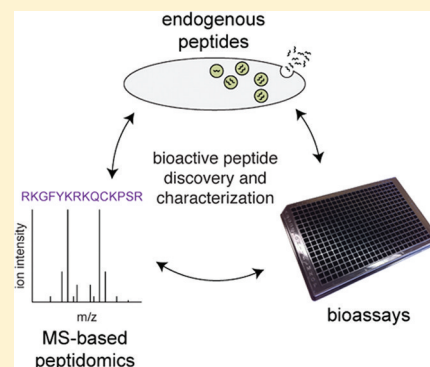


# Investigating Endogenous Peptides and Peptidases Using Peptidomics

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**ABSTRACT:** Rather than simply being protein degradation products, peptides have proven to be important bioactive molecules. Bioactive peptides act as hormones, neurotransmitters, and antimicrobial agents *in vivo*. The dysregulation of bioactive peptide signaling is also known to be involved in disease, and targeting peptide hormone pathways has been a successful strategy in the development of novel therapeutics. The importance of bioactive peptides in biology has spurred research to elucidate the function and regulation of these molecules. Classical methods for peptide analysis have relied on targeted immunoassays, but certain scientific questions necessitated a broader and more detailed view of the peptidome—all the peptides in a cell, tissue, or organism. In this review we discuss how peptidomics has emerged to fill this need through the application of advanced liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods that provide unique insights into peptide activity and regulation.



## BIOACTIVE PEPTIDE ACTION, PRODUCTION, AND SIGNALING

Organisms seamlessly integrate numerous classes of molecules, including bioactive peptides, into biochemical pathways that enable all the processes needed for life.<sup>1–3</sup> Insulin and glucagon, for example, are two of several well-known pancreatic peptide hormones that partake in hormonal regulation of physiological glucose metabolism.<sup>4,5</sup> Neuropeptides such as substance P<sup>6,7</sup> and the enkephalins<sup>8,9</sup> signal within the central nervous system (CNS) and mediate behavioral processes. There are also more than 20 known antimicrobial peptides (AMPs)<sup>10</sup> that have important roles in the innate immune system including the defensins. The defensin family of antimicrobial intestinal peptides thwart infection by serving as endogenous antibiotics,<sup>11</sup> and more recent experiments have also shown that the defensins regulate the intestinal microbiome,<sup>12</sup> all the bacteria present in the gut, to extend the physiological role of these peptides even further.

Moreover, the discovery of these peptides has begun to impact medicine as well. Liraglutide,<sup>13</sup> a glucagon like peptide 1 (GLP-1) mimetic, has been developed to treat diabetes and AMP mimics are promising antibiotic agents.<sup>14</sup> Additional peptides such as the conotoxins, a group of neurotoxic peptides produced by the venomous marine cone snail, are also emerging as potential therapeutics.<sup>15–18</sup> Here, we focus on the application of peptidomics to problems in mammalian biology, the exception being the work described on honeybee neuropeptidomics, but we recognize that peptidomics has the potential to impact many different areas of biology.

## PRODUCTION AND SIGNALING OF BIOACTIVE PEPTIDES

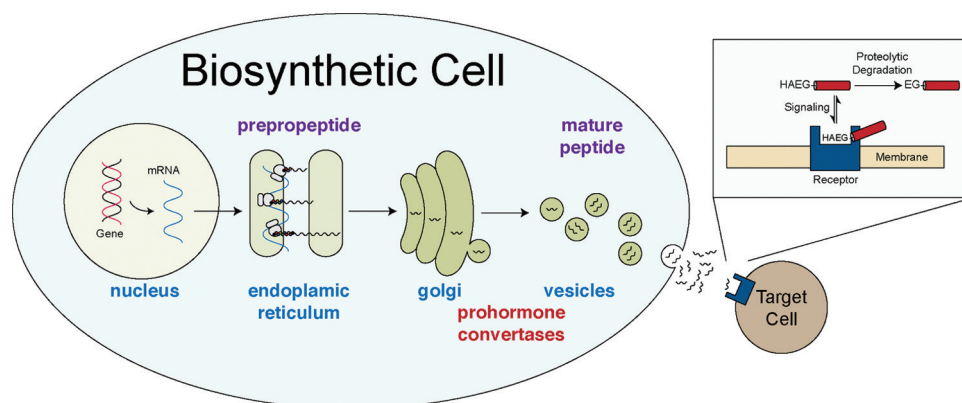
Though bioactive peptides have diverse functions and sequences, many of these peptides have analogous biochemical mechanisms that control their production, regulation, and signaling<sup>19</sup> (Figure 1). Bioactive peptide synthesis commences with the expression of a biologically inactive preprohormone that is typically 100–350 amino acids long.<sup>19</sup> A signal peptide at the N-terminus directs the peptide into the secretory pathway. The signal peptide is cleaved shortly after the peptide enters the lumen of the ER to afford the prohormone. Prohormones are then shuttled from the endoplasmic reticulum (ER) to the Golgi apparatus (Golgi). In the Golgi the prohormone begins its conversion into a mature bioactive peptide through proteolysis by a group of serine proteases called the prohormone convertases (PCs)<sup>20,21</sup> (Figure 1). This processing begins in the Golgi and continues into the secretory vesicles. Some peptides undergo additional proteolytic processing and post-translational modifications (acetylation, sulfation, and/or amidation) to produce the active peptide (Figure 2).<sup>22,23</sup>

Peptides are then released from cells into the extracellular milieu by fusion of secretory vesicles with the plasma membrane<sup>2,19</sup> (Figure 1). The secretion of some bioactive peptides can be regulated by an external stimulus that causes the acute release of the peptide as needed (e.g., glucose release of insulin).<sup>24,25</sup> After release, bioactive peptides travel to a

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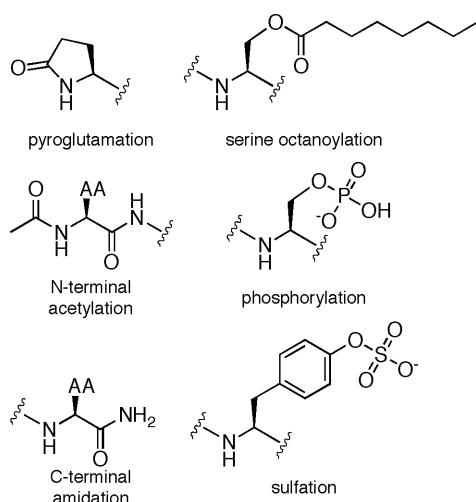


**Figure 1.** The key steps in the production and regulation of bioactive peptides. A prepropeptide is produced from mRNA encoding bioactive peptides. This peptide enters the secretory pathway through the ER and Golgi, before being packaged into secretory vesicles. In the trans Golgi as well as the secretory vesicles the prohormone encounters subtilisin-like proteases, called prohormone convertases, that process the prohormone to generate a mature form of the bioactive peptide. During this maturation process these peptides can also obtain additional post-translational modifications, such as C-terminal amidation and N-terminal acetylation. Stimulation of cells can lead to secretion of the peptides through fusion of the vesicles with the plasma membrane. Once released, the peptides can bind to receptors, typically G-protein coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs), to elicit a cellular or physiological response. Once released, peptides can also undergo proteolysis to regulate their activity.

target cell or tissue to bind their cognate membrane receptors, which initiates intracellular signal transduction and a biological response.<sup>26</sup> The principal receptors involved in bioactive peptide signaling include seven transmembrane G-protein coupled receptors (GPCRs)<sup>27</sup> and receptor tyrosine kinases (RTKs).<sup>28</sup> Peptide signaling is terminated by removal of the peptide through renal clearance<sup>29</sup> or proteolytic inactivation of a peptide<sup>30</sup> (Figure 1). By understanding the general framework for the production and signaling of bioactive peptides it has made it easier to discover and characterize additional peptides, receptors and peptidases involved in physiological peptide signaling.

## BIOACTIVE PEPTIDE DISCOVERY

The methods used to investigate bioactive peptides have evolved as new techniques have been introduced into biology.



**Figure 2.** Post-translational modifications found on bioactive peptides. Some modifications, such as N-terminal acetylation or C-terminal amidation, are common, while others such as serine octanoylation are rare.

Classical methods for bioactive peptide discovery rely on bioassays that enable the biochemical purification of bioactive peptides from tissues by identifying fractions with a desired bioactivity.<sup>31</sup> By performing multiple rounds of purification, bioactive peptides could be purified and subsequently identified. Many important peptides were discovered through this type of approach including insulin,<sup>32</sup> glucagon,<sup>33,34</sup> and the endorphins.<sup>35–37</sup> Modern variants of this approach have replaced a cellular or physiological phenotypic assays with a receptor activation assay to identify bioactive peptides and in some cases these experiments help characterize the function of the receptor as well. For example, the identification of novel GPCR ligands,<sup>38–40</sup> led to the discovery of sleep regulating orexins<sup>41</sup> and prolactin-releasing peptide,<sup>42</sup> which demonstrate the effectiveness of screening receptors in the discovery of new bioactive peptides.

To circumvent the need for bioassays, Tatemoto and Mutt developed an alternative approach for bioactive peptide discovery that relies on the biochemical enrichment of peptides with a C-terminal amide,<sup>43–48</sup> which are commonly found on bioactive peptides that are part of the secretory pathway.<sup>19</sup> Oxidative cleavage of a C-terminal glycine by peptidyl alpha-amidating monooxygenase (PAM)<sup>49–51</sup> produces the C-terminal amide-containing peptides. C-terminal amides are thought to protect bioactive peptides from carboxypeptidase-mediated degradation and in some cases are required for bioactivity.<sup>52</sup> Tatemoto and Mutt developed a biochemical method that enabled them to purify peptides with C-terminal amides from complex samples. This approach was highly successful and led to the discovery of neuropeptide Y,<sup>46</sup> peptide histidine isoleucine (PHI(1–27)),<sup>45</sup> and galanin,<sup>48</sup> which are now known to regulate a variety of physiological functions.<sup>3</sup>

Lastly, bioactive peptides have also been discovered through analysis of mRNAs, which may reveal the presence of a bioactive peptide within the protein-coding region. For example, alternative splicing of the calcitonin gene affords two different mRNA products,<sup>53</sup> with one splice form encoding the calcitonin peptide while the alternatively spliced form was predicted to encode an unknown peptide that was aptly named the calcitonin-gene related peptide (CGRP).<sup>54</sup> This prediction

**Table 1. Peptide Hormones and Physiological Processes**

| peptide     | physiological process |
|-------------|-----------------------|
| insulin     | glucose metabolism    |
| angiotensin | blood pressure        |
| oxytocin    | social behavior       |
| enkephalins | pain                  |
| ghrelin     | appetite              |
| orexins     | sleep                 |

was validated in subsequent experiments that demonstrated that CGRP was present in tissues.<sup>55,56</sup> Physiological experiments identified CGRP as a potent vasodilator.<sup>57</sup> Most importantly, CGRP signaling was recently linked to the onset of migraines,<sup>58,59</sup> and this insight led to the development of CGRP-receptor antagonists as a new class of antimigraine drugs in clinical trials.<sup>60,61</sup> CGRP exemplifies the basic as well as biomedical impact of bioactive peptide discovery.

## BIOACTIVE PEPTIDE FUNCTION

Pharmacology and genetics have been used to characterize the physiological functions for many peptides.<sup>9,12</sup> For instance, injection of GLP-1 reduces blood glucose levels to clearly define a role for GLP-1 in blood glucose homeostasis.<sup>62</sup> Using pharmacology to ascertain peptide function is limited due to the poor bioavailability of most peptides.<sup>63</sup> This is a particular problem in the study of neuropeptides since peptides do not readily cross the blood–brain barrier. Pharmacological studies with small-molecule receptor agonists (or antagonists) of neuropeptide receptors provide an alternative approach to understand the physiological function of a particular bioactive peptide.<sup>37,61,64</sup> The analgesic natural product alkaloid morphine, for example, is an agonist for the opioid receptors to indicate that the endorphins, natural peptide agonists of the opioid receptors, are involved in pain sensation.<sup>57</sup>

Genetics have proven to be a more general approach for studying bioactive peptide function *in vivo*.<sup>22,65–67</sup> For example, the role of the peptide YY (PYY), an intestinal hormone, in body mass regulation and feeding behavior was established by knocking this peptide out in a mouse model.<sup>68</sup> Mice lacking PYY were obese and ate more than their wildtype counterparts, which demonstrates that PYY is a peripheral signal that is able to promote satiety (the feeling of fullness). These experiments also revealed that mice that ate protein, but not fat or carbohydrate, released more PYY, and this finding provides a

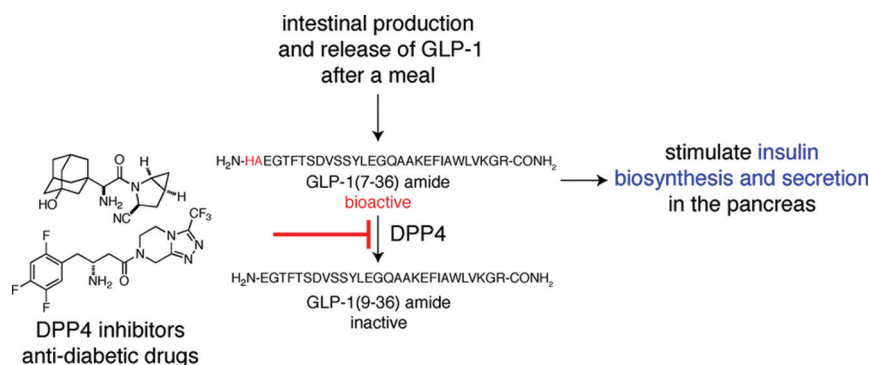
new model to explain why proteins are better appetite suppressants than fat or carbohydrate.

Knockout studies of peptides are often difficult, however, because a single gene sometimes produces more than one bioactive peptide.<sup>69–71</sup> For example, the glucagon gene controls the production of glucagon, GLP-1 and GLP-2.<sup>70,71</sup> As a result, knocking out the glucagon gene would result in loss of GLP-1 and GLP-2 as well. Glucagon and GLP-1 do have unique receptors though, and knocking out the peptide receptors instead of the peptides enables the physiological functions of the bioactive peptide pathways to be assessed.<sup>27,72,73</sup> To study glucagon signaling, for example, glucagon receptor knockout mice were generated, and these mice showed improved glucose tolerance relative to their wildtype counterparts.<sup>74</sup> The application of pharmacology and genetics has provided valuable information to further our understanding of the cellular and physiological roles of peptides (Table 1).

## PROTEOLYTIC REGULATION OF BIOACTIVE PEPTIDES

An approach for controlling endogenous peptide signaling in medicine is to target the proteases and peptidases that regulate these pathways with small-molecule inhibitors.<sup>30,75</sup> A prototype example of the success of this approach is the inhibition of the angiotensin converting enzyme (ACE), a well-studied exopeptidase, in the treatment of hypertension and cardiovascular disorders.<sup>75</sup> ACE is responsible for the cleavage and activation of the angiotensin I peptide to generate angiotensin II, a ligand for the angiotensin receptor and a potent vasoconstrictor that causes hypertension. Understanding this mechanism led to the development of small-molecule ACE inhibitors as anti-hypertensive drugs that function by preventing the activation of angiotensin I.<sup>75</sup>

The insulinotropic hormone GLP-1 is released from the gut after a meal<sup>30</sup> and activates its receptors in the pancreas where it stimulates insulin biosynthesis and secretion.<sup>76</sup> Detailed studies of GLP-1 signaling revealed that GLP-1 has a very short half-life in blood due to N-terminal processing of this peptide by a peptidase called DPP4.<sup>77,78</sup> DPP4 removes an N-terminal dipeptide to inactivate GLP-1. Importantly, this pathway has been harnessed in the development of a new class of antidiabetic drugs<sup>30,79</sup> that inhibit DPP4. By blocking DPP4 activity endogenous GLP-1 levels are increased, leading to higher levels of insulin and improved glucose tolerance (Figure 3). The development of ACE and DPP4 inhibitors underscores



**Figure 3.** Peptide hormone signaling regulates physiology and can be harnessed for therapeutic gain. DPP4 inhibitors extend the half-life of the bioactive form of the insulinotropic hormone GLP-1. In doing so, these inhibitors increase insulin secretion and improve physiological glucose tolerance, which is of tremendous value in the treatment of diabetes.



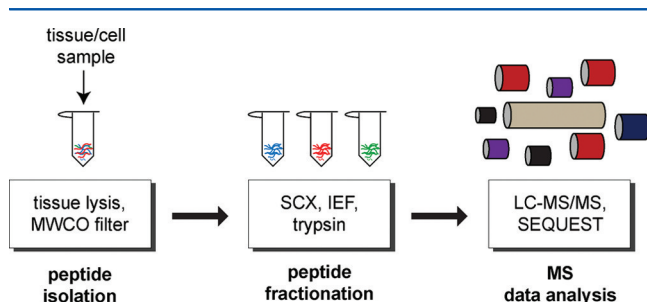
the importance of bioactive peptides in basic research and medicine.

## ■ THE CASE FOR PEPTIDOMICS

The importance of peptides supports the need for better methods to analyze these molecules in a biological setting. Classical methods for peptide analysis rely mostly on immunoassay approaches that require specific antibodies to recognize the peptide of interest.<sup>80,81</sup> Immunoassays are valuable because they provide a means to detect peptides from a variety of biological samples.<sup>80,82,83</sup> While effective, immunoassays do not provide a global analysis, require the time-consuming generation of antibodies, and can sometimes suffer from antibody cross-reactivity with other peptides.<sup>82</sup> For example, Jankowski et al. discovered a novel vasoconstrictive peptides angiotensin A by mass spectrometry and showed that this peptide cross-reacts with antibodies against angiotensin II.<sup>82</sup> More specifically, cross-reactivity is a particular problem when studying peptide proteolysis since shorter or longer peptides may still contain the antibody epitope, making it difficult to measure the peptide of interest. Peptidomics approaches can overcome the challenges associated with immunoassays because liquid chromatography–tandem mass spectrometry (LC-MS/MS) provides a global analysis of the peptidome. This attribute enables new types of experiments to elucidate important features of bioactive peptide regulation.

## ■ PEPTIDOMICS WORKFLOW, INSTRUMENTATION, AND ANALYSIS

Peptidomics refers to any method that provides a broad view of the entire peptide pool in a biological sample, the peptidome.<sup>84,85</sup> Modern peptidomics approaches rely heavily on LC-MS/MS to provide sensitive detection and identification of peptides from biological samples. The two predominant types of mass spectrometers used in peptidomics studies are quadrupole time-of-flight (Q-TOF) and ion trap (IT) instruments.<sup>86</sup> These instruments enable quantitation using several approaches and provide the tandem mass spectra that are necessary to identify the peptide sequence. The key steps in every peptidomics experiment are peptide isolation, peptide fractionation and processing, mass spectrometry, and data analysis (Figure 4). Svensson and colleagues provided an early



**Figure 4.** The key steps in any peptidomics experiment include sample preparation and peptide isolation, processing of the sample (including isotopic labeling), and finally detection and data analysis. While these steps vary in subtle ways from experiment to experiment, the overall workflow is consistent for most peptidomics experiments.

example of an effective peptidomics workflow during their discovery of neuropeptides.<sup>84</sup> Svensson and co-workers used focused microwave radiation of rodent brains<sup>84,87,88</sup> to ablate

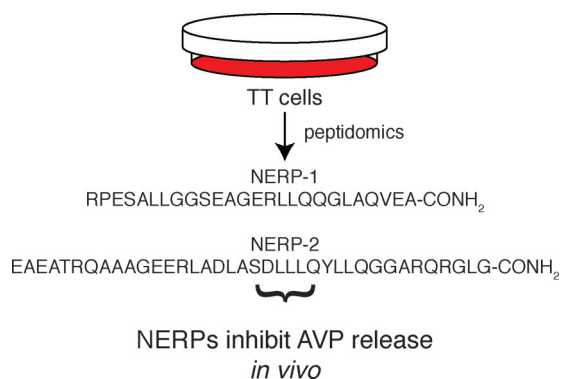
proteolytic activity by heat denaturing all proteins prior to peptide isolation. Heating tissues prior to isolation of the peptidome has become a standard process in peptidomics even though different heating methods are used (e.g., *ex vivo* heating of tissues by microwave<sup>89</sup> or boiling<sup>90–92</sup>).

Following microwave irradiation the brain tissue was homogenized in slightly acidic buffer and centrifuged to remove any insoluble debris. Next, Svensson and colleagues fractionated their sample by passing it through a molecular weight cutoff (MWCO) filter (10 kDa) to separate the peptides from larger proteins.<sup>84</sup> MWCO filters are used in almost every peptidomics experiment to enrich the peptidome. Other fractionation schemes can also be included to provide different views of the peptidome, including enzymatic digestion (e.g., trypsin), strong cation exchange (SCX), isoelectric focusing (IEF),<sup>92</sup> and reverse-phase (RP) chromatography (Figure 4). The use of additional peptide fractionation improves the peptidome coverage. In one example, a 10-fold increase in coverage was obtained through the addition of an SCX step into the workflow.<sup>92</sup> The isolated neuropeptides were then subjected to reverse phase LC-MS/MS using an electrospray ionization (ESI) Q-TOF-MS. The identification of peptides is generally carried out using SEQUEST<sup>93</sup> or Mascot<sup>94</sup> software packages.

Using this workflow Svensson and colleagues identified greater than 500 neuropeptides.<sup>84</sup> Some of these peptides are known bioactive neuropeptides, including substance P, beta-endorphin, and neurotensin, which validated the methodology. There were also a number of novel neuropeptides. Many of these novel peptides were derivatives of known neuropeptides, including longer or shorter peptide fragments and post-translationally modified forms. The common PTMs observed in this data set included acetylation, phosphorylation, and pyroglutamination (Figure 2). The characterization of these novel neuropeptides highlights the value of peptidomics to obtain information that is inaccessible by other approaches. More generally, Svensson et al. demonstrated how to obtain quality peptidomics data, and their approach has been emulated in subsequent peptidomics studies.

## ■ USING PEPTIDOMICS TO DISCOVER BIOACTIVE PEPTIDES

Yamaguchi and colleagues developed a peptidomics strategy to identify new bioactive peptides by detecting peptides that are secreted by TT cells, a human thyroid cell line.<sup>95</sup> By using peptidomics to detect amidated peptides in the conditioned media of TT cells, they were able to detect known bioactive peptides, such as CGRP.<sup>53,57</sup> In addition, these experiments detected two novel C-terminally amidated peptides derived from the VGF gene called neuroendocrine regulatory peptides-1 and -2 (NERP-1 and -2). Subsequent histological studies revealed that NERP-1 and NERP-2 are present *in vivo* and that they co-localized in the rat brain (supraoptic nucleus and paraventricular nucleus) with vasopressin, a multifunctional peptide hormone.<sup>96–101</sup> This led to the hypothesis that the NERPs and vasopressin may interact. This idea was validated when pharmacological delivery of the NERPs suppressed vasopressin release in a dose-dependent fashion to demonstrate a physiological activity for these peptides<sup>95</sup> (Figure 5). In this case, the detection of C-terminal amides using peptidomics led to the discovery of two novel bioactive peptides and provided a peptidomics version of the Tatamoto and Mutt approach.<sup>44</sup>



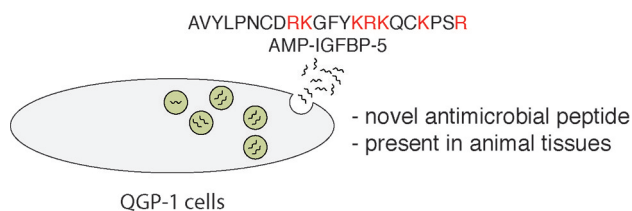
**Figure 5.** Discovery of novel bioactive peptides using structural features of the peptide as a guide. A number of bioactive peptides contain C-terminal amides, which result from enzymatic production in the secretory pathway. Analysis of peptides secreted by TT cells using a peptidomics approach led to the discovery of the neuroendocrine regulatory peptides (NERPs). These peptides were later shown to co-localize with arginine vasopressin (AVP), a hormone involved in blood pressure, and physiological studies demonstrated that NERPs control AVP release *in vivo*. In doing so, this approach demonstrates the utility of peptidomics in accelerating the discovery of novel bioactive peptides.

Sasaki et al. generalized this approach further to include peptides that lack C-terminal amides by performing cell-based assays to determine which of the peptides they detected were able to induce intracellular calcium release, which is a hallmark of receptor activation by a bioactive peptide.<sup>102</sup>

In a more focused search Osaki and Minamino developed a peptidomics strategy to discover novel mammalian antimicrobial peptides.<sup>103</sup> They took advantage of the fact that antimicrobial peptides are typically highly basic peptides—containing multiple lysines and arginines—and developed a cation exchange fractionation method that specifically enriched basic peptides. Treatment of QGP-1 cells, a human pancreatic endocrine cell line, with forskolin and carbachol promotes the secretion of intracellular peptides into the media. Secreted peptides were enriched by cation exchange, fractionated and desalted by HPLC, and analyzed by matrix-assisted laser desorption ionization (MALDI)-MS to reveal a novel peptide fragment of insulin-like growth factor-binding protein 5 (IGFBP-5).<sup>104,105</sup>

IGFBP-5 is a known secreted protein, but its proteolysis and antimicrobial activity had not been reported before. The peptide fragment of IGFBP-5 identified in this study was referred to as antimicrobial peptide-IGFBP-5 (AMP-IGFBP-5).<sup>103</sup> Synthesis of the AMP-IGFBP-5 enabled the antimicrobial activity of this peptide to be tested. AMP-IGFBP-5 had an IC<sub>50</sub> of ~1 μM with *E. coli* K12 and was on par or better than the established antimicrobial peptides cathelicidin<sup>106</sup> and β-defensin-2.<sup>107</sup> Additional experiments revealed that this peptide was active against a number of Gram-negative and Gram-positive bacteria as well as the fungus *Pichia pastoris* GS115. In addition, AMP-IGFBP-5 is present in the brain and gut of rats, suggesting a potential physiological role for this peptide in the innate immune system (Figure 6).

These examples highlight the utility of peptidomics to accelerate the discovery of novel bioactive peptides by enabling the detection and identification of these peptides. Most of these peptides would be undetectable using standard molecular



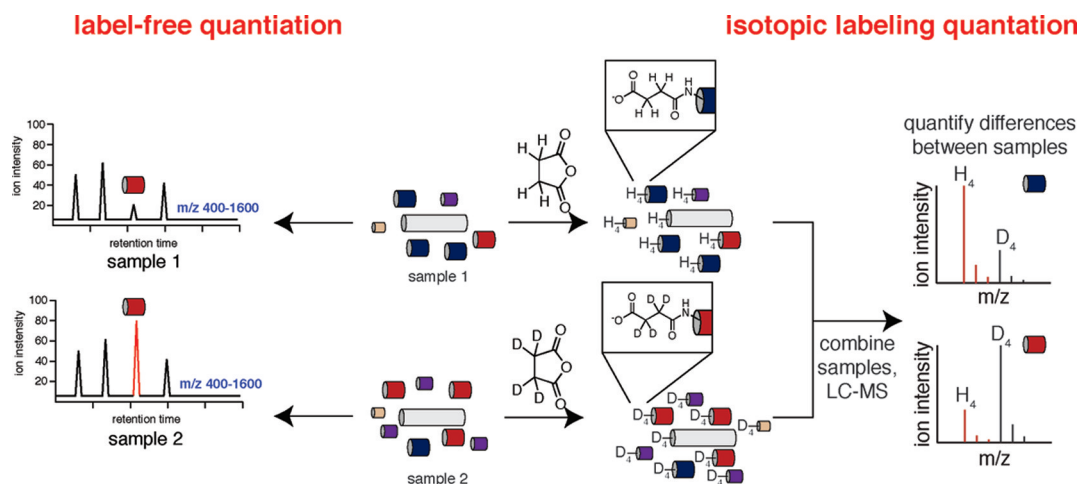
**Figure 6.** Peptidomics of highly basic peptides from QGP-1 cells revealed the production of a series of IGFBP-5 peptides. While this protein was predicted to be secreted, it had not previously been hypothesized to produce bioactive peptides. The structure of the antimicrobial-IGFBP-5 (AMP-IGFBP-5) was highly charged and reminiscent of known defensin antimicrobial peptides. Subsequent antimicrobial and antifungal assays demonstrated that AMP-IGFBP-5 has antimicrobial and antifungal activity equal to or better than that of defensins. Lastly, AMP-IGFBP-5 was present in the gut, which suggests that this peptide might play a role in innate immunity or regulation of the microbiome.

biology techniques used to identify proteins (e.g., silver stained SDS-PAGE gels or proteomics).<sup>108,109</sup> In addition, immunoassays are not a discovery-based approach because they require structural knowledge of the peptides first so that antibodies can be generated.<sup>110</sup> These studies also imply new functions for precursor proteins that generate these peptides, such as VGF<sup>111</sup> and IGFBP-5.<sup>104,105</sup> As additional assays are integrated with peptidomics approaches, the number of bioactive peptides discovered is bound to increase.

## ■ QUANTITATIVE PEPTIDOMICS

The above examples take advantage of the ability of peptidomics to identify peptides within complex mixtures.<sup>112</sup> In some cases, however, it is important to quantify peptides between two samples to correlate changes in peptide levels with changes in a phenotype or genotype.<sup>89–92,113–115</sup> Quantitative peptidomics methods enable differences in relative peptide concentrations to be determined and can be carried out using isotopic labeling methods<sup>113,114,116,117</sup> or a label-free approach<sup>90–92,118–121</sup> (Figure 7).

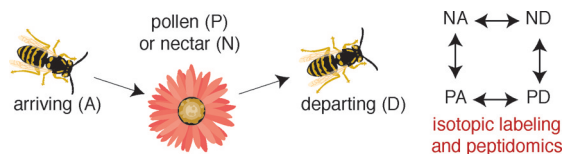
In one example of an isotope labeling approach, a light and heavy form (e.g., deuterium or carbon-13) of succinic anhydride is used to differentially label two peptidome samples. Succinic anhydride and the deuterated version of succinic anhydride, *d*<sub>4</sub>-succinic anhydride, are readily available. Labeling of two different peptidomes with succinic anhydride and *d*<sub>4</sub>-succinic anhydride results in a mass difference of 4 Da between the same peptide in each sample<sup>113</sup> (Figure 7). After separate labeling reactions the two samples are mixed and analyzed in the same LC-MS/MS experiment. Differences in relative peptide concentrations are measured by comparing the ratio of the light labeled peptide to the heavy labeled peptide in the mass spectrum (Figure 7). This approach enables the relative abundance of peptides between different samples to be determined, and additional labels can be created if more samples are to be included. By contrast, label-free approaches simply rely on the ion intensity, absolute signal at the MS detector, between two samples to provide relative changes in peptide levels between two samples (Figure 7).<sup>92</sup>



**Figure 7.** There are two primary approaches used for quantitative proteomics: stable isotope labeling methods and label-free approaches. Isotopic labeling relies on the chemical labeling of the peptidome using stable isotope variants of the same reagent. Analysis of the MS for each peptide should reveal a heavy and light labeled version of the peptide, and the ratio of these peptides enables quantitation. By contrast, in label-free approaches samples are run sequentially, and the peak intensities are used to determine changes in the concentration of the peptide between two samples.

## APPLICATION OF QUANTITATIVE PEPTIDOMICS TO BEHAVIORAL STUDIES

Using an isotopic labeling strategy, Sweedler and colleagues investigated changes in neuropeptides in the honeybee brain during behavioral assays.<sup>113</sup> Honeybees were chosen as the model system because they show amazingly sophisticated behavior.<sup>122,123</sup> They have ~100 neuropeptides, and in this report they were able to quantitatively measure about 50 of those peptides. Quantitation was accomplished using a chemical labeling approach with succinic anhydride and *d*<sub>4</sub>-succinic anhydride to differentially label the peptidomes in a series of pairwise comparisons. Changes in neuropeptide levels were measured as honeybees were foraging for food.<sup>124</sup> Four categories were created based on whether the honeybees preferred pollen (P) or nectar (N) as a food source or whether the honeybees were isolated when they arrived (A) or departed (D) from the food (Figure 8).



**Figure 8.** Neuropeptidomics of honeybees as they arrive (A) or depart (D) a food source (pollen (P) or nectar (N)) demonstrated that there are specific neuropeptides that are associated with food gathering in honeybees. By correlating changes in these peptide levels with phenotypes associated with foraging, these studies provided new insights into the functions of these peptides and revealed the ability of peptidomics to correlate changes in peptide levels with complex behaviors.

Combinations of these categories led to four different sample sets for peptidomics: nectar arriving (NA), nectar departing (ND), pollen arriving (PA), and pollen departing (PD) samples (Figure 8). Peptidomics analysis compared NA to ND, NA to PA, ND to PD, and PA to PD. Of the 50 peptides measured in these studies, eight were significantly different in one of the four pairwise comparisons. The changes were distributed between nectar (N) and pollen (P) foragers as well as

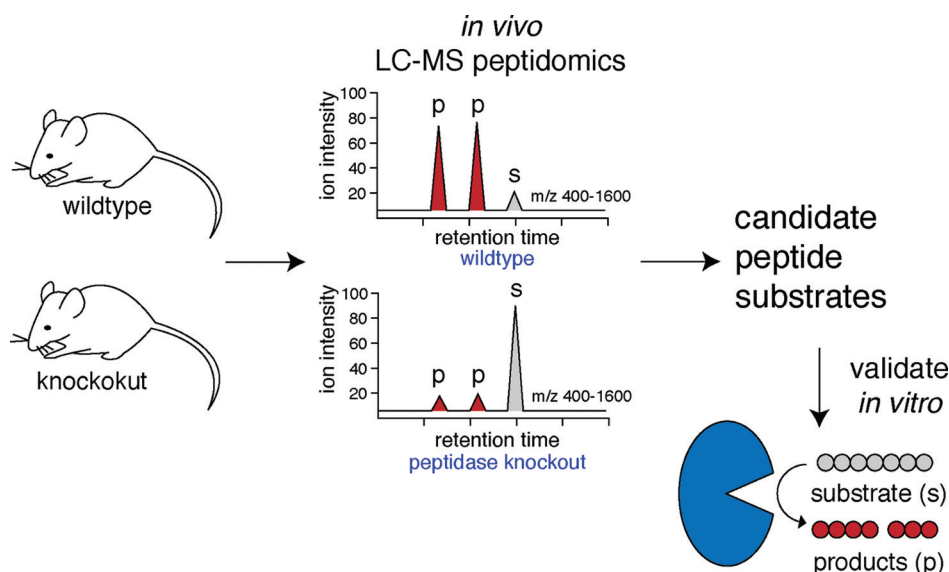
differences between arriving (A) and departing (D) honeybees. Together these changes were distinct enough to categorize the four different groups using the peptide signatures and canonical discriminant analysis.<sup>125</sup> Brockmann and colleagues pushed the frontiers of peptidomics by making measurements with small amounts of tissue and demonstrating that the honeybee neuropeptidome could be used to correlate neuropeptides with behavior. This work sets the stage to subsequently study these peptides and their functions in honeybees or other related organisms.

## QUANTITATIVE PEPTIDOMICS TO ELUCIDATE PEPTIDASES AND PROTEASES SUBSTRATES

One major question for every enzyme is the identification of natural substrates.<sup>126</sup> For most peptidases and proteases, the identification of candidate substrates is a two-step process. Traditionally, the substrate specificity of the peptidase or protease is determined *in vitro* using synthetic peptide libraries.<sup>127,128</sup> On the basis of the enzyme specificity, the next step is to look for candidate endogenous substrates and then test these natural substrates *in vitro*.<sup>129</sup> Finally, in the best case scenario, the activity of the enzyme is perturbed via genetics or chemical inhibition, and the endogenous levels of the substrate are evaluated to determine the physiological relevance of the prediction.<sup>77</sup>

While occasionally successful, this strategy often leads to many false positives because *in vitro* experiments are poor predictors of *in vivo* substrate utilization due to a number of factors that cannot be recapitulated *in vitro*.<sup>91,130</sup> First, *in vitro* experiments cannot account for the distribution of enzymes and substrates within cells and tissues. Therefore, enzyme–substrate relationships that are determined *in vitro* might not occur *in vivo* because the two molecules do not have the opportunity to interact. Next, assays with pure enzymes do not factor in the relative contributions of other enzymes in the proteome. As a consequence, even if an enzyme and substrate interact *in vitro*, other enzymes may be responsible for processing the peptide *in vivo*.<sup>126</sup> Lastly, the presence of competitive substrates *in vivo* can prevent an enzyme from cleaving an *in vitro* substrate *in vivo*.<sup>131</sup> Together these cases





**Figure 9.** Identification of endogenous substrates of peptidases relies on peptidomics. While there are subtle differences in the methods for quantitation and data analysis the overall workflows for these experiments are similar. In this approach, comparison of the peptidomes of mice that differ in the activity of a particular peptidase/protease can reveal peptides regulated by the enzyme. These peptides will include substrates and/or products of the enzyme, which, in turn, can be used to infer biochemical and biological function of the peptidase/protease.

highlight the difficulty in trying to predict endogenous peptidase–substrate relationships from *in vitro* experiments.<sup>91</sup>

Peptidomics offers an elegant solution to this problem by enabling the substrates of peptidases to be measured directly in cells and tissues.<sup>90–92,114,115,132,133</sup> In doing so, substrate discovery using peptidomics takes into account all of the aforementioned challenges presented by *in vitro* experiments. These peptidomics experiments rely on the fact that perturbation of the activity of a peptidase—either by using genetics to knock it out or pharmacological inhibition—results in different amounts of substrates and products in cells and tissues. Comparison of samples with differences in peptidase activity using quantitative peptidomics can identify these endogenous substrates and products (Figure 9). The identification of peptidase-regulated peptides can help explain the biochemical, cellular, and physiological functions of the peptidase. We provide several examples below that attempt to target particular enzymes involved in peptide biosynthesis, degradation, and signaling.

## PROHORMONE CONVERTASES

Fricker and colleagues pioneered efforts to identify substrates and products of the PCs using peptidomics.<sup>134</sup> As mentioned, PCs are the enzymes responsible for making the primary cut in the prohormone peptide in the secretory pathway that eventually leads to the production of the mature peptide.<sup>19</sup> The PCs cleave prohormones at dibasic sites (e.g., KK, KR, RR), a very distinctive sequence that has been used to predict the presence of peptide hormones within longer genes. The PCs are a family of subtilisin-like serine proteases with seven members in mammals.<sup>135</sup> PC1/3 and PC2 represent the majority of the PC activity in neuroendocrine cells and other tissues.<sup>135</sup> For example, PC2 is involved in the production of a number of neuropeptides as well as insulin and glucagon in the pancreas.

Mice lacking PC2 show impaired neuropeptide processing by radioimmunoassays (RIAs) that measured the levels of full-

length peptide.<sup>136,137</sup> Like all immunoassays, RIAs suffer from issues with cross-reactivity,<sup>82</sup> and this problem may be exacerbated in peptide processing studies where incompletely processed peptides are very similar in structure, which would favor cross-reactivity. As a result, Fricker and colleagues decided to employ a quantitative peptidomics approach to study PC2. In these experiments hypothalamic peptidomes from wildtype (PC2<sup>+/+</sup>) and PC2-null (PC2<sup>-/-</sup>) mice were compared by peptidomics to identify specific substrates of PC2 using an isotopic labeling method.<sup>134</sup> The substrates would be expected to be elevated in the knockout mice, while the products would be elevated in the wildtype mice.

A number of neuropeptides were identified in this study including fragments of cocaine and amphetamine regulated transcript (CART), chromogranins, preprotachykinins, provasopressin, and secretogranin (Table 2). The levels of the

**Table 2. Prohormone Convertase 2 Regulated Peptides Identified by Peptidomics**

| precursor                | PC2 <sup>-/-</sup> /<br>PC2 <sup>+/+</sup> |
|--------------------------|--|
| CART(33–52)              | <0.05                                      |
| chromogranin A (392–402) | <0.05                                      |
| proenkephalin(219–229)   | <0.05                                      |
| VGF(487–507)             | <0.05                                      |
| alpha-MSH                | <0.1                                       |

peptides detected in the PC2<sup>-/-</sup> sample were greatly reduced in comparison to the PC2<sup>+/+</sup> sample. This data supports a general role for PC2 in the production of many neuropeptides in the hypothalamus. In addition, a number of protein fragments, most likely from the breakdown of proteins within the cell, were also detected in these samples. These fragments are not part of the secretory pathway and the levels of these fragments did not change, demonstrating the specificity of PC2 for secreted hormones. Finally, this specificity supports the notion that the methodology is capturing changes that are

occurring *in vivo*, since any processing that occurred during the sample preparation would target secretory and non-secretory peptides equally. Fricker and colleagues also utilized their data to determine that aromatic residues or proline are preferred by PC2 at the P1' and P2' positions. In total, this work provided a physiological view of PC2 biochemistry and a template for comparative peptidomics studies to identify peptides that are regulated by peptidases and proteases.

## PROLYL PEPTIDASES

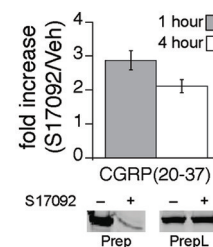
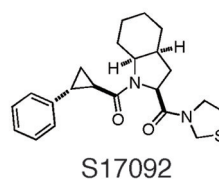
One of the benefits of peptidomics is that it is unbiased and can be used to discover novel endogenous substrates for peptidases, which can help define the cellular and/or physiological function of peptidases.<sup>77</sup> Examples of this are found in the analysis of members of the prolyl peptidase family. The prolyl peptidases are a family of serine peptidases that cleave peptides preferentially on the C-terminal side of proline residues (i.e., proline is strongly preferred at the P1 position). The prolyl peptidase family is composed of prolyl endopeptidases and dipeptidyl peptidases, which preferentially cut at proline residues at the penultimate position of the N-terminus (i.e., H<sub>2</sub>N-XP-peptide).<sup>78</sup>

Prolyl endopeptidase (PREP) is the founding member of the prolyl peptidase family.<sup>138,139</sup> Interest in this protein was derived from its unique selectivity for proline. Initial *in vitro* assays with PREP identified candidate substrates for the enzyme, such as vasopressin,<sup>139,140</sup> which led to new hypotheses about PREP function. Vasopressin was previously linked to memory performance and led to the development of selective PREP inhibitors, such as S17092,<sup>141,142</sup> as potential anti-amnesic compounds. Interestingly, while S17092 inhibitors showed improved cognitive function in monkeys<sup>143</sup> and humans,<sup>144</sup> PREP inhibition did not regulate physiological levels of vasopressin, highlighting the difficulty in using *in vitro* experiments to predict endogenous substrates.<sup>140,145</sup>

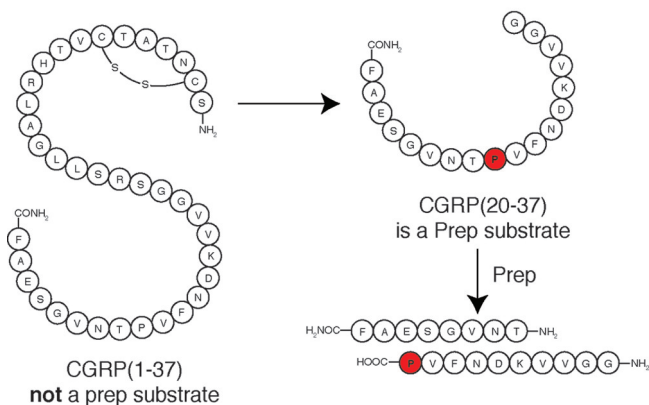
To gain a deeper insight in the biochemistry of PREP *in vivo*, two groups utilized comparative peptidomics to investigate PREP substrates in the nervous system using selective pharmacological inhibition of PREP. The first study utilized the commercially available inhibitor, Z-ProProlinal, to study PREP activity in a rat model.<sup>133</sup> In these experiments quantitation was performed using an isotopic labeling strategy termed iTRAQ.<sup>146</sup> Using this approach, Tenorio-Laranga and colleagues identified a number of novel PREP substrates in the brains of inhibitor-treated rats. These were proline-containing peptides elevated in the inhibitor-treated samples versus the untreated samples. These peptides were not explicitly tested as PREP substrates but fit into the known substrate profile for the enzyme. On the basis of their peptidomics data, Tenorio-Laranga and co-workers concluded that PREP is involved in the catabolism of peptides in the CNS.<sup>133</sup>

Nolte and colleagues utilized the PREP inhibitor S17092 to study the impact of PREP on the CNS peptidome of mice.<sup>90</sup> In this case, quantitation was performed using a label-free approach. The substrates identified in this study raised important questions about the PREP substrate specificity that were then studied in greater detail. Specifically, peptidomics was able to provide a possible explanation of why PREP had evolved a preference for cutting shorter peptides. One of the PREP substrates identified was a fragment of the bioactive peptide CGRP, CGRP(20–37), which was elevated in the S17092-treated sample (Figure 10). The full length CGRP(1–

### *in vivo* peptidomics



### *in vitro* experiments with recombinant Prep afford a model of CGRP proteolytic processing *in vivo*

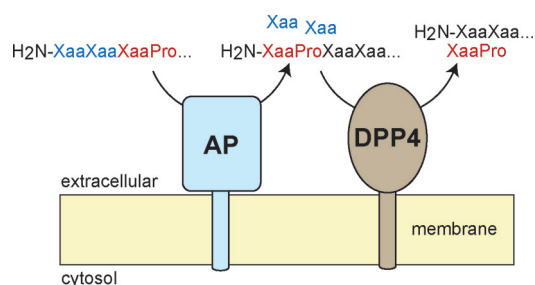


**Figure 10.** Peptidomics of Prep revealed that Prep regulates endogenous levels of CGRP(20–37). Follow-up *in vitro* experiments demonstrated the Prep is able to process the shorter CGRP(20–37) but not the longer, full-length, peptide CGRP(1–37). This length preference enables Prep to participate in the catabolism of CGRP without regulating the bioactive form of the molecule (i.e., CGRP(1–37)), even though the cut sites are identical within the two molecules. This data supports a model of CGRP proteolysis in the nervous system where the full-length CGRP is processed by unknown enzymes followed by Prep processing of the proline-containing CGRP(20–37) fragment.

37) was not a substrate, even though it contains the very same cut site. These data demonstrate that Prep uses sequence and length specificity to cleave a subset of proline-containing peptides in the nervous system.

Tagore and colleagues<sup>91,92</sup> extended peptidomics to study another prolyl peptidase, DPP4, in the kidney. While the role of DPP4 in plasma GLP-1 regulation was established<sup>77</sup> the function of this enzyme in other tissues, including the kidney, was less clear. Peptidomics comparison of wildtype (DPP4<sup>+/+</sup>) and DPP4 null (DPP4<sup>-/-</sup>) samples identified a number of DPP4 substrates elevated in the DPP4<sup>-/-</sup> samples. One interesting question that emerged from these studies was an attempt to explain how the penultimate proline containing substrates of DPP4 are generated *in vivo*. The data suggested that peptides are cleaved first by aminopeptidases until a penultimate proline is encountered. Penultimate proline peptides are not aminopeptidase substrates and are then released and cleaved by DPP4. In this model, aminopeptidase and DPP4 activities form a biochemical pathway that is responsible for the N-terminal degradation of proline-containing peptides (Figure 11). This model was tested by adding peptides with internal prolines to tissue lysates with and without aminopeptidase activity. In the absence of amino-





**Figure 11.** Peptidomics revealed an unappreciated physiological pathway for the renal catabolism of proline containing peptides that interlinks aminopeptidase (AP) and dipeptidyl peptidase 4 (DPP4) activities. These experiments demonstrate the value of peptidomics in understanding peptide processing and characterizing the biochemical and physiological functions of enzymes.

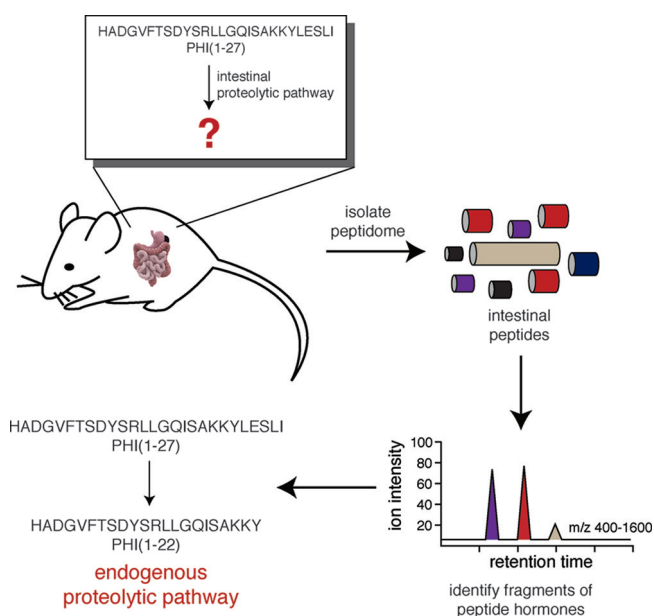
peptidase activity the production of penultimate proline containing peptides was reduced, which demonstrated a role for kidney aminopeptidase activity in the production of DPP4 substrates.<sup>91,92</sup> In total, these studies highlight the value of peptidomics in providing detailed information about peptidases *in vivo*.

## ■ A PEPTIDOMICS STRATEGY TO ELUCIDATE THE PROTEOLYTIC PATHWAYS THAT PROCESS BIOACTIVE PEPTIDES

As mentioned, the regulation of bioactive peptides by peptidases is of basic interest and can also be used to develop novel therapeutics (e.g., DPP4 and ACE inhibitors). Despite the interest in these pathways, we currently lack information about the proteolytic regulation of most bioactive peptides due to the lack of a general approach for elucidating these pathways *in vivo*. Specifically, any approach that can identify physiologically relevant peptide fragments can reveal the proteolytic pathways that process bioactive peptides and accelerate the discovery of the peptidases responsible for peptide processing. Tinoco, Kim, and colleagues developed a general peptidomics-based strategy to elucidate the endogenous pathways that regulate bioactive peptides coupling *in vitro* assays and *in vivo* peptidomics measurements of endogenous peptides<sup>131</sup> (Figure 12).

This peptidomics-based approach was applied to investigate the proteolysis of PHI(1–27),<sup>147</sup> an intestinal peptide hormone which has been linked to a number of biological functions, including prolactin secretion<sup>148</sup> and glucose stimulated insulin secretion (GSIS).<sup>149</sup> First, PHI(1–27) was incubated with intestinal lysates, and this revealed the production of PHI(3–27) and PHI(1–22) as the predominant fragments suggesting potential N- and C-terminus specific cut sites. *In vitro* experiments using chemical inhibitors demonstrated that the N-terminal processing is regulated by DPP4. Peptidomics experiments revealed the existence of PHI(1–22) but not PHI(3–27) in the intestine.<sup>150</sup> The absence of PHI(3–27) *in vivo* was explained by the presence of competitive DPP4 substrates which can attenuate DPP4 processing of PHI(1–27). This hypothesis was supported by *in vitro* experiments with recombinant DPP4 where the presence of additional DPP4 substrates greatly slowed PHI(1–27) processing.

Lastly, the functional impact of the C-terminal proteolysis of PHI(1–27) was assessed using a GSIS assay<sup>151</sup> with mouse pancreatic  $\beta$  islets. In these experiments PHI(1–27) was active



**Figure 12.** A peptidomics-based approach to identify physiologically relevant proteolytic pathways that process peptide hormones. In this approach, peptidomics identification of fragments of bioactive peptides enables physiological-relevant proteolytic pathways to be identified. Application of this approach to the peptide hormone PHI(1–27) identified a previously unappreciated pathway for the C-terminal proteolysis of this peptide. Interestingly, this pathway was also shown to regulate the activity of PHI(1–27) in a cell-based glucose-stimulated insulin secretion (GSIS) assay.

and able to promote insulin secretion, as reported, but PHI(1–22) was inactive to indicate that proteolysis of PHI(1–27) *in vivo* ablates the bioactivity of this peptide. Together this data demonstrates the utility of peptidomics in characterizing the endogenous proteolytic pathways that regulate bioactive peptide hormones. More generally, by coupling these experiments to improved strategies for peptidase discovery and bioassays, such as GSIS, additional enzymes and pathways that regulate important physiological pathways will be discovered.

## ■ CONCLUSIONS AND FUTURE DIRECTIONS

As a whole, the application of LC-MS peptidomics has had a major impact on redefining the outlook of the peptide field. Advancements in peptidomics workflows and analytical tools have enabled improved strategies for identifying bioactive peptides and characterizing their functions.<sup>92</sup> Peptidomics also helps to study peptidases and molecular pathways that regulate peptides in general.<sup>89–92,114,115,131,132</sup> Because peptides are such powerful regulators of physiology, this research will impact our basic understanding of peptide and enzyme function *in vivo* and may eventually provide novel insights that will be of benefit in the development of therapeutics like protease/peptidase inhibitors. Moreover, these same approaches can be extended from proteases to other classes of enzymes, such as kinases, that may modify bioactive peptides as well.

Looking forward there appears to be a number of opportunities for peptidomics to make an impact in biology and medicine. In the past decade, biologically active peptides encoded in short open reading frames (sORFs) have been serendipitously identified in mammals,<sup>152,153</sup> insects,<sup>154</sup> and bacteria.<sup>155</sup> These peptides lack signaling sequences and are

released directly into the cytoplasm.<sup>156</sup> Humanin was the first sORF derived peptide to be identified through a screen to identify genes that inhibit apoptosis, and it exhibits neuroprotective properties<sup>152,157</sup> by direct binding to proapoptotic proteins in the mitochondria. More recently, peptides encoded by the *polished rice* (*pri*) sORF gene have been shown to function as transcription factors that affect epidermal differentiation in *Drosophila*,<sup>158</sup> suggesting that such peptides may be more ubiquitous than suspected. Improvements in the endogenous identification of sORF encoded peptides can be made by integrating genomic approaches with peptidomics.<sup>159–161</sup>

Furthermore, as we improve our ability to deliver peptides *in vivo* the search for natural bioactive peptides is likely to ramp up. Natural product discovery was based on the fact that small-molecule drugs could be found by scouring nature's stockpile of biologically active chemicals.<sup>162</sup> Similar approaches for peptides have been hindered because peptides were traditionally considered to be poor drugs.<sup>163</sup> The development of biologics aims to overcome many of these issues.<sup>164–166</sup> Drugs such as liraglutide<sup>13</sup> and pramlintide<sup>167</sup> are peptides that are derived from GLP-1 and amylin, respectively. The emergence of these peptide drugs and improved methods for intracellular delivery of bioactive peptides<sup>168,169</sup> and proteins<sup>170</sup> suggests a bright future for bioactive peptide medicines. Much of this will be predicated on the discovery of new bioactive peptides and will be accelerated by the inclusion of peptidomics.

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## ABBREVIATIONS

LC-MS/MS, liquid chromatography–tandem mass spectrometry; ER, endoplasmic reticulum; Golgi, Golgi apparatus; AMP, antimicrobial peptide; GLP, glucagon-like peptide; GPCR, G-protein coupled receptor; RTK, receptor tyrosine kinase; PAM, peptidyl alpha-amidating monooxygenase; PHI(1–27), peptide histidine isoleucine; mRNAs, messenger RNAs; CGRP, calcitonin gene-related peptide; PYY, peptide YY; ACE, angiotensin converting enzyme; DPP4, dipeptidyl peptidase 4; Q-TOF, quadrupole time-of-flight; IT, ion trap; MWCO, molecular weight cutoff; SCX, strong cation exchange; IEF, isoelectric focusing; ESI, electrospray ionization; NERP, neuroendocrine regulatory peptide; MALDI, matrix-assisted laser desorption ionization; IGFBP-5, insulin-like growth factor-binding protein 5; AMP-IGFBP-5, antimicrobial peptide-IGFBP-5; PC, prohormone convertases; PREP, prolyl endopeptidase; iTRAQ, isobaric tags for relative and absolute quantitation; CNS, central nervous system; GSIS, glucose stimulated insulin secretion; sORF, short open reading frames; PRI, polished rice.

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