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Identifying receptors for neuropeptides and peptide hormones: challenges and recent progress

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Abstract

Intercellular signaling events mediated by neuropeptides and peptide hormones represent important targets for both basic science and drug discovery. For many bioactive peptides, the protein receptors that transmit information across the receiving cell membrane are not known, severely limiting these signaling pathways as potential therapeutic targets. Identifying the receptor(s) for a given peptide of interest is complicated by several factors. Most notably, cell-cell signaling peptides are generated through dynamic biosynthetic pathways, can act on many different families of receptor proteins, and can participate in complex ligand-receptor interactions that extend beyond a simple one-to-one archetype. Here, we discuss recent methodological advances to identify signaling partners for bioactive peptides. Recent advancements have centered on methods to identify candidate receptors via transcript expression, methods to match peptide-receptor pairs through high throughput screening, and methods to capture direct ligand-receptor interactions using chemical probes. Future applications of the receptor identification approaches discussed here, as well as technical advancements to address their limitations, promises to lead to a greater understanding of how cells communicate to deliver complex physiologies. Importantly, such advancements will likely provide novel targets for treatment of a number of human diseases within the central nervous and endocrine systems.

Graphical Abstract

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Introduction

Neuropeptides and peptide hormones are cell-to-cell signaling molecules abundant in the central nervous system (CNS) and endocrine system, where they act as key modulators of physiological processes such as body temperature,^{1, 2} energy homeostasis,³ circadian rhythm,⁴ and more.⁵ Mimicking or antagonizing peptide signaling represents a potential strategy for treating a number of human diseases, including epilepsy,⁶ depression,^{7, 8} diabetes,⁹ osteoporosis,¹⁰ and others.^{11, 12} Advances in mass spectrometry (MS)-based “peptidomics” methodologies has enabled the confident identification of a large number of endogenous peptides that correlate or appear to directly regulate normal physiology or disease states.^{13–15} However, for many functional peptides discovered, the receptor protein(s) responsible for transmitting their signals are not known.^{16–27} A lack of validated receptors for bioactive peptides severely limits the ability to rationally design drug molecules targeting these pathways.

The purpose of this review is to discuss two subjects critical to identify the receptor proteins for a given bioactive peptide. First, we outline some of the challenges associated with determining receptors for cell-cell signaling peptides, including those arising from their unique biosynthesis and complicated ligand-receptor interactions. We highlight that peptide-receptor interactions are often much more complex than a simple one peptide-one receptor paradigm, and that peptides may activate a number of classes of receptor other than “orphan” G protein-coupled receptors (GPCRs). These possibilities make identifying the receptor for a given peptide fundamentally distinct from research efforts that aim to “deorphanize” GPCRs.²⁸ Second, we provide an overview of recent methodological advances that have proven successful for identifying functional receptors for peptides of interest. Although this review focuses on endogenous cell-cell signaling peptides, many of the approaches described can also be applied to other ligands without known receptors, such as natural products or toxins. Recent progress and future work identifying receptors for bioactive peptides promises to open up new avenues of exploration in understanding normal cellular communication and in treating diseases caused by signal dysregulation.

Neuropeptide and peptide hormone discovery

Both neuropeptides and peptide hormones are generally synthesized from larger precursor peptides that undergo extensive post-translational modifications (Figure 1). In most cases, an amino acid sequence (termed a “signal sequence”) in the precursor targets the newly translated protein to the secretory pathway, where it is transported via secretory granules to the point of release on the outer membrane. Beginning during translation and continuing during trafficking, propeptides undergo a series of proteolytic processing steps to generate several smaller peptides, which generally involves cleavage at dibasic (e.g., KK, KR, etc.) or monobasic sites by endopeptidases (e.g., PC1/3, PC2)^{29, 30} followed by subsequent “trimming” by exopeptidases (e.g., carboxypeptidase E).^{31, 32} In addition to these well-established processing pathways, enzymes such as furin,^{33–35} carboxypeptidase D,³⁶ and cathepsins L, V, and H,^{13, 37–39} may also be involved in processing a subset of peptides, although the full roles of these enzymes in neuropeptide and peptide hormone processing remain unclear and warrant further study. Full maturation of cell-cell signaling peptides

can also include side-chain and backbone modifications, including C-terminal amidation,⁴⁰ terminal and side-chain acylation,^{41, 42} phosphorylation,⁴³ sulfation,^{43–45} glycosylation,⁴⁶ and others.^{13, 47–49} These post-translational modifications (PTMs) are often essential for the known functions of the mature peptides; deficiency in enzymes responsible for these PTMs often produce severe phenotypes^{50, 51} and exogenous administration of synthetic peptides lacking such PTMs often have significantly reduced biological activity.^{41, 52, 53}

The biosynthetic pathway common to most cell-cell signaling peptides leads to a number of important consequences that set these molecules apart from many other proteins. First, a given precursor often generates a number of unique mature peptides, many of which can have distinct functions.^{5, 13} Furthermore, precursor processing can differ significantly between tissues or across different physiological states. For example, the proopiomelanocortin (POMC) precursor is processed into over 10 distinct peptides in the pituitary, and the peptides produced differ significantly between the anterior and intermediate lobes.^{42, 54} The peptides produced from this differential processing then proceed to act on distinct subsets of receptors, ultimately leading to different physiological functions.⁵⁴ Similarly, proglucagon generates a number of distinct peptides in a tissue-dependent manner, including glucagon and glucagon-like peptide 1 (GLP-1), and these peptides activate distinct receptors.⁵⁵ Bioactive peptides can also be generated through pathways outside of the canonical secretory pathway, such as the hemorphins and hemopressins (generated from proteolytic cleavage of hemoglobin).^{56, 57} As another example of non-canonical signaling peptides, small open reading frames (smORFs) generate bioactive peptides through mechanisms distinct from secreted peptides,^{20, 21} and can play roles in cell-cell signaling.⁵⁸ However, because of their small size (<100 codons), smORFs are usually excluded from gene annotations and smORF-encoded peptides often evade detection in standard proteomic searches, suggesting that the majority of smORF-encoded peptides have yet to be fully characterized. As a result of the complexity of biosynthesis and post-translational processing, mature peptide products generated from a given tissue can rarely be predicted based on genome or transcript information alone, but instead must be characterized using techniques that can directly measure a peptide's final form, including all PTMs.

Mass spectrometry is currently the most comprehensive tool employed to identify and characterize peptide signaling molecules.^{13–15} Importantly, MS-based peptidomics analysis allows for the non-targeted identification of peptides from biological tissues, and the high resolving power of modern mass spectrometers allows for determination of precise sequence composition, including nearly all post-translational modifications. Most peptidomics workflows utilize computational algorithms to match experimental MS and MS/MS data to predicted spectra generated from genome- or transcriptome-derived protein databases, a method termed “probability sequence matching”.¹⁴ However, probability sequence matching requires a well curated and accurate protein database with which to generate the predicted peptide fragmentation spectra, and is unable to identify peptide matches not present in the database (including mutations). A distinct peptide sequencing approach, *de novo* sequencing, generates experimental sequence tags directly from experimental MS and MS/MS data, and then queries these tags against the target protein database.^{14, 59, 60} Because *de novo* sequencing first generates peptide sequences

based solely on the experimental data, this method allows the identification of peptides not present in the search databases, including mutations and errors in the deposited protein sequences. Advances in sampling have enabled characterization of peptides within specific tissues,^{61–63} released from tissues into extracellular space,^{4, 64} from an individual cell,^{65, 66} and even within individual organelles.⁶⁷ Thus, the technical advancements in measurement science coupled to bioinformatics have enabled the identification and quantification of a large number of endogenous peptides from a wide array of organisms and tissues. Because these advancements in peptide identification tend to detect peptides based on abundance rather than specific functions, many peptides discovered through peptidomics approaches do not have well-understood biological activity at the time of their discovery.^{16, 21} One of many key pieces of information that contributes to a full understanding of a cell-cell signaling peptide's biological function is insight into what receptor proteins are engaged and activated to transmit signals. However, identifying receptor proteins for a given endogenous peptide is often complicated by several factors arising from the biology of these molecules.

Challenges in neuropeptide and hormone receptor identification

The unique biosynthetic, structural, and functional properties of precursor-derived peptides offer a number of challenges for identifying receptor proteins that differ from those of many other classes of protein-protein interactions. For one, a single precursor often gives rise to many different peptide sequences (distinct peptides and alternatively processed forms), which can have distinct biological functions and receptor specificities (Figure 1). For many precursors, there is a bias by researchers to focus on one or two peptides with very obvious bioactivity, while other peptides generated from the precursors are considered byproducts from biosynthesis or completely ignored. However, these understudied peptides may prove to play important roles in cell-cell signaling. For example, research on proenkephalin-A has focused primarily on Met-enkephalin and Leu-enkephalin, potent opioid peptides that are agonists of the δ -opioid receptor (δ OR). Over 30 years after the discovery of Met-enkephalin and Leu-enkephalin,⁶⁸ proenkephalin-A-derived bovine adrenal medulla peptide 22 (BAM22) was found to be a potent agonist for previously orphan receptors in the Mas-related GPCR family termed “sensory neuron-specific GPCRs” (SNSRs), which are localized to sensory neurons in the dorsal root ganglia and are potential targets for the treatment of chronic pain.^{69–71} Interestingly, BAM22 has also been shown to interact with SNSR- δ OR heterooligomers to activate SNSR signaling while simultaneously inhibiting δ OR signaling,⁷² despite this peptide being a potent δ OR agonist when this receptor is expressed alone.⁶⁹ In addition, not every peptide produced from a given precursor is expected to signal through a canonical ligand-receptor interaction, and some may serve alternative functions. As some examples, the C peptide from proinsulin is well-known to play a critical role in insulin folding,⁷³ and the peptide opiorphin's primary role is suspected to be as a protease inhibitor.^{16, 74} Nevertheless, a number of well-studied precursors produce peptides which appear to have biological activity, but for which no receptor has been unambiguously identified, including several peptides from the proSAAS precursor,¹⁶ cerebellin peptide,^{17–19} several granin-derived peptides,²² glicentin-related pancreatic polypeptide,²³ synenkephalin,²⁴ GnRH-associated peptide 1,²⁵ N-terminal POMC peptides,²⁶ and others.^{16, 27}

In addition to the fact that precursors often generate many peptides, neuropeptide and peptide hormone signaling is complicated by complex peptide-receptor dynamics (Figure 2). Although some peptide-receptor systems adhere to a simple one peptide-to-one receptor paradigm, most families feature multiple discrete ligands and several distinct receptors, each with specific selectivities and biological functions. A recent analysis of known human peptide-GPCR interactions found that each receptor responds to an average of 2.9 peptide ligands, while each peptide was able to activate an average of 1.9 receptors.⁷⁵ For example, POMC generates several distinct peptides, including adrenocorticotrophic hormone (ACTH), β -lipotropin (LPH), γ -LPH, α -melanocyte stimulating hormone (MSH), β -MSH, γ -MSH, β -endorphin, and several additional peptides with less well-defined functions.⁴² ACTH and the MSH peptides can activate five different melanocortin receptors (MCRs), with each of these receptors having distinct selectivities for each peptide, while β -endorphin potentially activates both the μ -opioid receptor and the δ -opioid receptor. An individual peptide can also engage multiple unrelated receptors. For example, the peptide hormone relaxin-3, which was initially identified as a ligand for the receptor LGR7,⁷⁶ was also found to interact with additional receptors GPCR135 and GPCR142 (now called RXFP3 and RXFP4).^{77, 78} Similarly, several opioid peptides have recently been demonstrated to interact with both the opioid receptor family (κ , δ , and μ)^{79, 80} and the atypical chemokine receptor ACKR3/CXCR7.^{81, 82} A single receptor can also engage multiple peptides from distinct precursors. For example, the parathyroid hormone receptor 1 (PTH1R) is potentially activated by peptide ligands generated from distinct precursors (parathyroid hormone and parathyroid hormone-related protein), and these ligands interact and induce different conformations of the receptor to enact distinct functions.⁸³ Similarly, the *Drosophila* sex peptide receptor is activated by peptides from two distinct families.⁸⁴ The complexity of neuropeptide and peptide hormone signaling strongly suggests that for some known and well-studied peptides and receptors, there may be additional interactions waiting to be discovered.

Only orphan GPCRs?

To identify the receptor for a given biologically active peptide, a common assumption is that the peptide must act through an “orphan” GPCR, a term denoting the over 100 GPCRs that do not have known endogenous ligands.¹⁶ This assumption is rooted in the notion that signaling systems follow a one ligand-to-one receptor rule, and thus a peptide of interest is likely to signal through a receptor that currently has no clearly identified ligand. However, as outlined above, there are many examples in which a given receptor engages several peptides from diverse precursors, indicating that even well-studied (non-orphan) receptors may recognize additional ligands not yet characterized. As a result, a given peptide of interest may signal through non-orphan receptors (Figure 3). Limiting a search to exclusively orphan receptors risks overlooking important interactions with established receptors which may be critical for biological function.

Another potential weakness in targeting exclusively orphan GPCRs for receptor identification is the assumption that peptides only interact through proteins in the GPCR family, and not with other families of membrane-bound receptors. Precursor-derived peptides can signal through a variety of different proteins that are not members of the GPCR family. For example, big-dynorphin (one of several peptides produced from prodynorphin)

is a highly potent modulator of the acid-sensing ion channel ASIC1a, in addition to the κ -opioid receptor.^{79, 85} Consistent with this observation, secreted peptides have been shown to be direct modulators of receptors other than GPCRs, including ion channels,^{86–89} receptor tyrosine kinases,⁹⁰ receptor guanylyl cyclases,^{91, 92} and others.^{93–95}

For many approaches to identify receptors for a given peptide, it is practical to use various criteria to narrow down candidate receptors, and examining only orphan GPCRs is a common strategy to generate this “short list”. Although there have been successes focusing exclusively on orphan GPCRs (some of which are discussed in this review), this strategy may miss physiologically important interactions. As new approaches to identify receptors for peptides are developed, methods should strive to remain relatively unbiased and actively explore many different classes of receptor proteins, both “well-studied” and “orphan”.

Identifying receptor candidates based on expression

When faced with a specific peptide of interest without a known receptor, a careful examination of gene expression between cell lines and tissues can be used to identify receptor candidates (Figure 4). For this approach, several cell lines or tissues that show response to the peptide (e.g., cAMP accumulation, Ca^{2+} influx, etc.) are collected and the mRNA expression for potential receptors compared. Receptors that are expressed in all reactive cells/tissues are then tested individually (usually in cell-based receptor activation assays) to determine if the candidate receptor is responsible for the signaling event.

Two important examples of using gene expression to guide receptor identification approach involve the identification of receptors for proSAAS-derived peptides PEN and BigLEN.^{96, 97} PEN and BigLEN are highly abundant peptides found in the hypothalamus with proposed roles in feeding regulation and metabolism. For each of these peptides, ligand binding and ligand-induced cell signaling experiments suggested receptors present on both the Neuro2A neuroblastoma cell line and on hypothalamic membranes. The signaling behavior in both the cell line and tissue were consistent with activation of a GPCR. The researchers then compared a list of orphan GPCRs enriched in both Neuro2A and in hypothalamus to generate a candidate list of receptors. For BigLEN,⁹⁶ four candidate GPCRs were each recombinantly expressed in Chinese hamster ovary (CHO) cells along with a promiscuous $\text{G}\alpha$ subunit and individually tested for activation by exogenous BigLEN. Of the four receptors tested, GPR171 showed a response consistent with agonism by BigLEN. The three other candidate GPCRs evaluated showed no such activity from BigLEN, highlighting the specificity of this identified interaction. Follow up experiments in this recombinant system, as well as in cell lines and animals expressing endogenous receptor, demonstrate GPR171 plays a major role in biological function BigLEN, including feeding behavior *in vivo*. For PEN,⁹⁷ five candidate receptors were identified and evaluated using the same process as described for BigLEN, and only one receptor (GPR83) was activated by exogenous PEN peptide. It is important to note that despite BigLEN and PEN arising from the same precursor these two peptides were found to activate distinct receptors, again highlighting the complexity of neuropeptide signaling. Interestingly, follow-up experiments demonstrated that GPR171 and GPR83 can interact and influence each other's signaling,⁹⁷ and these interactions may differ between tissues.

More recently, an expression-based approach was used to identify a receptor for maturation-inducing hormones (MIHs) in the jellyfish *Clytia hemispherica* responsible for oocyte maturation.⁹⁸ In this study, the researchers focused their search for MIH receptors on GPCRs due to prior evidence showing an increase in cAMP concentration upon MIH stimulation, consistent with signaling through a $G\alpha_s$ -mediated pathway. To identify potential MIH receptor candidates, these researchers first generated a list of all predicted GPCRs in this organism and then selected their candidate receptors from Class A GPCRs with transcripts enriched in oocytes. From this analysis, 16 candidate GPCRs were individually expressed in CHO cells along with a promiscuous $G\alpha$ protein subunit and tested for activation by a mixture of 33 predicted *Clytia* neuropeptides, including four MIHs. Of the 16 receptors tested, only one receptor (designated MIHR) was activated by the peptide mixture, and subsequent experiments demonstrated that this receptor was specifically activated by the four MIH peptides. Experiments utilizing *MIHR* knock-out jellyfish generated from CRISPR/CAS9 revealed critical roles for both MIHR and MIH peptides in oocyte maturation, consistent with the finding of MIHR as a critical MIH receptor.

Similar to the approaches described above to identify the receptors for PEN, BigLEN, and MIHs, an approach termed a “deductive ligand-receptor matching strategy” has been used to identify potential receptors for several peptides based on gene expression across cell types.^{99–103} Using PCR, the mRNA levels for orphan GPCRs are evaluated across several cell lines and tissues responsive to stimulation by the peptide of interest. Receptors which show mRNA expression in all responsive cell lines are then identified as initial candidates, and a further curated to generate a “short list” based on criteria such as sequence homology and tissue distribution. To evaluate candidate receptors, researchers employing this technique generally utilize siRNA-mediated knockdown of each candidate receptor, monitoring peptide-induced downstream signal. Using this approach, putative receptors have been identified for neuronostatin (derived from the somatostatin precursor),⁹⁹ the insulin C peptide,¹⁰⁰ phoenixin,¹⁰¹ adropin,¹⁰² and CART(55–102)/CART(65–102).¹⁰³ However, initial studies employing this deductive ligand-receptor matching approach often monitor signaling events that are significantly downstream from the ligand receptor interaction, such as changes in *cFos* mRNA expression,^{99, 102, 103} changes in ERK phosphorylation,¹⁰³ and *in vivo* effects.^{99–103} Furthermore, these initial studies rarely recombinantly express their candidate receptors in non-responsive cell lines to provide “gain-of-function” activity to peptide stimulation, as was performed in the PEN, BigLEN, and MIH examples described above. Due to these limitations, it is more difficult to determine if responses using this deductive ligand-receptor matching strategy are due to direct ligand-receptor interactions, or if the identified proteins influence the observed signaling indirectly through alternative mechanisms. In one case, follow-up studies using recombinant receptor provide conflicting data for a ligand-receptor pair identified through a primarily siRNA-based strategy (adropin with GPR19). Although Rao and Herr reported a adropin-mediated reduction of cAMP in HEK293 cells expressing GPR19,¹⁰⁴ Foster et al. report no activity for this ligand-receptor pair by dynamic mass redistribution or internalization assays using Flp-In T-Rex 293 cells expressing recombinant GPR19.⁷⁵ Although the origin of this discrepancy is unclear, this may suggest that the adropin-GPR19 interaction is more complex than a one-to-one peptide-receptor interaction. In some instances, follow-up studies demonstrating co-localization by

microscopy^{103, 105} or co-immunoprecipitation experiments¹⁰³ provide some evidence of a peptide-receptor interaction. However, in many cases further studies must be performed to more definitively validate putative peptide-receptor interactions identified using the deductive ligand-receptor matching strategy.

As described in the examples above, examining mRNA expression across cells and tissues has proven successful to identify receptors for specific peptides of interest. In contrast to high-throughput methods, the approaches discussed in this section tend to focus on one peptide ligand of interest, allowing for more careful follow-up studies to validate the ligand-receptor hit and examine its effects *in vivo*. Although in theory examining gene expression could be a non-targeted method to identify candidates, in practice researchers have generally limited their candidate receptors to orphan GPCRs when generating short lists to evaluate experimentally. As a result, any interactions between peptides of interest and non-orphan receptors (or receptors of other classes) will not be detected using these strategies.

Large-scale screening approaches

In principle, one method to identify a receptor for a given peptide would be to individually express every possible receptor protein and test for activation. In the spirit of this approach, high-throughput cellular screens have been developed to survey a large array of GPCRs for activation by a given peptide or collections of peptides (Figure 5). As one prominent example, a large-scale Tango β -arrestin recruitment assay was developed that facilitates the simultaneous screening of a compound of interest against over 300 GPCRs.¹⁰⁶ Advantages of this powerful platform include the ability to screen both orphan and non-orphan GPCRs, capability to screen for antagonists (if agonists are known), and open-access availability to the scientific community. However, this platform screens against GPCRs exclusively, so ligand interactions with non-GPCR proteins will be missed. In addition, any signaling systems that require co-receptors for function, such as the receptor activity modifying proteins,¹⁰⁷ would likely not be functionally active in this panel. Finally, it is important to note that over 50% of the GPCRs present in the panel (including all orphan GPCRs) were not able to be functionally validated.¹⁰⁶ Although a lack of validation does not necessarily preclude identification of putative agonists for these receptors, it does increase the likelihood of false negatives if using the platform to identify a receptor for a peptide of interest. Complementary to the individual expression approach, genetic knock-out screens can also identify novel ligand-receptor interactions by looking for loss of function upon ligand stimulation.^{108, 109} However, knock-out screens can suffer from limitations, including a dependency on endogenous receptor expression in the host cell line and inability to identify receptors with functional redundancy. Although fully comprehensive screens are powerful, in practice it is not possible to test every possible ligand with every possible receptor. An alternative to a truly comprehensive high-throughput screen is to use some criterion to preselect pools of candidate peptides and receptors for screening.

Invertebrates such as are *Platynereis dumerilii* are important systems to understand neurotransmission and its influence on behavior, as well as the evolution of cell-cell signaling systems.^{87, 110, 111} However, very few neuropeptide receptors have been identified in non-model organisms, limiting the utility of homology searching as a means to

identify receptors for given peptides of interest. To overcome this limitation, Bauknecht and Jékely recently employed a novel combinatorial strategy to match peptides with putative neuropeptide GPCRs.¹¹⁰ In this approach, 87 predicted *Platynereis* GPCRs were individually expressed in CHO cells along with a promiscuous Gα protein (to facilitate signaling through the phospholipase C pathway) and exposed to each of three synthetic peptide mixtures containing 32–48 predicted neuropeptides from *Platynereis*. For a given expressed receptor, an increase in intracellular calcium concentration in response to one of the three peptide mixtures suggested that an agonist was present in that mixture. Receptors showing hits in this initial screen were further subjected to peptide sub-mixtures to facilitate identification of the agonist peptide(s). Using this approach, these researchers identified 19 novel ligand-receptor pairs, as well as several additional pairs predicted based on orthology. Many of identified peptide-receptor pairings were not related to known peptide/receptor families, highlighting the importance of pursuing a relatively unbiased approach. Using a combinatorial screening strategy, these researchers were able to evaluate 10,962 peptide-receptor combinations without testing each pair individually. Similar strategies have subsequently been used to identify peptide-receptor pairs in other non-model organisms.^{112, 113}

Computational approaches can also provide valuable insight to identify putative neuropeptides and receptors prior to combinatorial screening. Foster et al. employed bioinformatics methods to predict candidate neuropeptides and receptors by taking advantage of common sequence motifs present in both.⁷⁵ Candidate peptide ligands were predicted from mining genomic information for specific sequence motifs (e.g., secretion signal sequences and dibasic sites) and evolutionary conservation, while candidate peptide receptors were predicted based on examining conserved sequence or structural motifs. Constructs encoding for putative peptide receptors were expressed in mammalian cell lines and evaluated for peptide ligand interactions using several different techniques. In total, 218 predicted peptides were screened against 21 predicted peptide receptors (in addition to known peptide receptors) and assayed for multiple signaling pathways associated with ligand binding or stimulation: mass redistribution, receptor internalization, β-arrestin recruitment, and second messenger stimulation. From these assays, peptide-mediated responses were identified for all 21 predicted peptide receptors, demonstrating the utility of this bioinformatics approach to accurately predict cell-cell signaling peptides and peptide receptors. Interestingly, some signals were only seen in a subset of assay platforms (e.g., mass redistribution and receptor internalization but not β-arrestin recruitment), highlighting the importance of screening multiple pathways in these types of studies. In addition, this study identified 9 new peptides (and several known peptides) that activate multiple receptors, again emphasizing the complexity of peptide-receptor interaction networks.

Combining machine learning with experimental validation can be an effective approach to identify receptors for novel peptides. Shiraishi et al. recently demonstrated that machine learning could predict novel peptide-receptor pairs even for ligand sequences that share no sequence homology with known neuropeptides.¹¹⁴ In this study, these authors aimed to identify receptors for neuropeptides from the invertebrate chordate *Ciona intestinalis*, an important organism for the study of neuropeptide evolution in both invertebrates and vertebrates. In addition to homologs of vertebrate neuropeptides, this animal also

produces many *Ciona*-specific neuropeptides that share no sequence homology to known peptides. While some neuropeptide-receptor pairs in this animal have been identified based on sequence homology, this strategy is not possible for the *Ciona*-specific peptides. To develop a machine-learning approach to identify novel peptide-receptor pairs, novel Peptide Descriptors (derived from amino acid physiochemical properties) were developed and incorporated into a support vector machine with known active and inactive ligand-receptor pairs as a training set. Prediction performance in this model was improved over conventional machine-learning approaches by incorporating a novel genetic algorithm-based feature selection. The optimized method was used to computationally screen interactions between 140 putative GPCRs with 19 known neuropeptides, resulting in 29 predicted peptide-receptor pairs. Predicted ligand-receptor pairs were then evaluated using Ca^{2+} mobilization assays. Overall, 12 peptide-receptor pairs were validated, representing a 41% success rate for this approach. Importantly, 11 of the 12 identified pairs are non-homologous to previously identified peptide-GPCR interactions.

One major advantage of large-scale cell-based screening approaches is their ability to experimentally screen many individual ligand-receptor interactions for specific downstream responses of interest, which can minimize false-positives from non-specific interactions that may be detected in affinity-based approaches. In addition, application of genetic knockouts or advances in machine learning can allow one to evaluate ligand-receptor interactions without prior knowledge of receptor class. However, many screening approaches require the assembly of large libraries of genetically manipulated cell lines, which can be time consuming and difficult to validate.

Chemical capture-based approaches

The membrane-bound nature of the majority of cell-surface receptors presents technical challenges for classical affinity-driven approaches to identify protein-protein interactions. For neuropeptides and peptide hormones, these challenges are often exacerbated due to the fact that peptide receptors often lack large extracellular domains (severely limiting commonly used approaches involving recombinant protein libraries of receptor ectodomains¹⁰⁸) and are present at very low abundances. Peptide residence time at ligand-binding sites can also vary considerably between systems,¹¹⁵ which may limit traditional pull-down approaches to characterize protein interaction networks.

Despite the technical challenge, classic affinity-enrichment approaches, often with covalent crosslinking, have been utilized to isolate receptors for peptide ligands. For example, early studies to elucidate the receptors for vasoactive intestinal polypeptide¹¹⁶ and calcitonin gene-related peptide¹¹⁷ made use of chemical crosslinking and enrichment to aid in characterizing peptide receptors and associated proteins. However, correctly identifying the sequences of these receptors was difficult based on crosslinking and pull-down alone, and often was accomplished using gene expression-based approaches.¹¹⁸ Advancements in protein sequencing by mass spectrometry have offered increased ability to determine the primary sequence of receptors directly after pull-down. For example, photoreactive crosslinking, protein isolation, and identification by MS identified an *Arabidopsis* leucine-rich repeat receptor kinase called PEPR1 as the receptor for the endogenous peptide *AtPep1*,

which triggers activation of the plant's innate immune response.¹¹⁹ Despite progress in this methodology to identify novel peptide-receptor interactions,¹²⁰ some disadvantages include non-selectivity of photoreactive groups toward residues on the receptor protein and with off-target proteins, high background labeling,¹²¹ short half-lives of reactive groups,¹²² and inherently short reactive interaction window.¹²³

Proximity-induced reactivity offers an alternative to the highly reactive and transient activation window of photoreactive groups. Proximity-induced approaches employ functionalities with significantly reduced reactivity relative to traditional crosslinking approaches, and rely on protein binding by the ligand to bring these reactive groups into close proximity to protein for covalent bond formation.¹²⁴ Perhaps one of the most notable of these approaches for labeling and identifying extracellular peptide receptors involves proximity-induced covalent bond formation between ligands labeled with hydrazine derivatives and oxidized glycan functionalities on carbohydrate-containing receptors (Figure 6).¹²⁵ After covalent bond formation on living cells, azide-alkyne cycloaddition facilitates biotinylation of the peptide-receptor complex. Biotin then allows for the enrichment of labeled proteins, which are then identified via MS analysis. Early examples of this technology demonstrated its effectiveness to identify receptors for transferrin, apelin, growth factors, insulin, and others as model ligands.¹²⁵ Since its initial discovery, this technology has been used to identify a number of peptide-receptor interactions, including identifying LINGO2 as a primary receptor for the secreted cytokine Trefoil factor family 3 (TFF3) involved in gastrointestinal wound healing,¹²⁶ identifying thrombospondin 4 as a major interaction partner for a serum peptide derived from high-molecular-weight kininogen involved in regulating immune cell chemotaxis,¹²⁷ and more.^{128–132} Recently, this ligand-receptor capture approach has been further developed and refined to overcome some prior limitations.^{133, 134} It is important to note that the hydrazine chemistry utilized in this approach is limited to glycosylated proteins and requires glycoprotein oxidation, which may disrupt some ligand-receptor interactions and are likely not compatible with *in vivo* studies. Despite these limitations, spontaneous ligand-receptor capture approaches have demonstrated effectiveness in identifying novel interactions, and are likely to continue to be powerful tools for identifying peptide receptors in future studies.

Protein capture-based methods have several distinct advantages over alternative approaches for the identification of receptors for ligands of interest. Most notably, these approaches are fairly unbiased in the types of proteins they can hit; one generally does not need to preselect receptor classes of interest beforehand. The unbiased nature of chemical capture allows the discovery of unexpected interactions with a wide variety of different protein classes. For proximity-driven ligation approaches, the spontaneous reaction between the ligand and receptor may prove advantageous to minimize off-target interactions, simplify MS data analysis, and build more productive crosslinks relative to more highly reactive groups.¹²³ However, all capture-based approaches suffer from a high potential for false-positive hits, and careful experimental controls are required to delineate true ligand-receptor interactions from non-specific hits. Even with these essential controls, protein binders identified using capture-based approaches may not always represent biologically interesting interactions that account for a peptide's bioactivity. Follow-up studies to investigate the

physiological relevance of interactions identified in this way are critical to drawing meaningful conclusions.

Challenges and concluding remarks

Recent methodological advancements, including those outlined above, have directly led to the discovery of new ligand-receptor interactions, opening the doors for future studies to explore these ligand-receptor interactions in physiology and disease. However, for a researcher aiming to identify new peptide-receptor interactions, challenges remain. For one, MS-based peptidomics experiments often identify hundreds of peptides within a given tissue. Even for a given peptide, multiple forms (e.g., with different post-translational modifications) often exist. With all of these peptides present, how does one choose the most promising sequences to pursue for receptor identification? For these situations, the high throughput methods above may be the best choice, although these methods have drawbacks, particularly in having to choose a given receptor subclass and signal output to monitor for activation.

For all approaches discussed, false positive or false negative hits can occur, and experimental design must be carefully considered to minimize false hits. Even with the appropriate controls, identification of a ligand-receptor pair should include rigorous validation experiments, preferably recapitulation of activity in a recombinant system. Perhaps most critically, identifying a putative peptide-receptor pair using many of the above methods does not permit one to draw definitive conclusions on the function of this pair *in vivo*. Extensive follow-up experiments often must be performed to assess the contributions of the identified interaction in normal physiology and disease.

To fully uncover the molecular mechanisms underlying peptide signaling, future efforts to identify receptors would benefit from an “unbiased” approach that does not pre-select a subset of receptors or signal outputs to investigate. Chemical capture-based approaches excel in this regard, although current methods still have limitations in the types of functionalities they can label, which may limit their general application. Unbiased chemical capture approaches also have the advantage of being able to identify non-traditional modes of peptide-protein interactions, such as natural peptides that engage in allosteric or antagonistic interactions, peptides that participate in multiprotein complexes, or peptides that act as enzyme inhibitors.^{16, 74} Powerful methods to rigorously identify the protein interactions for specific peptides of interest should enable a more thorough understanding of cell-to-cell signaling in both normal physiology and disease, which may directly identify novel therapeutic targets.

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Keywords

Peptide

A biopolymer comprised of amino acid residues.

Neuropeptide	An endogenous signaling peptide produced and secreted by neurons.
Peptide hormone	An endogenous signaling peptide produced and secreted by cells other than neurons (e.g., from endocrine cells). The same peptide sequence can be produced by multiple different cell types, and thus can be both a neuropeptide and a peptide hormone.
Receptor	A protein that recognizes and responds to a specific compound or an alternative stimulus. Compounds that bind to receptors are termed ligands. Upon ligand binding, receptors may initiate downstream signaling events.
Cell-cell signaling	Communication between two or more cells, often through the release of signaling molecules by one cell to activate receptor proteins on another cell.
Peptidomics	Methods that aim to comprehensively analyze the peptide content of a given biological sample, usually using mass spectrometry-based methods.
GPCR	Abbreviation for G protein-coupled receptor, a large family of membrane-embedded signaling proteins characterized by seven transmembrane helices and their association with heterotrimeric G proteins.
Orphan ligand	A bioactive molecule (or suspected bioactive molecule) without an established receptor.
Orphan receptor	A receptor protein without an established ligand.

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Figure 1.

Biosynthesis of precursor-derived peptides involves extensive post-translational modifications. As a result, a given precursor often generates many different mature peptide products, and these products can differ based on tissue or physiological state. The mature forms of these peptides often cannot be predicted from genomic or transcriptomic information. PCs, prohormone convertases. CPE, carboxypeptidase E. PTMs, post-translational modifications.

Figure 2.

Endogenous peptide-receptor interactions are often more complex than a one peptide-one receptor model. Shown is a cartoon representation showing types of peptide-receptor dynamics common for known neuropeptide and peptide hormone systems.

Figure 3.

Cartoon depiction of some possible receptors for a given bioactive peptide of interest.

Peptides can signal through receptors that are not orphan GPCRs. Receptor identification studies may benefit from fully exploring these possibilities.

Figure 4.

Identifying peptide receptors based on tissue expression. First, cell lines or tissues are identified that show biological response to the peptide of interest. Then, receptor transcripts (typically for orphan GPCRs only) are compared among the identified tissues to identify receptors candidates. A select number of candidate receptors are chosen for direct evaluation in recombinant receptor activation assays.

Figure 5.

Identifying peptide receptors using high-throughput screening. A variety of methods can be used to identify a library of putative neuropeptides and a library of putative receptors. Putative neuropeptides are chemically synthesized, and cell lines expressing each putative receptor are generated. Following this, each candidate receptor is screened against each of the candidate peptides (or pools of peptides) and downstream signaling is measured to identify hits. Putative ligand-receptor pairs can then be validated in subsequent cell-based assays.

Figure 6.

Identifying receptors using chemical capture.^{125, 133} In this example, cells natively expressing glycoproteins are treated with oxidant to generate reactive aldehyde functionalities. These chemically modified cells are then treated with ligand analogue bearing an aldehyde-reactive functionality and a handle for enrichment (e.g., azide, alkyne, or biotin). Covalent crosslinking of ligand to receptor proteins allows for enrichment after cell lysis and membrane solubilization, and identification of captured proteins is accomplished through MS-based proteomics methods. The white circle in ligand analogue structure represents variable chemical structure used to link ligand directing group, aldehyde-reactive group, and enrichment handle.