Aplysia allatotropin-related peptide and its newly identified D-amino acid–containing epimer both activate a receptor and a neuronal target

Received for publication, June 8, 2018, and in revised form, September 4, 2018. Published, Papers in Press, September 7, 2018, DOI 10.1074/jbc.RA118.004367

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1- to D-residue isomerization is a post-translational modification (PTM) present in neuropeptides, peptide hormones, and peptide toxins from several animals. In most cases, the D-residue is critical for the biological function of the resulting D-amino acid–containing peptide (DAACP). Here, we provide an example in native neuropeptides in which the DAACP and its all-L-amino acid epimer are both active at their newly identified receptor in vitro and at a neuronal target associated with feeding behavior. On the basis of sequence similarity to a known DAACP from cone snail venom, we hypothesized that allatotropin-related peptide (ATRP), a neuropeptide from the neuroscience model organism Aplysia californica, may form multiple diastereomers in the Aplysia central nervous system. We determined that ATRP exists as a D-amino acid–containing peptide (D2-ATRP) and identified a specific G protein–coupled receptor as an ATRP receptor. Interestingly, unlike many previously reported DAACPs and their all-L-residue analogs, both L-ATRP and D2-ATRP were potent agonists of this receptor and active in electrophysiological experiments. Finally, D2-ATRP was much more stable than its all-L-residue counterpart in Aplysia plasma, suggesting that in the case of ATRP, the primary role of the L- to D-residue isomerization may be to protect this peptide from aminopeptidase activity in the extracellular space. Our results indicate that L- to D-residue isomerization can occur even in an all-L-residue peptide with a known biological activity and that in some cases, this PTM may help modulate peptide signal lifetime in the extracellular space rather than activity at the cognate receptor.

Prohormone-derived peptides, such as neuropeptides and peptide hormones, undergo a number of post-translational modifications (PTMs) that greatly impact their biological functions. One unusual PTM is the enzyme-catalyzed conversion of a residue from the L-stereoisomer to the D-stereoisomer to generate a D-amino acid–containing peptide (DAACP). DAACPs have been identified in animals across several phyla, acting as neuropeptides, hormones, and toxins (1–9). Despite considerable progress in developing methods to identify DAACPs (8, 10–13), L- to D-residue isomerization remains difficult to detect de novo because this PTM does not change a peptide’s mass or chemical composition. As a result, relatively little is known about the full biological consequences of isomerization on peptide function.

In most cases of L- to D-residue isomerization, the DAACP appears to be significantly more biologically active than its all-L-residue diastereomer (for a list of known cell–cell signaling DAACPs; Table S1). Consistent with this observation, the only identified receptors for cell–cell signaling DAACPs, the achatin-like neuropeptide receptors, are activated by their DAACP ligands and not by their all-L-residue analogs (14, 15). In fact, most DAACPs have been identified based on studies in which the synthetic all-L-residue peptide fails to recapitulate the biological activity of the isolated endogenous compound. However, these bioactivity tests would fail to identify peptides in which both the DAACP and the all-L-residue isomer have similar activities, and this possibility is rarely explored. To our knowledge, outside of the achatin receptors found in several animals (14), no additional receptors for DAACPs have been identified and studied. As a result, it is unclear whether L- to D-residue isomerization is always a critical mediator of receptor

This work was supported by National Institutes of Health Grant P30 DA018310 from the National Institute on Drug Abuse and National Institutes of Health Grant R01 NS031609 from the NINDS (to J. V. S.); by the National Resource for Aplysia, funded by Public Health Service Grant P40 OD010952; and by National Natural Science Foundation of China Grants 31671097 and 313711104 (to J. J.), J1103512, and J1210026 (to the School of Life Sciences). The authors declare that they have no conflict of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains supporting methods, supporting references, Table S1, and Figs. S1–S12.

1 Supported in part by a Beckman Institute Postdoctoral Fellowship, funded by a Beckman Foundation gift to the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign.

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The abbreviations used are: PTM, post-translational modification; DAACP, D-amino acid–containing peptide; ATRP, allatotropin-related peptide; CNS, central nervous system; aPTPR, Aplysia allatotropin-related peptide receptor; APM, aminopeptidase M; GPCR, G protein-coupled receptor; IP1, inositol 1-monophosphate; MRM, multiple reaction monitoring; CHH, crustacean hyperglycemic hormone; FA, formic acid; ACN, acetonitrile; Fmoc, N-(9-fluorenyl)methoxycarbonyl; DMF, dimethylformamide; FBS, fetal bovine serum; PyBOP, benzotriazole-1-yl-oxy-tris(pyrryldino)-phosphonium hexafluorophosphate.
activation, as is the case in the achatin-like neuropeptide system. In addition to altering receptor activity, \( \delta \)-residues can also decrease the susceptibility of peptides to protease relative to their all-L-residue counterparts (3, 6, 16, 17), although few studies have directly examined how \( L \)- to \( D \)-residue isomerization influences the stability of cell-cell signaling DAACPs to endogenous proteases (15).

Allatotropins are a family of prohormone-derived peptides that play roles in multiple biological processes. The first allatotropin peptide was identified from the insect *Manduca sexta*, where it was isolated based on its stimulation of juvenile hormone secretion (18). Since this initial discovery, allatotropins have been found to act in a variety of biological processes in neuropeptide, hormonal, and myoactive functions (19–22). Allatotropin-related peptides have been identified in many animals across phyla, including insects, mollusks, and annelids, although the roles of these peptides remain poorly understood (19, 23, 24). Allatotropin receptors have been characterized from insects (25) and annelids (14) and predicted in several additional phyla based on sequence similarity (14, 24, 26). Furthermore, phylogenetic analyses show that allatotropin prohormones and their receptors are evolutionarily related to orexin prohormones and receptors found in vertebrates (24, 27). We recently reported an allatotropin-related peptide (hereafter referred to as ATRP) from the model organism *Aplysia californica* and identified physiological roles for the all-L-residue form of this peptide as a regulator of peripheral and central nervous system functions in the feeding network (23). A receptor for ATRP, or to our knowledge any other molluscan allatotropin-related peptide, has not been experimentally verified.

Predatory cone snails produce venom consisting of hundreds of compounds, including disulfide-constrained conotoxins and neuropeptide/hormone-like compounds, which act on a variety of molecular targets (28–30). Several peptides from cone snail venom have been identified as DAACPs (31, 32), including the peptide Cononap-Vt (here called \( \delta 2 \)-Conp; see Fig. 1) from *Conus vitulinus*, which shows high sequence similarity to allatotropin and ATRP (see Fig. 1) (33). The similarity in sequences of the DAACP \( \delta 2 \)-Conp in the cone snail venom and ATRP in *Aplysia* CNS led us to hypothesize that allatotropin-related peptides in some species may undergo \( L \)- to \( D \)-residue isomerization. Here, we determined that ATRP exists in the *Aplysia* CNS as two diastereomers, \( L \)-ATRP and \( \delta 2 \)-ATRP. We examined the signaling of \( L \)- and \( \delta 2 \)-ATRP through their newly identified receptor and in physiological experiments and studied their relative susceptibilities to action by endogenous proteases. Overall, our results show that \( L \)- to \( D \)-residue isomerization in cell-cell signaling DAACPs is not always critical for receptor activation, demonstrating that even “well-characterized” peptides may exist as multiple diastereomers and that \( L \)- to \( D \)-residue isomerization could be more widespread than previously thought.

**Results**

**ATRP is a DAACP in the CNS**

The high sequence similarity between ATRP and \( \delta 2 \)-Conp led us to hypothesize that ATRP may exist as two diastereomers bearing either \( L \)- or \( D \)-Phe at position 2 (\( L \)- or \( \delta 2 \)-ATRP; Fig. 1). To determine the chirality of ATRP in the *Aplysia* CNS, we examined the chromatographic properties, mass spectral profiles, and protease stabilities for peptides extracted from ganglia by LC–MS and LC–MS/MS. Consistent with the hypothesis that ATRP exists in multiple diastereomers, LC–MS analysis of cerebral ganglia peptide extracts identified two major peaks with different retention times that each matched the predicted \( m/z \) value for ATRP of 511.9 for \( z = +3 \) (Fig. 2, A and B). The MS/MS fragment spectra for the two peaks were very similar (Fig. 2C), and the majority of the peaks present in these MS/MS spectra matched fragments predicted for ATRP and matched those of synthetic \( ^{13} \mathrm{C} \cap \delta 2 \)-ATRP (Fig. S1). Consistent with previously reported expression revealed by Northern blotting (23), ATRP was detected in all major ganglia by LC–MS (Fig. S2) and showed similar behavior.

To relatively rapidly test whether one of the peaks identified as ATRP may contain a \( \delta \)-residue, we incubated *Aplysia* cerebral ganglia peptide extracts with aminopeptidase M (APM), a relatively nonspecific peptidase that rapidly degrades most all-L-residue peptides but degrades DAACPs bearing a \( \delta \)-residue near their N terminus at a reduced rate. In fact, we have previously used APM digestion as a screening tool to identify putative DAACPs in complex biological mixtures (8, 34). When incubated with APM for 15 h, we found that the later-eluting ATRP peak was completely degraded (Fig. 3). In contrast, the earlier-eluting ATRP peak resisted degradation over this time, consistent with the hypothesis that this species bears a \( \delta \)-residue near its N terminus (Fig. 3 and Fig. S3).

To directly test our hypothesis that the two major peaks present in the chromatograms corresponded to the two predicted diastereomers of ATRP, we spiked into cerebral ganglia extracts \( ^{13} \mathrm{C} \cap L \)-ATRP or \( ^{13} \mathrm{C} \cap \delta 2 \)-ATRP, ATRP analogs bearing two \( ^{13} \mathrm{C} \)-glycine residues (Fig. 1), and analyzed the resulting spiked samples by LC–MS and LC–MS/MS (Fig. 4). The results from this experiment demonstrate that the two peaks observed indeed correspond to the two predicted diastereomers of ATRP, with the earlier-eluting peak co-eluting with \( ^{13} \mathrm{C} \cap \delta 2 \)-ATRP and the later-eluting peak co-eluting with \( ^{13} \mathrm{C} \cap L \)-ATRP.

To add support to our assignment of stereochemistry for ATRP, with the earlier-eluting peak co-eluting with \( ^{13} \mathrm{C} \cap \delta 2 \)-ATRP, we also synthesized analogs of ATRP with a \( \delta \)-residue at position 3 (\( ^{13} \mathrm{C} \cap \delta 3 \)-ATRP) or position 4 (\( ^{13} \mathrm{C} \cap \delta 4 \)-ATRP). \( ^{13} \mathrm{C} \cap L \)-ATRP, \( ^{13} \mathrm{C} \cap \delta 2 \)-ATRP, \( ^{13} \mathrm{C} \cap \delta 3 \)-ATRP, and \( ^{13} \mathrm{C} \cap \delta 4 \)-ATRP

**Figure 1. Primary sequences of peptides relevant to this study.** *df*, highlighted in **bold type** indicates a \( \delta \)-Phe residue. A red *G* indicates a \( ^{13} \mathrm{C} \)-Gly residue containing one \( ^{13} \mathrm{C} \) atom at the carbonyl carbon, incorporated to distinguish synthetic peptides from endogenous peptides.
were resolved by LC, and spiking experiments indicated that the endogenous signals correspond to L-ATRP and D2-ATRP and not to D3-ATRP or D4-ATRP (Fig. S4).

The percentage of D2-ATRP (mean ± standard deviation) calculated from the above independent experiments was 30 ± 10% in the cerebral ganglia extracts, as determined by relative peak area in the LC–MS chromatograms (from Figs. 2 and 3 and Figs. S2 and S4, A and B; n = 5 experiments on different biological sets). It is important to note that our extraction protocol isolates peptides present inside cells at a particular time point, not those released from the neuron. Because L- to D-residue isomerization is a late-stage PTM (35), we regularly see both the DAACP form and the partially processed all-L-residue form in these extracts (see Fig. S3 for examples of other DAACPs).

Thus, the ratio detected from the ganglia extracts may not be representative of the ratio ultimately released by the neurons for cell–cell communication or what may be present in the extracellular space after peptide release.

To demonstrate that the formation of D2-ATRP was not a result of spontaneous epimerization resulting from peptide extraction or sample handling, we performed a separate experiment in which 13C-L-ATRP was spiked into the peptide extraction solution before ganglia dissection and then processed as normal. When analyzed by LC–MS, the results from this experiment showed signals for both 13C-L-ATRP and L-ATRP (distinguished by isotope pattern), whereas the endogenous D2-ATRP peak showed no signal from 13C-D2-ATRP (Fig. S5), demonstrating that no significant conversion of 13C-L-ATRP to 13C-D2-ATRP occurred during peptide extraction and sample preparation. Together, the results from these experiments demonstrate that ATRP exists endogenously as both L-ATRP and D2-ATRP in the CNS of Aplysia.

**Identification and characterization of a receptor for ATRP**

Insect allatotropin receptors have been identified and characterized (25), as have two receptors for an allatotropin-related peptide from Platynereis (14). Based on homology, several candidate allatotropin-related peptide receptors have been proposed in other phyla, including mollusks (14, 26). However, to our knowledge, no allatotropin-related peptide receptor has been experimentally confirmed in a mollusk.

To identify candidate ATRP receptors, we performed a translated BLAST search of the NCBI Aplysia nucleotide library using one of the Platynereis allatotropin receptors as a query; several putative receptors were identified (Fig. S6). We amplified a sequence containing the predicted ORF from the top BLAST hit (XM_005106157.2, here referred to as...
activates apATRPR in a dose-dependent manner, with an EC_{50} value of 200 nM. In contrast, the endogenous G protein-coupled receptor (GPCR) apATRPR indicates that apATRPR is able to associate with the accumulation of D-phosphatidylinositol 1-monophosphate (IP1) in cells in the presence of LiCl. IP1 is a byproduct of phospholipase C activation through the canonical G\alpha_{q} signaling pathway (36). We found that 13C-L-ATRP was able to activate apATRPR in a dose-dependent manner, with an EC_{50} value of 40 nM in our assay, consistent with the hypothesis that apATRPR is a receptor for ATRP. In contrast, the truncated analog 13C-ATRP(5–14) showed no ability to stimulate B61/62 (23, 37). We found that both 13C-L-ATRP and 13C-D2-ATRP were strongly active at stimulating B61/62 in a dose-dependent manner (Fig. 6, A, B, and F). We also evaluated the ability of 13C-L-Conp and 13C-d2-Conp for activation of B61/62 and found that both of these peptides were able to activate this neuron (Fig. 6, C, D, and F), although the effect was weaker than for 13C-L- or 13C-d2-ATRP. In contrast, the truncated analog 13C-ATRP(5–14) showed no ability to stimulate B61/62 (Fig. 6E), which is consistent with our results showing that this peptide was unable to activates apATRPR in the IP1 accumulation experiments. For active peptides, it is important to note that the concentrations of peptides used to elicit physiological responses in the isolated ganglia were higher than the EC_{50} values determined by IP1 assay in CHO-K1 cells overexpressing apATRPR. Peptide potencies can differ significantly among different overexpression systems (e.g., between different cell types) or when measuring downstream responses in biological tissues (38–40). Thus, the EC_{50} value generated from measuring IP1 accumulation in the overexpression system is not necessarily indicative of the concentration needed to achieve physiological response from intact ganglia.

The physiological experiments on B61/62 activity were performed using CNS preparations of buccal and cerebral ganglia. Consistent with these electrophysiology results, real-time PCR experiments demonstrated that apATRPR was expressed in the buccal and cerebral ganglia (Fig. S10). However, we cannot exclude the possibility that the activities of ATRP and Conp analogs in B61/62 may also be mediated through receptors other than apATRPR, because there are several putative recep-
**ATRP is an active α-amino acid–containing peptide**

![Figure 5. Peptide activation of apATRPR, as determined by IP1 accumulation assay in CHO-K1 cells transiently transfected with apATRPR.](image)

**Figure 5.** Peptide activation of apATRPR, as determined by IP1 accumulation assay in CHO-K1 cells transiently transfected with apATRPR. A, representative experiment showing dose-response curves for P3H-ATRP, P3C-2-ATRP, P3C-2-Comp, or GdFFD. Each point represents the value from an individual well on the plate, with each condition run in duplicate. B, potency values for peptides tested for activation of apATRPR, as determined by IP1 accumulation assay. For active peptides, log E₅₀ values are reported as the means ± S.D. from at least three independent experiments, rounded to one significant figure. For inactive peptides, the log E₅₀ is listed as being greater than the highest concentration tested, from at least two independent experiments (see Fig. 5 for all dose-response curves used to calculate potency values). C, for peptides that were agonists of apATRPR shown in B, log E₅₀ values were compared via one-way analysis of variances and Bonferroni post hoc test. **, *p < 0.01; ***, *p < 0.001. For each peptide, each point represents the log E₅₀ from an independent experiment, and lines represent means for these points. Note that peptides that showed no activity are unable to be compared in this analysis.

Discussion

We have identified a novel DAACP, P2-ATRP, from the CNS of *Aplysia*, and have identified a GPCR, named apATRPR, which is activated by both L- and P2-ATRP. To our knowledge, apATRPR represents only the second receptor for endogenous cell–cell signaling DAACPs identified, after the achatin-like neuropeptide receptor, which also has a homolog in *Aplysia* (14, 15). Like many other DAACPs that have been identified (3, 4, 6, 7, 14, 21), both L- and P2-ATRP are present in ganglia extracts. This finding is consistent with studies demonstrating that L- to P-residue isomerization occurs during the latter stages of prohormone processing and only in specialized cells (35). For toxins like P2-Comp (33), detected predominantly in the DAACP form, it is possible that proteases present in the venom may be responsible for degrading the remaining all-L-residue peptide that did not undergo L- to P-residue isomerization.

In contrast to the L- versus P-residue receptor specificity observed for several other DAACPs (14, 15), we found that the two diastereomers of ATRP were active both at an identified receptor, apATRPR (Fig. 5), and in physiological experiments on motor neuron B61/62 (Fig. 6). The selectivity of apATRPR for ATRPs (and not for truncated ATRP(5–14) or for GdFFD, another DAACP that occurs in this animal), along with the high homology between the ATRP/apATRPR sequences and other allatotropin/receptor sequences (14, 25), suggest that ATRPs are indeed cognate ligands for apATRPR. The discovery that both L- and P2-ATRP are agonists of apATRPR, along with results demonstrating that L- and P2-Comp (which differ from ATRPs at their N termini) are also agonists of the receptor, demonstrate that apATRPR is relatively tolerant of substitutions near the N termini of its ligands. However, the inactivity of ATRP(5–14) suggests that some component of the N-terminal region of ATRP (e.g. the peptide backbone) makes critical contacts with the receptor, even if the identity of the N-terminal residues is not critical. Alternatively, the N-terminal residues of ATRP may be important for maintaining ligand conformation, required for activation of apATRPR. Identifying the role of the
N terminus in apATRPR activation may be addressed in future studies. Ultimately, these findings suggest that both L- and D2-ATRP are active in *Aplysia*. Our study constitutes one of few examples known of neuropeptides where both the DAACP and its all-L-residue diastereomer are active (Table S1) and is the first to demonstrate activity for both diastereomers at both the receptor and circuit levels.

In contrast to many other DAACPs, which appear to be restricted to a single phylum (1, 4, 5, 41–43), allatotropins and allatotropin-related peptides are present across several different phyla. Allatotropins play a variety of roles in a number of invertebrates, and recent phylogenetic analysis has revealed orthology between allatotropin neuropeptides and their receptors with vertebrate orexins and orexin receptors (24). The finding that ATRP exists as a DAACP in *Aplysia* immediately suggests that other allatotropins or allatotropin-related peptides may exist as multiple diastereomers in other phyla, such as insects or annelids. Determining whether other allatotropin-related peptides are present as DAACPs may provide insights into the evolution of L- to D-residue isomerization throughout the metazoan.

We chose to investigate the stereochemistry of ATRP because of its high sequence similarity to D2-Conp, a neuropeptide-like DAACP toxin found in the venom of the predatory cone snail *C. vitulinus* (33). Like allatotropins, D2-Conp was found to be myoactive in mollusk buccal mass preparations, whereas L-Conp had moderately weaker myoactive properties in *Pomacea bridgesii*, although the receptor mediating signaling in this species has not been identified. Our results show

**Figure 6. Modulation of B61/62 excitability by peptides.** A–E, representative electrophysiology traces showing the excitability of B61/62 under different conditions, tested using 3-s current pulses applied every 30 s in high-divalent saline, for 13C-L-ATRP (A), 13C-D2-ATRP (B), 13C-L-Conp (C), 13C-D2-Conp (D), and 13C-ATRP(5–14) (E). Control, activity before peptide perfusion. Wash, activity after washout of peptide. F, collected data comparing the number of spikes for B61/62 during 3-s current pulses under different conditions. Each point represents the average of two technical replicates in an independent experiment for 13C-L-ATRP (F3, 12) = 18.92, p < 0.001, n = 5), 13C-D2-ATRP (F3, 12) = 36.41, p < 0.001, n = 5), 13C-L-Conp (F3, 12) = 17.60, p < 0.001, n = 5), or 13C-D2-Conp (F3, 12) = 17.94, p < 0.001, n = 5). The values were compared by repeated measures analysis of variances and Bonferroni post hoc test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
ATRP is an active α-amino acid–containing peptide

![Hemolymph plasma stability](image)

Figure 7. Stability of 20 μM peptides in Aplysia hemolymph plasma at room temperature, as determined by LC–MRM. Each point is derived from the peptide peak area in the LC–MRM chromatogram (normalized to the 1-min time point and to an internal standard) incubated in the hemolymph of one animal, run in two biological replicates. The curves show the fit of the data to an exponential decay model. All points come from the same experiments and are plotted on separate panels (A–C) for clarity.

that L- and D2-Comp are ligands for the identified receptor apATRPR and can also act in the CNS, suggesting that ATRP receptors may be molecular targets for D2-Comp during prey capture. Consistent with this hypothesis, we have previously shown that L-ATRP is myoactive on Aplysia muscle (23). Interestingly, in contrast to a previous report showing that D2-Comp was more myoactive than L-Comp on mollusk muscle, we found that L-Comp and D2-Comp were similarly potent at directly activating apATRPR and that both L- and D2-Comp activated motoneurons B61/62 to similar levels. The difference between the modulating activities for L- and D2-Comp at B61/62 versus their previously reported activities on mollusk muscle may reflect differences between the species used in the experiments (A. californica versus P. bridgesii) (24) or differences in receptor expression in CNS versus muscle tissue. It is also interesting to note that both L- and D2-ATRP showed similarly high levels of activity at modulating B61/62 neuron excitability, whereas L- and D2-Comp were each weaker at modulating B61/62 excitability, despite the similar EC_{50} values for D2-ATRP and L-Comp at activating apATRPR in the IP1 accumulation assay. This discrepancy between the relative potencies in the IP1 accumulation assay in CHO-K1 cells transfected with apATRPR versus activity in living Aplysia neurons in intact ganglia may indicate that there are additional receptors for ATRPs in the Aplysia CNS with somewhat different specificities than the one investigated here. Indeed, our initial BLAST search to identify apATRPR indicated multiple proteins with similar sequences, strongly suggesting that there are additional receptors for these ligands (Fig. S6). In addition, these ligands might also differentially activate distinct signaling pathways through the same receptor (e.g. G protein signaling versus β-arrestin signaling), which may lead to differences in physiological outcomes (44, 45). It will be of great interest to examine the differences in ligand specificity and gene expression of the different putative ATRP receptors and to fully characterize the signaling pathways activated by different ligands in future studies.

Compared with other functional DAACPs identified from Aplysia (e.g. GdFFD, NdWF-NH2 (5, 7, 8)), ATRP is a relatively long peptide (14 residues). Thus, the D-Phe residue at position 2 in DAACPs would be predicted to provide protection from aminopeptidases (8, 34) but likely not from endopeptidases or carboxypeptidases, which would be able to attack sites distant from the D-residue. The much higher stability of D2-ATRP relative to L-ATRP in plasma is consistent with the predominantly aminopeptidase activity previously reported in hemolymph (46) and suggests that D2-ATRP is significantly more stable to endogenous aminopeptidases than is L-ATRP. In addition to their known presence in the hemolymph plasma, membrane-bound aminopeptidases are also found to be significant contributors to neuropeptide degradation in the CNS (46–49). Together, these observations suggest that one of the primary roles of L- to D-residue isomerization in the case of ATRP is to protect this peptide from degradation by aminopeptidases present in the extracellular space (50). Although CNS membranes also contain endopeptidases in addition to aminopeptidases (51), stability to aminopeptidases likely provides regulation of signaling duration among different cells or tissues (50). Furthermore, the stability of D2-ATRP may also promote its ability to diffuse longer distances to signal at receptors distant from the point of neuropeptide release (52–54). It is also possible that D2-ATRP may act as an inhibitor of aminopeptidases to alter the signaling duration of other neuropeptides released in the same vicinity, as has been shown for opiorphin (55, 56). Despite its high stability in hemolymph plasma, D2-ATRP was readily degraded in soluble ganglia homogenate (which contains intercellular enzymes but likely no membrane-bound enzymes), demonstrating that the nervous system is capable of degrading this molecule to terminate the signal, as is required for cell–cell communication.

D2-ATRP’s stability in plasma also suggests that ATRP may play more hormonal roles in the physiology of Aplysia, and the D-residue provides extra stability to prolong signal lifetime. Consistent with this hypothesis, insect allatotropins have been determined to have both endocrine and paracrine roles (19–22), and several crustacean DAACPs act as hormones (4, 41, 57, 58). In fact, both diastereomers of crustacean hyperglycemic hormone (CHH) were found to induce a hyperglycemic response but differed in the time course of their physiological
actions (4). However, in contrast to our study on ATRP, the activity of CHH diastereomers has not been evaluated at their receptor, so it is not known whether the physiological differences attributed to L- and D-CHH result from receptor activity, biological stability, or both. Our results suggest that L- and D2-ATRP may behave similarly to the CHH diastereomers in that both are physiologically active yet may produce different responses in vivo and that proteolytic stability may contribute to these differences.

Overall, our results add critical information with regards to the functions of DAACPs, their receptors, and the role of L- to D-residue isomerization in cell-to-cell signaling. Importantly, the similar activity for both L- and D2-ATRP at their cognate receptor apATRPR has two major implications. First, functionally, it indicates that L- to D-residue isomerization may not always be critical for receptor activity but may still have important implications for biological stability and the duration of signal lifetime in the extracellular space. Second, practically, because most DAACPs have been identified when the synthetic all-L-residue analog fails to recapitulate the bioactivity of the endogenous compound, the finding that both L- and D2-ATRP are present and active at the receptor and in physiological experiments suggests that even “well characterized” peptides may exist as multiple diastereomers. Therefore, the presence of DAACPs is likely underestimated. Our results emphasize an important need to develop unbiased tools to identify DAACPs without activity-guided screening, as well as to add DAACP screening to more standard LC–MS peptidomic measurements.

Experimental procedures

General

NovaPEG Rink Amide resin was purchased from NovaBiochem (855047). Protected amino acids were purchased from Novabiochem, Chem Impex, and AnaSpec. Fmoc-^{13}C-Gly-OH was purchased from AnaSpec (61572). Benzotriazole-1-yl-oxytris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was purchased from Novabiochem. 1-Hydroxybenzotriazole was purchased from AnaSpec. Oligonucleotide primers were purchased from Integrated DNA Technologies. All other solvents and reagents used, unless specified differently below, were purchased from Thermo Fisher Scientific or MilliporeSigma. A. californica were obtained from the National Institutes of Health/University of Miami National Resource for Aplysia (Miami, FL) or Marinus Scientific (Long Beach, CA). The animals were maintained at 14 °C in an aquarium filled with Instant Ocean (Aquarium Systems Inc.). Before dissection, the animals were anesthetized by injecting 50% (volume/body weight) 333 mM MgCl₂ (e.g. 50 ml MgCl₂ solution for a 100-g animal) into the body cavity.

Peptide synthesis

Peptides were synthesized using Fmoc solid-phase peptide synthesis using NovaPEG Rink Amide resin. For each coupling reaction, the resin was treated with a solution of 4 molar equivalents Fmoc-protected amino acid (with the appropriate side-chain protecting group) activated with 4 molar equivalents of PyBOP and 8 molar equivalents of N,N-diisopropylethylamine in a solution of 0.1 M 1-hydroxybenzotriazole in N-methyl-2-pyrrolidone and stirred at room temperature for 40 min to overnight. After coupling, the resin was washed three to five times with dimethylformamide (DMF). For deprotection of the Fmoc protecting groups, the resin was treated with a solution of 20% piperidine in DMF while stirring at room temperature for 20 min. After each deprotection reaction, the resin was washed three to five times with DMF. After the assembly of the peptide chain, the peptides were cleaved from the resin, and side-chain protecting groups were removed with a solution of 95% TFA, 2.5% triisopropylsilanate, and 2.5% H₂O₂ for >3 h. The peptides were then precipitated with cold methyl tert-butyl ether and purified by reversed-phase HPLC. Peptide purity was assessed by analytical-scale HPLC, and peptide identity was confirmed by MALDI–TOF MS (see “Peptide characterization” in the supporting information). GFFD and GdFFD, used for controls in several experiments, were synthesized as previously described (15).

Analysis of neuropeptides by LC–MS

For a typical experiment, two or three A. californica (70–160 g) were dissected, and ganglia were placed into 500 μl of acidified acetone (40:6:1 acetone:H₂O:concentrated HCl) on ice. The ganglia were cut into smaller pieces with scissors and then homogenized with a pestle and centrifuged (14,000 × g, 20 min, 4 °C). The supernatant was dried and then resuspended in 500 μl of H₂O or 0.1% TFA or 500 μl of H₂O or 0.1% formic acid (FA) + 0.01% TFA and desalted using HyperSep C18 Cartridges (Thermo, 60108–302). Importantly, because ATRP analogs bound C18 relatively weakly (i.e. were early eluting), salts and nonbinding compounds were washed with H₂O or 0.1% FA (without organic solvent) to reduce sample loss, and then peptides were eluted using a stepwise gradient of 25% methanol/H₂O, 50% methanol/H₂O, and 75% methanol/H₂O. These eluted fractions were combined and dried, and the resulting extracts were dissolved in H₂O or 0.1% FA (20 μl/animal) and centrifuged (>15,000 × g, 5 min) prior to analysis by LC–QTOF using a Dionex Ultimate 3000 RSLC with a nano-flow selector (Thermo) coupled to a Bruker Impact HD UHR-QqTOF mass spectrometer with a CaptiveSpray NanoSource. For the experiments shown in Figs. 2 and 4, 4.5 μl of peptide extract was spiked with either 2 μl of H₂O (for Fig. 2) or 2 μl of 250 nm {^{13}}C-ATRP analog (for Fig. 4), and 5 μl of the resulting solution was injected onto the LC–QTOF system. For the experiment shown in Fig. S5, {^{13}}C-L-ATRP was spiked into the acidified acetone solution prior to animal dissection (final concentration = 20 nm peptide in 500 μl of acidified acetone), and then the samples were processed as described above.

For LC separations by LC–QTOF, the sample was loaded onto a C18 Acclaim PepMap precolumn trap (5 μm; Thermo Scientific; part 164564-CMD) with a loading solvent of 98% H₂O, 2% acetonitrile (ACN), 0.1% FA, 0.01% TFA with a flow rate of 15 μl/min for 3 min. The line was then switched to an Acclaim PepMap RSLC column (C18, 75 μm × 150 mm, 2-μm
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The sample was separated using a gradient elution with the following conditions: solvent A = 95% H₂O, 5% ACN, 0.1% FA; solvent B = 95% ACN, 5% H₂O, 0.1% FA; flow rate = 300 nl/min; temperature = 30 °C; gradient = 0–3 min, 4% B; 3–90 min, 4–50% B; 90–93 min, 50–90% B; 93–100 min, 90% B; 100–110 min, 90–4% B; 110–120 min, 4% B.

For MS and MS/MS (collision-induced dissociation fragmentation) analysis by LC–QTOF, data were acquired in positive ion mode over a range of 300–3000 m/z with an MS scan rate of 1 Hz and a variable MS/MS scan rate of 1.5 Hz for high intensity (over 1,000,000/1000 sum) ions. For each MS scan, five precursors were selected for MS/MS in a data-dependent manner with a preferred mass list including m/z 511.45–512.45 (corresponding to endogenous ATRP with z = +3) and m/z 766.89–767.89 (corresponding to ATRP with z = +2). To plot extracted ion chromatograms, the data were exported into the .mzML format using Bruker Compass DataAnalysis 4.2. These .mzML files were then opened in TOPPView 2.3 (.mzML format using Bruker Compass DataAnalysis 4.2). These .mzML files were then opened in TOPPView 2.3 (59, 60), and the visible data range was set to the mass window of interest. The resulting visible layer data for the chromatogram was saved as an .mzML file, which was then imported into OriginPro 2018 for plotting. The resulting graphs were exported in the .eps format and imported into Adobe Illustrator to generate the final figures.

APM digestion of peptide extracts

Six *A. californica* (80–130 g) were dissected, and peptides from the cerebral ganglia were extracted in acidified acetone, as described above. After evaporation of the acidified acetone solution, the dried peptide extracts were dissolved in 300 μl of APM buffer (25 mM Tris, 500 mM NaCl, pH 7.5), and the solution was adjusted to pH 7.5, as judged by pH paper. The solution was split into two conditions: “Extracts” = 140 μl of peptide extracts + 10 μl of APM buffer, or “Extracts + APM” = 140 μl of peptide extracts + 10 μl of aminopeptidase M (EMD Millipore, 164598–50U, final activity = 0.37 unit/ml) in APM buffer. These solutions were incubated at 37 °C for 15 h. After incubation, the solutions were acidified by the addition of 1% TFA and desalted with HyperSep C18 Cartridges, as described above. For each condition, the eluted fractions were combined, dried, resuspended in 40 μl of H₂O + 0.1% FA, and centrifuged (13,200 × g, 5 min). The supernatant (5 μl) was analyzed by LC–QTOF, as described above. Identification of the ~27 min peak in Fig. 3 was carried out using PEAKS Studio 8.0 (Bioinformatics Solutions Inc.). For this analysis, the compounds were identified using the AutoMS(n) tool in Compass DataAnalysis 4.2, exported as .mgf files, loaded into PEAKS Studio 8.0, and searched against an *A. californica* prohormone database maintained in our laboratory.

Cloning of apATRPR

*A. californica* (120–200 g) were dissected, and RNA from the ganglia were extracted using TRIzol reagent (Invitrogen, 15596018), exactly as previously described (15), using aqueous solutions prepared with nuclease-free H₂O or H₂O treated with diethyl pyrocarbonate. Synthesis of cDNA was carried out using the iScript cDNA synthesis kit (Bio-Rad, 1708890), using 2 μg of RNA for each tissue in a 40-μl scale reaction. After synthesis, the reaction was diluted to 100 μl with H₂O. cDNA samples were stored at -20 °C until use.

cDNA from the five major ganglia (buccal, cerebral, pedal, pleural, and abdominal) were combined, and the predicted ORF of *apATRPR* (XM_005106157.2) was amplified by PCR using a Phusion high-fidelity DNA polymerase kit (New England Biolabs, E0553S). The primers are as follows: forward, 5′-CCTCGAGTCC-AACATGGGCTGAACGATAC-3′; and reverse, 5′-CTCGC-GGCCGGTTATGACGACCGGCTGAAG-3′. The resulting PCR product was purified from agarose gel with a Zymo DNA clean and concentrator kit with agarose dissolving buffer and then ligated into pMiniT 2.0 vector using an NEB PCR cloning kit (New England Biolabs, E1202S) following the manufacturer’s instructions. The resulting plasmid was transformed into NEB 10-β competent *Escherichia coli* (provided with the above kit) using heat shock and grown on Lennox formulation broth (LB: 10 g/liter tryptone, 5 g/liter NaCl, 5 g/liter yeast extract)-agar plates supplemented with 100 μg/ml ampicillin at 37 °C. A single colony was grown in 5 ml of LB with 100 μg/ml ampicillin at 37 °C overnight with shaking, and then the plasmid was isolated with a mini-prep kit (Qiagen, 27104). The resulting plasmid containing the ORF for *apATRPR* and pcDNA3.1(+) vector were each double digested with BamHI-HF (New England Biolabs, R3136S) and NotI-HF (New England Biolabs, R3189S). The bands corresponding to the *apATRPR* and the pcDNA3.1(+) were each purified from an agarose gel using a Zymo DNA clean and concentrator kit. The restriction enzyme-digested insert containing the *apATRPR* sequence (22 fmol) was ligated to the restriction enzyme-digested pcDNA3.1(+) vector (66 fmol) with T4 DNA ligase (New England Biolabs, M0202L) in rapid ligation buffer (2 × concentrations = 132 mM Tris-HCl, 20 mM MgCl₂, 2 mM DTT, 2 mM ATP, 15% PEG 6000, pH 7.6) at room temperature for 10 min. The ligation product was then transformed into chemically competent *E. coli* (DH5α) using heat shock and then grown on LB-agar plates with 100 μg/ml ampicillin at 37 °C overnight. A single colony was grown in 5 ml of LB with 100 μg/ml ampicillin at 37 °C for 12 h with shaking, and then 2 ml of this starter culture was diluted into 200 ml of LB with 100 μg/ml ampicillin and incubated at 37 °C overnight with shaking. The plasmid was then isolated by maxi-prep (Omega Bio-tek, D6922). The insert sequence in the isolated plasmid was confirmed by Sanger DNA sequencing (Fig. S7).

apATRPR activation assays

CHO-K1 cells (ATCC, CCL-61) were cultured in F-12K medium (Gibco, 21127–022) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (HyClone, SV30010) at 37 °C in 5% CO₂. Cells were grown to 85–100%
confluence in 35-mm diameter tissue culture-treated dishes (Falcon, 353001) before transfection. For transfection (day 1), the medium was exchanged for F-12K medium with 10% FBS and no antibiotics. For each dish, 2 μg of apATRPR plasmid (in pcDNA3.1(+) vector) was mixed with 200 μl of Opti-MEM (Gibco, 11058021), followed by the addition of 8 μl of Turbofect (Thermo Fisher Scientific, R0531), and the mixture was incubated at room temperature for 15 min. After this incubation, the DNA/Turbofect mixture was added dropwise to the cell dish, and the cells were incubated at 37 °C in 5% CO₂ overnight. The next day (day 2), the cells were trypsinized and reseeded in opaque white 96-well half-area tissue culture-treated plates (Corning, 3688) at a density of 20,000 cells/well in F-12K and 10% FBS and allowed to recover at 37 °C in 5% CO₂ overnight.

Activation of apATRPR (day 3) was detected by monitoring IP1 accumulation using IPOne detection kit (Cisbio, 62IPA-PEB). The medium was removed from each well and replaced with 14 μl of stimulation buffer (provided with the kit). Appropriate peptide dilutions (14 μl in stimulation buffer) were then added to each well. When making serial dilutions of peptide, we found it was critical to exchange pipette tips in between each dilution. The plates were then incubated at 37 °C in 5% CO₂ for 1–1.5 h. After this incubation, 6 μl of IP1-d2 and 6 μl of anti–IP1-cryptate in lysis buffer were added to each well and incubated at room temperature for at least an hour, using 0.5× the manufacturer’s recommended concentration of IP1-d2 and anti–IP1-cryptate. After this incubation, homogeneous time-resolved fluorescence signal from the IPOne detection kit was read using an Analyst HT plate reader and the following settings: optics = top, dichroic mirror = 400 nm, excitation at 330 ± 40 nm, emission at 665 ± 5 nm and at 620 ± 17.5 nm, Z height = 1.2 mm, 100 flashes/well, 400 μs of integration time, 10-ms interval between flashes, 50-μs delay after flash, raw data units = counts, attenuator mode = out, PMT setup = digital. IP1 standards (10.7–11,000 nM) were included in each experiment, and the ratio of fluorescence intensities for 665 nm/620 nm was fit to a sigmoidal dose response (four parameters) in GraphPad Prism 7 to generate a standard curve. The resulting IP1 standard curve was used to calculate the experimental IP1 concentration in each well based on the 665-nm/620-nm ratio.

IP1 accumulation data were then fit to a sigmoidal dose response (three parameters) in GraphPad Prism 7 to calculate EC50 values. LogEC50 values reported in the main text are the means ± S.D. from at least three independent experiments. Inactive peptides were tested in at least two independent experiments. All dose-response curves used to generate values reported in the main text are shown in Fig. S59. For active peptides shown in Fig. 5, mean values were compared using one-way analysis of variances with Bonferroni post test in GraphPad Prism 7.

Electrophysiology

Recordings of the physiological activity from CNS preparations of cerebral and buccal ganglia were performed as described previously (23). Recordings were made in high-divalent saline (368 mM NaCl, 10 mM KCl, 18.3 mM CaCl₂, 101 mM MgCl₂, 10 mM HEPES, pH 7.6) to suppress polysynaptic pathways. For each experimental condition on a given CNS preparation, 3-s current pulses were applied and repeated every 30 s to form technical replicates. After recordings stabilized, the values for two technical replicates were averaged to give the number of spikes for that experiment, plotted as an individual point in Fig. 6F. Mean values for each condition across five independent experiments were compared using repeated measures one-way analysis of variances, assuming sphericity, with Bonferroni post test in GraphPad Prism 7.

Peptide stability assays

For peptide stability assays in Aplysia plasma, Aplysia (70–100 g) were placed in artificial sea water and cooled in a ~20 °C freezer for ~1 h to anesthetize. The animal was then pinned to a dissection plate and placed vertically to allow hemolymph to collect in the head region. Hemolymph was then extracted from the animal by puncturing just behind the head with a needle and collecting the hemolymph in a clean tube. The animal was immediately sacrificed after hemolymph collection. The hemolymph was centrifuged (15,000 × g, room temperature, 12 min), and the plasma (supernatant) was moved to clean tubes. A stock solution containing 333 μM of each peptide was prepared in H₂O. The peptide stock (30 μl, final concentration = 20 μM each peptide) was added to the plasma (470 μl), and the resulting mixture was incubated at room temperature.

At each time point during a digestion experiment, 10 μl of the reaction mixture was removed and quenched with 20 μl of a solution containing 10 μM GdYFD (as an internal standard) in 50% methanol/H₂O + 1% TFA and stored at −20 °C until analysis. For analysis, each quenched time point was thawed; diluted to ~0.1% TFA, 0.1% FA in 4% methanol/H₂O; and desalted with C18 solid-phase exchange centrifuge spin columns (Thermo Fisher Scientific, 89870). Desalted and dried samples were dissolved in H₂O + 0.1% FA and analyzed using a Bruker EVOQ Elite triple-quadrupole mass spectrometer coupled to a Bruker Advance UHPLC in MRM mode in positive ion mode. Chromatograms were generated from the sum of multiple fragment ions. The parent ion (m/z, mass window, 0.7)/fragment ions (m/z, mass window, 2) used for each peptide were [13C]-ATRP = 512.6/(566.2, 580.8, 495.8, 110.1, 136.1); [13C]-Conp = 497.6/(136.1, 219.7, 237.6); GFFD = 485.2/(120.2, 281.1, 177.1), and GYFD = 501.2/(120.2, 281.1, 193.1). For each time point, the peak area in the resulting LC–MRM chromatogram for the peptide of interest was first normalized to the peak area for GdYFD, and the amount of peptide peak remaining subsequently calculated relative to the 1-min time point for each biological set. The data were plotted in GraphPad Prism 7.

ATRP is an active d-amino acid–containing peptide

Acknowledgment—We thank the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign for providing resources and instrument time, as well as general advice.
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