTMs) that significantly influence their biological activity.\(^1\)\(^-\)\(^3\) One poorly understood PTM is the enzyme-catalyzed isomerization of one amino acid residue from the \(L\)-stereoisomer to the \(D\)-stereoisomer (Figure 1a) to form a \(d\)-amino acid-containing peptide (DAACP).\(^3\)\(^-\)\(^10\) DAACPs have been identified from diverse animals in multiple phyla, where they act as neuropeptides,\(^6\)\(^-\)\(^9\) hormones,\(^10\) and toxins\(^6\)\(^-\)\(^11\) and, in many cases, are significantly more biologically active than their all-\(L\)-residue analogues.\(^5\)\(^,\)\(^8\)\(^,\)\(^12\) DAACPs are difficult to identify by commonly used mass spectrometry (MS)-based peptide characterization techniques because...
DAACPs and their all-L-residue diastereomers have identical masses. Methods have been recently developed to address this challenge and enhance the identification of DAACPs. Nonetheless, the full functions of identified DAACPs are still unknown, and many DAACPs may remain unidentified. In addition, although D-residues have been shown to increase the protease resistance of synthetic peptides and several DAACP toxins and toxin fragments, relatively few studies have directly examined the influence of D-residues on the stability of cell–cell signaling DAACPs to central nervous system (CNS) proteases.

The marine mollusk *Aplysia californica* is an excellent model for investigations of learning and memory, neural circuits, neurochemistry, and neural signaling because it possesses a range of functions mediated through ALNRs in the CNS. Understanding the tissue localization of receptor expression (e.g., which specific cells transcribe mRNA for the receptor) is a prerequisite for understanding the physiological functions enacted through the receptor. In *Aplysia*, regions of the CNS (and even many specific neurons) are mapped to well-known physiological and behavioral functions. Because GdFFD-OH is active in the feeding and locomotor circuits to control other behaviors throughout the CNS. Ultimately, our results expand the roles that DAACPs play in the *Aplysia* CNS and help to clarify the role of 1- to 3-residue isomerization in both the functional and biological stability of these neuropeptides.

### RESULTS AND DISCUSSION

#### Expression of apALNR across the CNS

Understanding the tissue localization of receptor expression (e.g., which specific cells transcribe mRNA for the receptor) is a prerequisite for understanding the physiological functions enacted through the receptor. In *Aplysia*, regions of the CNS (and even many specific neurons) are mapped to well-known physiological and behavioral functions. Because GdFFD-OH is active in the feeding and locomotor circuits, controlled primarily by neurons in the buccal and cerebral, pedal, pleural, and abdominalganglia, respectively, we predicted that apALNR would be expressed in these ganglia. The extent of apALNR expression in other tissues is not known.

To gain insight into the relative expression of apALNP and apALNR across different tissues, we performed quantitative polymerase chain reaction (qPCR) experiments to measure gene expression for each major ganglion in the CNS (buccal, cerebral, pedal, pleural, and abdominal (Figure 2a) as well as other non-CNS tissues (the buccal mass and gill). In contrast to the pedal ganglia-specific expression for apALNP (Figure 2b), which is consistent with previous in situ hybridization (ISH) experiments, we observed that apALNR mRNA was present at relatively uniform levels across each ganglion of the CNS (Figure 2c). As expected for a GPCR, the expression level of apALNR is low compared to that of the neuropeptide prohormone *apALNP*, whose products are targeted for release and are generally not recycled.
apALNR mRNA. ISH is a useful qualitative technique for identifying specific cells that express a given transcript, and the results complement the quantitative information gained from qPCR measurements. Consistent with gene expression measurements by qPCR, our ISH experiments for apALNR mRNA revealed cell-specific staining in the buccal, cerebral, pedal, and pleural ganglia (Figure 3 and Figure S6). In the buccal ganglia, staining was most obvious in several of the large motor neurons located at the ventral surface. This staining in the buccal ganglia is consistent with the activity reported for GdFFD-OH and GdYFD-OH in the feeding network.8,9 In the cerebral ganglia, staining was most pronounced in the metacerebral cells (MCCs) and the region corresponding to the H and right G clusters. Weaker staining was observed on the outer lateral edge in the A/B cluster and in a small number of stained cells in the E clusters. In the pedal ganglia, expression was broadly distributed, with the most intense staining in septa IIIb and IIIc of the dorsal surface, consistent with the effects of GdFFD-OH on locomotor programs and locomotor behavior previously observed.8,9 Staining was weaker in neurons of the pleural ganglia than in the pedal ganglia, except for noticeable staining of LPl1, a giant neuron in the left pleural ganglion. The pattern of staining in the abdominal ganglia was not as consistent between preparations as it was for other ganglia examined (Figure S7), which could reflect technical issues or biological variability in expression between animals.

Figure 2. (a) Cartoon representation of the Aplysia CNS, highlighting the relative positions of each major ganglion and major connective nerves (cartoon not drawn to scale). Relative expression of (b) apALNP (neuropeptide prohormone) and (c) apALNR (receptor) in various tissues, as determined by qPCR. The buccal mass and gill were included as non-neural tissue. Note the differences in the vertical axes. Individual points represent each of three biological sets of five animals. Bars represent the mean ± the standard deviation (SD) of these three biological sets.

Figure 3. Representative images showing the localization of apALNR mRNA by ISH across different ganglia in the Aplysia CNS. Antisense probes revealed apALNR expression (left), while sense probes were included as negative controls (right). (a) Buccal ganglia (caudal surface). (b) Cerebral ganglia (dorsal surface). MCCs highlighted with arrows. (c) Pedal and pleural ganglia (dorsal−lateral view). Legend: LPed, left pedal; RPed, right pedal; LPl, left pleural; RPl, right pleural. LPl1 highlighted with an arrow. Scale bars are 500 μm.
Together, the qPCR and ISH experiments confirmed the predicted expression of apALNR in the buccal and pedal ganglia but also revealed expression of apALNR in the cerebral, pleural, and abdominal ganglia, regions that were not previously anticipated to express receptors for apALNP-derived DAACPs. The cellular expression of apALNR immediately suggests that apALNP-derived DAACPs may play additional roles beyond the specific feeding and locomotor circuits previously identified. For example, the MCCs of the cerebral ganglia enhance the strength of buccal muscle contractions and modulate the output of the central pattern generator (CPG) for biting movements. Cell-specific expression of apALNR in the MCCs suggests that apALNP-derived DAACPs may modulate these specific feeding behaviors through these cells. Similarly, apALNR expression in LPl1 suggests that apALNP-derived DAACPs may help regulate defensive mucus release, a behavior mediated by this neuron.

**GdFFD-OH Increases the Excitability of LPl1.** The presence of the apALNR transcript in LPl1, an easily identifiable cell asymmetrically present in the left pleural ganglion, made this cell a good candidate for testing the activity of apALNP-derived DAACPs for physiological functions outside of the feeding and locomotor circuits previously tested. Neuropeptides, through activation of cell-surface receptors, can increase or decrease the excitability of a neuron. To evaluate the effect of GdFFD-OH on LPl1, which expresses apALNR, we examined the excitability of the LPl1 neuron under different conditions by electrophysiology. In these experiments, a constant current pulse was applied to LPl1 every 60 s and LPl1 excitability was measured by the number of spikes evoked by the current pulses during the 3 s. Consistent with its positive staining by apALNR, ISH, we found that the excitability of LPl1 was increased by a perfusion of GdFFD-OH, but not by GFFD-OH, in electrophysiology experiments on isolated pleural—pedal ganglion preparations (Figure 4 and Figure S8). Recordings on LPl1 were performed in high-divalent saline, which limits polysynaptic influences. Of course, receptors other than apALNR may also be activated by GdFFD-OH, contributing to the observed modulation. Nevertheless, this finding confirms that apALNP-derived peptides can modulate the activity of neurons outside of the feeding and locomotor circuits, including a cell type known to function in defensive mucus release.

**Activation of apALNR and the Feeding Network by apALNP-Derived Peptides.** We next sought to determine whether peptides from apALNP other than GdFFD-OH were ligands for apALNR. Post-translational processing of apALNP (Figure 1b,c), along with DAACP analogues, to test for direct activation of apALNR in CHO-K1 cells expressing apALNR. For peptides that have not been detected as DAACPs, predicted DAACP analogues were designed with the D-residue at position 2, the location of the D-residue in all known molluscan DAACPs. Three predicted N- and C-terminal peptides, which lack structural similarity to known DAACPs from mollusks, were not evaluated. To determine apALNR activation in response to potential agonists, we transfected CHO-K1 cells with plasmids for the expression of apALNR and also Gtα16, a promiscuous Gt subunit that associates with most GPCRs and activates the phospholipase C signaling pathway. Activation of the canonical Gtα1 signaling was then measured using a commercially available assay that measures the accumulation of IP1 in the presence of LiCl upon activation of phospholipase C. Consistent with a prior report, we found that GdFFD-OH activates apALNR whereas GFFD-OH does not, using the IP1 accumulation assay (Table 1 and Figure S9). Interestingly, we found that GdYFD-OH was a potent agonist, with potency virtually identical to that of GdFFD-OH. The high apALNR potency for GdYFD-OH is consistent with the fact that this peptide displays high biological activity in physiological networks associated with feeding and locomotor behavior, similar to GdFFD-OH. In contrast, GYFD-OH, the all-L-residue analogue, was completely devoid of apALNR activity, indicating that the D-Tyr residue is critical for receptor activation for GdYFD-OH. Neither SDYADSDKDEESNALSDFAED-OH nor SYADSDKDEESNALSDFAED-OH activated apALNR up to 50000 nM (Table 1).

![Single cell excitability in intact ganglia](image)

Figure 4. LPl1 excitability (tested with 3 s current pulses every 60 s) after perfusion of isolated pleural and pedal ganglia of *Aplysia* with (a) GdFFD-OH [F(3,12) = 39.33; p < 0.001; n = 5] or (b) GFFD-OH [F(3,15) = 1; p > 0.05; n = 6] (see Figure S8). Control: activity before peptide perfusion. Wash: activity after washout of peptide. Bars represent the mean ± the standard error of the mean (SEM). Repeated measures analysis of variance and Bonferroni post hoc test: *p < 0.05; ***p < 0.001. Recordings were made in high-divalent saline.
in homogenized ganglion extracts by liquid chromatography (LC)—electrospray ionization mass spectrometry (MS) or with single-cell matrix-assisted laser desorption/ionization (MALDI) MS, but the chirality of the endogenous peptides is currently unknown. We found that none of the structural variants of GFF-NH$_2$, GDAS-OH, or YYGS-OH that we tested activated apALNR (Table 1), though some variants appeared to show minor activity at the highest concentration tested (500,000 nM). Thus, the presence of a d-residue at position 2 in a short peptide, even one as structurally similar to GdFFD-OH as GdFF-NH$_2$, is not sufficient to confer apALNR activity.

We previously found that GdFFD-OH and GdYFD-OH were both potent activators of the CPG associated with feeding behavior in electrophysiology experiments, while their all-L-residue analogues, GFFD-OH and GYFD-OH, were not (Table 1)\(^8,9,24\). In the study presented here, we evaluated the ability of SdYADSKDEESNAALSDFAED-OH, SYADSKDEESNAALSDFAED-OH, GdFF-NH$_2$, and GFF-NH$_2$ to induce circuit activity in the feeding network by electrophysiology. These electrophysiology experiments differ from the LP1 experiments described above in that we measured the cyclic activity bursts of the I2 nerve of the buccal ganglion and corresponding well-defined cycles of activity in several specific buccal neurons that are known to lead to stereotyped feeding responses.\(^36,37\)

Upon evaluating activity in the feeding network, we found that all four peptides were inactive (Table 1, Figure S10, and Table S1). Thus, for each peptide tested, in vitro activation of apALNR (or lack thereof) determined by the IP1 accumulation assay in cells transfected with apALNR matched their physiological effects in the Aplysia feeding network.

Together, our results support the identification of both GdFFD-OH and GdYFD-OH as key apALNP-derived agonists

**Table 1. apALNR Activation and Feeding Network Activity for apALNP-derived Peptides**

| Peptide          | apALNR EC$_{50}$ (nM) (IP1 assay) | Feeding network (electrophys.) |
|------------------|-----------------------------------|--------------------------------|
| GdFFD-OH         | 30                                | active                         |
| GFFD-OH          | >2,000,000                        | not active                     |
| GdYFD-OH         | 30                                | active                         |
| GYFD-OH          | >500,000                          | not active                     |
| SdYADSKDEESNAALSDF-AED-OH | >500,000                          | not active                     |
| SYADSKDEESNAALSDF-AED-OH | >500,000                          | not active                     |
| GdFF-NH$_2$      | >500,000                          | not active                     |
| GFF-NH$_2$       | >500,000                          | not active                     |
| YdYGS-OH         | >500,000                          | NT                             |
| YYGS-OH          | >500,000                          | NT                             |
| GdDAS-OH         | >500,000                          | NT                             |
| GDAS-OH          | >500,000                          | NT                             |

\(^*\)Primary sequences of select peptides predicted from processing of apALNP, along with associated apALNR activation EC$_{50}$ values, as determined by the IP1 accumulation assay in CHO-K1 cells transiently transfected with apALNR and G$_\alpha$-16 (see Figure S9 for all dose-response curves). For apALNR-active compounds, EC$_{50}$ values are the mean from at least three independent experiments. See Table S1 for the error associated with these measurements. For apALNR-inactive compounds, the EC$_{50}$ is listed as being greater than the highest concentration tested, from at least two independent experiments showing no or negligible activity. Activity in the feeding network is determined by electrophysiology measurements on intact buccal and cerebral ganglia (Figure 6, Figure S10, and Figure S11). Active: perfusion of the peptide induced statistically higher circuit activity relative to that of control conditions with no peptide perfusion. Not active: no increase in activity was detected. NT: not tested. Feeding network activity values for GdFFD-OH/GFFD-OH and GdYFD-OH/GYFD-OH are from previous reports.\(^8,9\)

**Figure 5.** Primary sequences of GdFFD-OH analogues made through single-residue substitutions (sub.) or terminal modifications (mod.), along with associated EC$_{50}$ values for apALNR activation (in parentheses) as determined by the IP1 accumulation assay in CHO-K1 cells transiently transfected with both apALNR and G$_\alpha$-16 (see Figure S9 for all dose-response curves and Table S2 for errors associated with these measurements). For non-natural analogues of GdFFD-OH, the substituted residue or terminus is colored red. For apALNR-active compounds, EC$_{50}$ is the mean from at least three independent experiments. For apALNR-inactive compounds, the EC$_{50}$ is listed as being greater than the highest concentration tested, from at least two independent experiments showing no or negligible activity. EC$_{50}$ values for GFFD-OH, GYFD-OH, and GdYFD-OH, which are included in Table 1, are repeated here for comparison.
of apALNR. The matching activity for apALNP peptides in apALNR activation as determined by the IP1 accumulation assay and in the feeding network as determined by electrophysiology measurements supports the hypothesis that apALNR may mediate the effects of apALNP-derived peptides in the feeding circuit. Furthermore, our results suggest that endogenous peptides, such as GYFD-OH, SdYADSKDEESNAALSDFAED-OH, and SYADSKDEESNAALSDFAED-OH, are inactive in the feeding network because of their inability to activate a key receptor, and not because of other factors such as proteolytic degradation.

Activation of apALNR by GdFFD-OH Analogues. To gain more insight into how apALNR recognizes its ligands and to determine if other peptides may be ligands for the receptor, we designed a library of GdFFD-OH analogues bearing single-residue substitutions or terminal modifications and evaluated the ability of each of these peptides to activate apALNR in CHO-K1 cells transiently transfected with both apALNR and Gα-16 using the IP1 accumulation assay described above (Figure S5, Figure S9, and Table S2). The results, summarized in Figure 5, reveal the specificity of the receptor and indicate that each residue of GdFFD-OH and both terminal charges make important contributions to apALNR activity, as judged by apALNR activation potency (EC₅₀ values). For example, position 2 appears to require an extended hydrophobic D-residue, position 3 can tolerate smaller hydrophobic residues but not charged residues, and position 4 requires a residue with a carbonyl group on its side chain (see the Supporting Information for more details). These results, which show that even relatively minor modifications to GdFFD-OH lead to dramatic losses of potency in most cases, suggest that GdFFD-OH and GdYFD-OH are likely the primary agonists for this receptor in vivo, although unidentified or unrelated sequences may also be endogenous agonists.

Physiological Activity of GdFFD-OH Analogues. To test the hypothesis that apALNR is a mediator of the physiological effects of GdFFD-OH, we examined whether a subset of the GdFFD-OH analogues described could directly induce feeding circuit activity in isolated buccal and cerebral ganglia. For these studies, we chose GdFFD-OH analogues with high apALNR potency (dAdFFD-OH), intermediate potency (GdLFD-OH, GdFAD-OH, Ac-GdFFD-OH, and GdFFD-NH₂), or no activity (AdFFD-OH) in the IP1 accumulation assay. Consistent with its high apALNR potency in the IP1 accumulation assay, we found that dAdFFD-OH activated the Aplysia feeding network in a manner similar to that of GdFFD-OH and GdYFD-OH (Figure 6 and Figure S11).8,9 GdLFD-OH, GdFAD-OH, Ac-GdFFD-OH, and GdFFD-NH₂ were each weaker at inducing circuit activation than dAdFFD-OH was, consistent with their reduced apALNR potency relative to that of dAdFFD-OH in the IP1 accumulation assay. Interestingly, we found that AdFFD-OH, which showed no ability to activate apALNR in the IP1 accumulation assay experiments, was modestly active in the feeding network. This contrasts with several other neuro-peptides evaluated (e.g., GdFF-NH₂ and SdYADSKDEESNA-
ALSDFDAED-OH, described above), which were unable to activate apALNR by the IP1 accumulation assay and showed no activity in the feeding circuit. The inconsistency between apALNR activation in cell-based assays with a transiently transfected receptor and electrophysiology activity in intact ganglia for AdFFD-OH might indicate the inability of the apALNR IP1 accumulation assay to completely recapitulate the complex biological interactions present in living neural networks. Indeed, peptide potencies can differ significantly among different expression systems and intact tissues.

Alternatively, activity for AdFFD-OH in the feeding network may indicate that alternative or modified isoforms of apALNR exist in vivo with altered selectivity or that additional unrelated receptors are activated by AdFFD-OH. Overall, the concordant activity for the analogues tested, both for receptor activation by the IP1 accumulation assay and in the feeding network by electrophysiology (including apALNP-derived peptides, described above), is consistent with apALNR as a mediator of the biological activity of this family of DAACPs, although the unexpected activity of AdFFD-OH in the feeding network leaves open the possibility that additional receptors for apALNP-derived DAACPs may also be present.

**Stability of DAACPs in CNS Homogenates.** D-Residues are known to enhance the stability of engineered peptides to proteases,11,12,17 but little is known about how D-residues influence the lifetime of naturally occurring DAACPs that act as cell–cell signaling peptides. To gain insight into the relative stability of DAACPs from *Aplysia* to endogenous proteases, we incubated exogenous GdFFD-OH, GdYFD-OH, GFFD-OH, GYFD-OH, and NdWF-NH$_2$ in ganglion homogenate extract and monitored the time course of peptide degradation by LC–MS with multiple-reaction monitoring (MRM) (Figure 7). We found that the all-L-residue peptides GFFD-OH and GYFD-OH were relatively rapidly degraded, with half-lives of 8.4 and 8.7 min, respectively. NdWF-NH$_2$, a cardioactive DAACP, was more stable under these conditions (half-life of 27 min). Previous studies have shown that the stability of NdWF-NH$_2$ is similar to that of NWF-NH$_2$ in ganglion membrane fractions,40 so we did not evaluate NWF-NH$_2$ in this experiment. Interestingly, both GdFFD-OH and GdYFD-OH were even more stable than NdWF-NH$_2$, with half-lives of 530 and 1100 min, respectively, in the ganglion homogenate.

Unlike classical neurotransmitters, neuropeptides are released from both synaptic and nonsynaptic sites and can travel relatively long distances (on the order of micrometers) from their site of release to activate distal receptors.41 This volume transmission mode of signaling within the CNS, and even hormonal-like roles traveling longer distances, are possible for transmission mode of signaling within the CNS, and even more stable than NdWF-NH$_2$, with half-lives of 530 and 1100 min, respectively, in the ganglion homogenate.

Further studies will be required to assess if GdFFD-OH and GdYFD-OH and mediates multiple physio-

**Figure 7.** Stability of 50 μM neuropeptides in *Aplysia* cerebral and abdominal ganglion homogenate extracts in PBS (pH 7.4) as determined by LC–MRM. Each point represents the mean ± the standard error of three biological replicates. Panel c shows calculated half-life values for each peptide.

and another known DAACP (NdWF-NH$_2$) from the same animal. This suggests that in some cases the D-residue can greatly extend the lifetime of cell–cell signaling DAACPs, in addition to being critical for receptor activation.

**Conclusions.** Prior to our work, Bauknecht et al.27 showed D-Phe-specific activation of apALNR by GdFFD-OH, but little else about this ligand–receptor interaction has been studied. Although previous studies44,45 have explored how DAACP structure relates to specific physiological functions (e.g., neuronal activity or muscle contraction), the receptors for most cell–cell signaling DAACPs have not been identified, and thus, little is known about the interactions of DAACPs with their receptors throughout the CNS. Here, we provide multiple compelling lines of evidence demonstrating that apALNR does indeed function as a receptor for the apALNP-derived DAACPs GdFFD-OH and GdYFD-OH and mediates multiple physiological effects throughout the CNS. First, qPCR and ISH analyses demonstrate, for the first time, that apALNR is expressed in the *Aplysia* CNS. Interestingly, whereas apALNP expression is largely restricted to the pedal ganglia, apALNR expression is distributed throughout the CNS. These results indicate that apALNP-derived DAACPs may be produced in one central ganglion (i.e., pedal) to affect targets throughout the CNS. Indeed, the apALNR expression data enabled us to
identify a novel cellular target of apALNP-derived DAACPs (LPI1), in addition to known targets in feeding and locomotor networks. The broad distribution of apALNR expression across the entire CNS suggests that many other roles could be played by this interesting DAAC receptor family. Receptor activation experiments with non-natural GdFFD-OH analogues (Figure 5) revealed that each residue of this ligand makes important contributions to potency and strongly suggest that GdFFD-OH and GdYFD-OH are likely the primary ligands for apALNR. Finally, GdFFD-OH and GdYFD-OH were more stable than their all-L-residue analogues and NdWF-NH2 in ganglion homogenate extract, providing evidence that the lifetimes of some cell–cell signaling peptides are significantly increased in vivo because of the incorporation of the D-residue, and these peptides may diffuse relatively long distances from their point of release throughout the CNS to activate distal receptors.

The largely concordant activity of peptides in both receptor activation by IP1 accumulation assays and physiological experiments is consistent with the hypothesis that apALNR mediates known physiological activities of GdFFD-OH and GdYFD-OH. However, there may be other receptors that contribute to the physiological functions of GdFFD-OH and GdYFD-OH. In fact, the physiological activity of AdFFD-OH in the feeding network, despite no apALNR activity in the IP1 accumulation assay, suggests that there are additional receptors (or isomers of apALNR) with differing ligand specificities that are activated by GdFFD-OH or GdYFD-OH. In the future, it will be of great interest to knock down apALNR expression to determine the specific contributions of this receptor to single-neuron excitability and/or network activity. Regardless of whether apALNR is the sole receptor for GdFFD-OH and GdYFD-OH, our results provide strong evidence that GdFFD-OH and GdYFD-OH are major ligands for apALNR, and that apALNR is expressed across many regions of the CNS, including in regions associated with feeding (buccal ganglia) and locomotion (pedal ganglia), and in specific cells known to control additional functions, ultimately suggesting that GdFFD-OH and GdYFD-OH play roles in a variety of behaviors.

Despite extensive efforts, there are still a large number of orphan GPCRs in vertebrates, including mammals. The identification of ALNRs as receptors whose endogenous ligands require a D-residue for activation suggests that some orphan GPCRs may be selective for DAACPs, and deorphanization efforts that do not consider L- to D-residue isomerization may miss ligand–receptor identifications. Overall, our results represent one of the first in-depth explorations of a receptor that recognizes a DAACP as its native ligand and provide evidence that apALNP-derived DAACPs play a variety of functions throughout the CNS, either by directly activating neurons or by acting as neuromodulators. Given the growing number of DAACPs that continue to be identified among a wide variety of organisms, our findings may be of significance for understanding L- to D-residue isomerization in other animals, including vertebrates.

## METHODS

Detailed procedures can be found in the Supporting Information.

**qPCR.** qPCR experiments were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, 4368708), according to the manufacturer’s specifications. The relative expression level for each gene was calculated using the ΔΔCt method. For each gene, ΔCt was calculated as the difference between the Ct values (mean of technical triplicates) for the gene of interest and GAPDH. Relative expression values were calculated using 2−ΔΔCt. See the Supporting Information for more details.

**In Situ Hybridization (ISH).** The method for the ISH experiments was adapted from ref 49 using antisense or sense digoxigenin-labeled RNA probes corresponding to an ∼750 bp region of the apALNR mRNA (XM_005106549.2). Riboprobes were synthesized using a SP6/T7 DIG RNA labeling kit (Roche), following the manufacturer’s instructions. See the Supporting Information for expanded details.

**Peptide Synthesis and Purification.** Peptides GdAS-OH and GdDAS-OH were purchased from CPC Scientific. All other peptides were synthesized by solid-phase peptide synthesis based on Fmoc protection of the main chain amine. Peptides with a C-terminal acid were synthesized on the solid phase using Wang resin preloaded with the C-terminal residue (Novabiochem or AnaSpec). Peptides with a C-terminal amide were synthesized on NovaPEG Rink Amide resin (Novabiochem, 855047). Coupling reactions were performed by treating the resin with a solution of 24 molar equivalents of Fmoc-protected amino acid with appropriate side chain protecting groups, activated with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and N,N-diisopropylethylamine (DIEA) (1:1:2 amino acid:PyBOP:DIEA molar ratio) in a solution of 0.1 M N-hydroxybenzotriazole in N-methyl-2-pyrrolidone. Coupling reactions were allowed to proceed at room temperature (RT) for >40 min with stirring or gentle shaking, after which the resin was rinsed with three to five washes of dimethylformamide (DMF). Deprotection of the Fmoc protecting group was performed in a solution of 20% piperidine in DMF for 20 min at RT with gentle stirring or shaking, after which the resin was rinsed with three to five washes of DMF. Acetylation of the N-terminus for Ac-GdFFD-OH was achieved by treating the resin with an 8:2.1 DMF/DIEA/acetic anhydride solution for 10 min. After the completion of the synthesis, peptides were cleaved from the resin and side chain protecting groups were removed using a solution of 95% trifluoroacetic acid, 2.5% H2O, and 2.5% triisopropylsilane for >3 h. After cleavage, most of the cleavage solution was removed by evaporation, and the peptides were dissolved in a water/acetonitrile (ACN) mixture or dimethyl sulfoxide for high-performance liquid chromatography (HPLC) purification. For SdYADSDKEEYNALSDFAED-OH and SYADSDKEEYNALSDFAED-OH, peptides were precipitated by the addition of cold methyl tert-butyl ether. Crude peptide mixtures were purified by reversed-phase HPLC and then dried under vacuum. Peptides were dissolved in a water/ACN solution and concentrations determined by UV absorbance and extinction coefficients calculated from the primary sequence at 214 nm or 280 nm. On the basis of this calculated concentration, peptides were aliquoted and dried under vacuum. The final peptide purity was assessed by reversed-phase HPLC and the identity confirmed by MALDI-TOF MS (see the Supporting Information).

**IP1 Accumulation Assays for apALNR Activation.** CHO-K1 cells (ATCC, CCL-61) were transiently transfected with apALNR [region corresponding to XP_005106606.1, in pcDNA3.1(+)] and Gin-16 [in pcDNA3.1(+)] using TurboFisher transfection reagent (ThermoFisher Scientific, R0531). After exposure to potential agonist peptides for 1 h, activation of apALNR was detected by monitoring IP1 accumulation using an IPOne Detection Kit (Cisbio, 62PAPBE), following the manufacturer’s instructions with minor modification. While we found that the recommended amount of IP1-d2 and anti-IP1-creptate (1X) worked well, we also obtained comparable EC50 values using half of these amounts (0.5X), so many assays were performed using 0.5X reagents. See the Supporting Information for more details.

**Electrophysiology.** Intracellular and extracellular recordings of the physiological activity from CNS preparations (either the cerebral and buccal ganglia or the pleural and pedal ganglia) were made as described previously. Mean values were compared using repeated measures one-way analysis of variance, assuming sphericity, with the Bonferroni post test in GraphPad Prism 7. See the Supporting Information for notes on the statistical analyses and more details.
Peptide Stability Assay. Cerebral and abdominal ganglia were isolated from *Aplysia* (65–85 g) and homogenized in phosphate-buffered saline (PBS) (pH 7.4). Each biological set included cerebral and abdominal ganglia from two animals. Tissue was centrifuged (14000g and 4 °C for 10 min), and the supernatant was removed and the protein content estimated using a BCA Protein Assay Kit (ThermoFisher Scientific, 23235). The homogenate from each biological set was diluted to 1000 μg/mL protein. A stock solution containing each peptide at 200 μM was prepared in PBS. The peptide stock in PBS (125 μL) was added to the ganglion homogenate (375 μL) and the resulting mixture incubated at 37 °C (final conditions being 50 μM peptide and 750 μg/mL homogenate protein). At each time point, 10 μL of the reaction mixture was removed, the reaction quenched with 20 μL of a solution containing 25 μM GdFF-NH₂ (as an internal control) and 1% formic acid (FA) in a 50% ACN/water mixture, and the quenched reaction stored at −20 °C until analysis. For analysis, each quenched solution was thawed, diluted to 0.1% FA in a 4% ACN/water mixture, and desalted with C18 solid-phase exchange centrifuge spin columns (ThermoFisher Scientific, 98970). Desalted and dried samples were dissolved in 0.1% FA in water and analyzed using a Bruker EVOQ Elite triple-quadrupole mass spectrometer coupled to a Bruker Advance UHPLC instrument, in MRM mode in positive ion mode. Channels of parent and fragment ion pairs were identified using the MRM Builder in the MS Workstation software for the EVOQ instrument or were manually entered on the basis of the predicted fragment ions. The parent ion (m/z, mass window of 0.7)/fragment ion (m/z, mass window of 2) pairs for each peptide were as follows: GFFD-OH, GdFFD-OH = 485.2/120.2; GYFD-OH, GdYFD-OH = 501.2/120.2; NDFW-NH₂ = 465.2/448.6; GdFF-NH₂ (synthesized with [13C]Gly) = 370.2/120.1. For each time point, the peak area in the resulting LC−MRM chromatogram for the peptide of interest was subsequently calculated relative to the “1 min” time point for each biological set. Data were plotted and half-life values calculated using a one-phase exponential decay model in GraphPad Prism 7.

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**REFERENCES**

(1) Fricker, L. D., Lim, J., Pan, H., and Che, F. Y. (2006) Peptidomics: identification and quantification of endogenous peptides in neuroendocrine tissues. Mass Spectrom. Rev. 25, 327–344.

(2) Mzhavia, N., Berman, Y., Che, F. Y., Fricker, L. D., and Devi, L. A. (2001) ProSAAS processing in mouse brain and pituitary. J. Biol. Chem. 276, 6207–6213.

(3) Hook, V., Funkelstein, L., Lu, D., Bark, S., Wegzyn, J., and Hwang, S. R. (2008) Proteases for processing proteuropeptides into peptide neurotransmitters and hormones. Annu. Rev. Pharmacol. Toxicol. 48, 393–423.

(4) Heck, S. D., Siok, C. J., Krapcho, K. J., Kelbaugh, P. R., Thadeio, P. F., Welch, M. J., Williams, R. D., Ganong, A. H., Kelly, M. E., Lanzetti, A. J., Gray, W. R., Phillips, D., Parks, T. N., Jackson, H., Ahlijanian, M. K., Saccamono, N. A., and Vollmann, R. A. (1994) Functional consequences of posttranslational isomerization of Ser(46) in a calcium channel toxin. Science 266, 1065–1068.

(5) Torres, A. M., Menz, I., Alewood, P. F., Bansal, P., Lahnstein, J., Gallagher, C. H., and Kuchel, P. W. (2002) D-amino acid residue in the C-type natriuretic peptide from the venom of the mammal, *Orothorhynchus anatinus*, the Australian platypus. FEBS Lett. 524, 172–176.

(6) Kamatani, Y., Minakata, H., Kenny, P. T. M., Iwashita, T., Watanabe, K., Funase, K., Sun, X. P., Yongsiri, A., Kim, K. H., Novales-Li, P., Novales, E. T., Kanapi, C. G., Takeuchi, H., and Nomoto, K. (1989) Achatin-I, an endogenous neuroexcitatory tetrapeptide from *Achatina fulica* containing a D-amino acid residue. Biochem. Biophys. Res. Commun. 160, 1015–1020.

(7) Morishita, F., Nakashiy, Y., Kaku, S., Furukawa, Y., Ohta, S., Hirata, T., Ohtani, M., Fujisawa, Y., Muneeoka, Y., and Matsushima, O. (1997) A novel D-amino-acid-containing peptide isolated from *Aplysia* heart. Biochem. Biophys. Res. Commun. 240, 354–358.

(8) Bai, L., Livnat, I., Romanova, E. V., Alexeeva, V., Yau, P. M., Vilam, F. S., Weiss, K. R., Jing, J., and Sweedler, J. V. (2013) Characterization of GdFFD, a D-amino acid-containing-neuropeptide that functions as an extrinsic modulator of the *Aplysia* feeding circuit. J. Biol. Chem. 288, 32837–32851.

(9) Livnat, I., Tai, H. C., Jansson, E. T., Bai, I., Romanova, E. V., Chen, T. T., Yu, K., Chen, S. A., Zhang, Y., Wang, Z. Y., Liu, D. D., Weiss, K. R., Jing, J., and Sweedler, J. V. (2016) A D-amino acid-containing neuropeptide discovery funnel. Anal. Chem. 88, 11868–11876.

(10) Soyez, D., Van Herp, F., Rossier, J., Le Caer, J. P., Tensen, C. P., and Lafont, R. (1994) Evidence for a conformational polymorphism of invertebrate neurohormones: D-amino acid residue in crustacean hyperglycemic peptides. J. Biol. Chem. 269, 18295–18298.

(11) Richter, K., Egger, R., and Klei, G. (1987) D-alanine in the frog skin peptide dermorphin is derived from L-alanine in the precursor. Science 238, 200–202.

**ASSOCIATED CONTENT**

Supporting Information The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.8b00167.

Detailed materials and methods, notes on apALNR characterization, statistical analyses, and peptide selection/activity/inactivity, Tables S1 and S2, Figures S1–S11 (as noted in the text), Figures S12–S14; peptide characterization data, and supporting references (PDF)

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**Notes**

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(12) Miller, S. M., Simon, R. J., Ng, S., Zuckermann, R. N., Kerr, J. M., and Moos, W. H. (1995) Comparison of the proteolytic susceptibilities of homologous L-amino acid, D-amino acid, and N-substituted glycine peptide and peptoid oligomers. Drug Dev. Res. 35, 20–32.

(13) Tao, Y., Quebbemann, N. R., and Julian, R. R. (2012) Discriminating D-amino acid-containing peptide epimers by radical-directed dissociation mass spectrometry. Anal. Chem. 84, 6814–6820.

(14) Tao, Y., and Julian, R. R. (2014) Identification of amino acid epimerization and isomerization in Crystallin proteins by tandem LC-MS. Anal. Chem. 86, 9733–9741.

(15) Adams, C. M., and Zubarev, R. A. (2005) Distinguishing and quantifying peptides and proteins containing D-amino acids by tandem mass spectrometry. Anal. Chem. 77, 4571–4580.

(16) Jia, C., Lietz, C. B., Yu, Q., and Li, L. (2014) Site-specific characterization of D-amino acid containing peptide epimers by ion mobility spectrometry. Anal. Chem. 86, 2972–2981.

(17) Milton, R. C. D., Milton, S. C. F., and Kent, S. B. H. (1992) Total chemical synthesis of a D-enzyme: The enantiomers of HIV-1 protease show reciprocal chiral substrate specificity. Science 256, 1445–1448.

(18) Hawkins, R. D., Kandel, E. R., and Bailey, C. H. (2006) Molecular mechanisms of memory storage in Aplysia. Biol. Bull. 210, 174–191.

(19) Gardner, D. (1971) Bilateral symmetry and interneuronal organization in the buccal ganglia of Aplysia. Science 173, 550–553.

(20) Jacob, M. H. (1984) Neurogenesis in Aplysia californica resembles nervous system formation in vertebrates. J. Neurosci. 4, 1239–1239.

(21) Church, P. J., and Lloyd, P. E. (1991) Expression of diverse neuropeptide co-transmitters by identified motor neurons in Aplysia. J. Neurosci. 11, 618–625.

(22) Jing, J., Gillette, R., and Weiss, K. R. (2009) Evolving concepts of arousal: Insights from simple model systems. Rev. Neurosci. 20, 405–427.

(23) Kandel, E. R. (2001) The molecular biology of memory storage: A dialogue between genes and synapses. Science 294, 1030–1038.

(24) Yang, C. Y., Yu, K., Wang, Y., Chen, S. A., Liu, D. D., Wang, Z. Y., Su, Y. N., Yang, S. Z., Chen, T. T., Livnat, I., Vilm, F. S., Cropper, E. C., Weiss, K. R., Sweeney, J. V., and Jing, J. (2016) Aplysia locomotion: Network and behavioral actions of GdFFD, a D-amino acid-containing neuropeptide. PLOS One 11, No. e0147335.

(25) Southey, B. R., Amare, A., Zimmerman, T. A., Rodriguez-Zas, S. L., and Sweeney, J. V. (2006) NeuroPedom: A tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides. Nucleic Acids Res. 34, W267–272.

(26) Maurer, R., Gaehwiler, B. H., Buescher, H. H., Hill, R. C., and Roemer, D. (1982) Opiate antagonistic properties of an octapeptide somatostatin analog. Proc. Natl. Acad. Sci. U. S. A. 79, 4815–4817.

(27) Burkepacht, P., and Jekely, G. (2015) Large-scale combinatorial deorphanization of Platynereis neuropeptide GPCRs. Cell Rep. 12, 684–693.

(28) Gomes, I., Bobeck, E. N., Margolis, E. B., Gupta, A., Sierra, S., Fakira, A. K., Fujita, W., Müller, T. D., Müller, A., Tschöp, M. H., Kleinau, G., Fricker, L. D., and Devi, L. A. (2016) Identification of GPR83 as the receptor for the neuroendocrine peptide PEN. Sci. Signaling 9, ra43.

(29) Gomes, I., Ayal, D. K., Wardman, J. H., Gupta, A., Gagnidze, K., Rodriguez, R. M., Kumar, S., Wetsel, W. C., Pintar, J. E., Fricker, L. D., and Devi, L. A. (2013) GPR171 is a hypothalamic G protein-coupled receptor for BigLEN, a neuropeptide involved in feeding. Proc. Natl. Acad. Sci. U. S. A. 110, 16211–16216.

(30) Fredriksson, R., and Schioth, H. B. (2005) The repertoire of G-protein-coupled receptors in fully sequenced genomes. Mol. Pharmacol. 67, 1414–1425.

(31) Rosen, S. C., Weiss, K. R., Goldstein, R. S., and Kupfermann, I. (1989) The role of a modulatory neuron in feeding and satiation in Aplysia: Effects of lesioning of the serotoninergic metacerebral cells. J. Neurosci. 9, 1562–1578.

(32) Weiss, K. R., Cohen, J. L., and Kupfermann, I. (1978) Modulatory control of buccal musculature by a serotonergic neuron (metacerebral cell) in Aplysia. J. Neurophysiol. 41, 181–203.

(33) Rayport, S. G., Ambron, R. T., and Babiarz, J. (1983) Identified cholinergic neurons R2 and LPl1 control mucus release in Aplysia. J. Neurophysiol. 49, 864–876.