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Neuropeptide-Processing Enzymes: Applications for Drug Discovery

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ABSTRACT

Neuropeptides serve many important roles in communication between cells and are an attractive target for drug discovery. Neuropeptides are produced from precursor proteins by selective cleavages at specific sites, and are then broken down by further cleavages. In general, the biosynthetic cleavages occur within the cell and the degradative cleavages occur postsecretion, although there are exceptions where intracellular processing leads to inactivation, or extracellular processing leads to activation of a particular neuropeptide. A relatively small number of peptidases are responsible for processing the majority of neuropeptides, both inside and outside of the cell. Thus, inhibition of any one enzyme will lead to a broad effect on several different neuropeptides and this makes it unlikely that such inhibitors would be useful therapeutics. However, studies with mutant animals that lack functional peptide-processing enzymes have facilitated the discovery of novel neuropeptides, many of which may be appropriate targets for therapeutics.

KEYWORDS: carboxypeptidase, peptidomics, prohormone convertase, peptide biosynthesis

NEUROPEPTIDES AND THEIR BIOSYNTHETIC PATHWAYS

Neuroendocrine peptides function in a large number of physiological processes including feeding and body weight regulation, fluid intake and retention, pain, anxiety, memory, circadian rhythms and sleep/wake cycles, and reward pathways.[1,2] Altogether, there are hundreds of peptides that have been detected in brain and other tissues, although only a fraction has known biological functions. The biological activity of peptides is usually mediated by G-protein coupled receptors, or in some cases, by enzyme-linked receptors (such as the insulin receptor). Because there are a large number of orphan G-protein coupled receptors, it is likely that there are peptides with undiscovered functions.[2,3] One approach to identifying novel neuropeptides makes use of a mutant animal lacking a peptide-processing enzyme, discussed in more detail after an overview of the peptide-processing enzymes.

When the amino acid sequences of peptide precursors were first discovered in the 1970s, it was immediately clear that generation of the active forms of the peptides required processing of the precursor at sites containing 2 or more basic amino acids.[4] In most cases, the sites were either Lys-Arg or Arg-Arg. Analysis of hundreds of peptide precursor cleavage sites in a variety of organisms has extended these earlier observations and confirmed that Lys-Arg and Arg-Arg constitute the vast majority of all cleavage sites, while Arg-Lys and Lys-Lys are found with much less frequency.[5] In addition to these sites, some cleavages occur at sites containing pairs of basic amino acids separated by 2, 4, or 6 other residues.[6] For many years, these sites were described as "monobasic" and the enzymes responsible for cleavage were thought to be distinct from the "dibasic" site enzymes. However, in the past decade it has become clear that the enzymes capable of processing precursors at Arg-Arg sites can also cleave at Arg-X-X-Arg sites, and the general consensus has recently been described as Arg-X_n-Arg, where n is either 0, 2, 4, or 6.[7,8] Several different peptide precursors are also cleaved at sites containing a single basic residue, with no upstream Arg in the appropriate position.[9] Often, these single basic sites are Pro-Arg sequences, and it is not yet clear if any of the identified prohormone convertases can cleave at these sites.

In addition to the classical pathway that involves cleavage at basic amino acids, several peptides have been detected that arise from cleavage at nonbasic residues or at basic residues that don't fit the typical consensus site.[9-16] In some cases, these peptides may be formed after secretion when the peptides are exposed to extracellular peptidases. However, several peptides that are formed from cleavage at nontypical sites appear to be present within purified secretory vesicles.[10] The enzymes responsible for these nontraditional cleavages are not currently known, although there are candidates (described below).

PEPTIDE-PROCESSING ENZYMES

The finding that peptide precursors contain basic amino acids separating the various bioactive and/or spacer regions

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led to the prediction that a trypsin-like endopeptidase initially cleaved the precursors, thereby generating intermediates with C-terminal basic residues.[4] These basic residues would then be removed by a carboxypeptidase B-like enzyme, in many cases producing the final peptide (Figure 1). In some cases, additional posttranslational processing steps are required such as C-terminal amidation, N-terminal acetylation, or other modifications (glycosylation, sulfation, and phosphorylation).

Two "trypsin-like" endopeptidases appear to be involved in the production of neuropeptides.[7,8] These enzymes, designated prohormone convertase 1 (PC1, also known as PC3) and prohormone convertase 2 (PC2), are broadly expressed in the neuroendocrine system.[17-20] Although some cell types express only 1 of the 2 enzymes, many neuroendocrine cells express both. Within the cell, PC1 and PC2 are enriched in peptide-containing secretory vesicles.[7,8] The pH optima of these enzymes are in the 5 to 6 range, corresponding to the moderately acidic pH of the interior of the secretory vesicles. These enzymes are serine proteases of the subtilisin family and are strongly activated by Ca^{2+}.[7,8] The combination of decreasing pH and increasing calcium levels activates PC1 and PC2 once they leave the Golgi apparatus. In addition, there are specific inhibitors of PC1 (named proSAAS) and PC2 (named 7B2) that also function to block enzyme activation early in the secretory pathway.[21,22] Both proSAAS and 7B2 bind tightly to their respective enzymes and are slowly cleaved by them. Interestingly, the products of this cleavage remain potent inhibitors until a carboxypeptidase removes the C-terminal basic residues, thereby rendering the inhibitor inactive.[23,24]

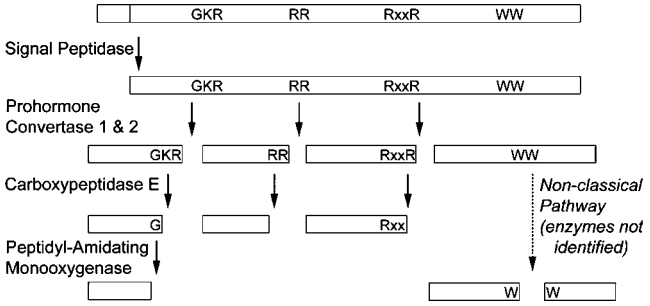

Figure 1. Classical and nonclassical neuropeptide processing scheme. First, the N-terminal sequence that drives translocation of the protein into the lumen of the endoplasmic reticulum is co-translationally removed by a signal peptidase. Then, in the classical scheme, the prohormone is typically processed at sites containing Lys-Arg (KR), Arg-Arg (RR), or Arg-Xaa_n-Arg, where n is 2, 4, or 6 (RxxR shown in figure). Processing at these basic amino acids involves endopeptidase action by an enzyme such as prohormone convertase 1 or 2 followed by the removal of the C-terminal basic residue(s) primarily by carboxypeptidase E, although an additional enzyme (carboxypeptidase D) is also able to contribute to processing. An amidating enzyme that is broadly expressed in the neuroendocrine system converts C-terminal Gly residues into a C-terminal amide. In addition to this classical pathway, a large number of peptides have been found that result from cleavage at nonbasic residues. An example of this nonclassical pathway for the generation of a peptide previously found in brain is indicated; this fragment of chromogranin B involves cleavage between 2 adjacent Trp residues (WW). Many other nonbasic cleavage sites have been reported, including other hydrophobic residues, short chain aliphatic residues, and acidic residues. The enzymes responsible for the nonclassical pathway are not clear. Some of these nonclassical processing events may occur after secretion and be mediated by extracellular peptidases, although some of the nonbasic mediated cleavages appear to occur within the secretory pathway.

The substrate specificities of PC1 and PC2 have been studied with purified enzymes, in cell culture, and in mice with disruptions of either one of the 2 genes.[15,25-27] Based on these studies, it appears that both PC1 and PC2 are able to cleave many of the same substrates although there are some sites cleaved preferentially by each. For example, PC2 cleaves the Lys-Lys sites in proopiomelanocortin (necessary to generate α-melanocyte-stimulating hormone, corticotrophin-like intermediate lobe peptide, and β-endorphin 1–27), whereas PC1 has negligible activity toward these sites in proopiomelanocortin but cleaves Lys-Lys sites in proenkephalin.[28-30] As predicted from the early studies on prohormone processing, PC1 and PC2 cleave on the C-terminal side of the basic residue(s); these residues often need to be removed by a carboxypeptidase before the peptide has biological activity.

The major peptide-processing carboxypeptidase was discovered in 1982 and named enkephalin convertase as well as carboxypeptidase E (CPE).[31,32] Because the enzyme is broadly expressed in the neuroendocrine system and processes many peptides in addition to enkephalin, the latter name is more appropriate. Subsequently, the name carboxypeptidase H was used for this enzyme, although this is rarely used in recent publications. CPE is a member of the metallocarboxypeptidase family, requiring Zn^{2+} in the active site for catalysis.[32,33] Unlike most other metallocarboxypeptidases, CPE also binds Ca^{2+} and this causes a slight activation and increase in stability.[34] CPE cleaves C-terminal Lys, Arg, and His residues from the C-terminus of a large number of peptides including those ending with Pro-Arg, although these bonds are cleaved orders of magnitude more slowly than peptides with penultimate amino acids other than Pro.[35-37]

For many years, CPE was thought to be the only carboxypeptidase involved in neuropeptide processing because it was the sole activity detected in purified secretory vesicles. However, studies on the *Cpe*$^{fat/fat}$ mice challenged this assumption: these mice lack functional CPE due to a point mutation in the *Cpe* gene that changes a Ser into a Pro and

destabilizes the protein structure.[38,39] Despite the absence of CPE activity in these mice, detectable levels of the mature forms of peptides are produced, albeit at reduced levels.[14,38,40-43] This result suggested that an additional carboxypeptidase was involved in peptide processing. A search for CPE-like enzymes led to the discovery of carboxypeptidase D (CPD).[44] However, CPD is primarily localized to the *trans* Golgi network and although some CPD can enter the immature secretory vesicles, no CPD is detectable in the mature secretory vesicle.[45,46] Thus, only the processing reactions that are initiated in the *trans* Golgi network and immature vesicles can be completed by CPD to produce the mature peptide. Because the majority of peptide processing occurs later in the secretory pathway, CPD is only able to play a moderate role and CPE is the major peptide-processing carboxypeptidase.

In addition to the endopeptidase and carboxypeptidase processing steps, some peptides require further processing reactions. It has been estimated that ~50% of all known bioactive peptides have a C-terminal amide residue.[47] However, if one considers all peptides that have been detected in brain, pituitary, and other tissues, and not just those peptides with known biological activities, the fraction of amidated peptides is considerably smaller. Still, this is an important modification that is essential for the biological activity of many peptides. In order for a peptide to get amidated it must have a C-terminal Gly residue. This Gly is converted to an amide residue by 2 distinct enzyme activities that are contained within a single multifunctional protein, peptidyl-glycine-α-amidating monooxygenase (PAM).[48] The N-terminal domain is a hydroxylating monooxygenase that oxidizes the alpha carbon of the Gly in a reaction that uses copper, oxygen, and ascorbate.[49] Then, the C-terminal lyase domain releases glyoxylate, leaving behind the nitrogen of the Gly as the C-terminal amide residue.[50] PAM is broadly distributed throughout the neuroendocrine system and appears to be the only amidating activity present in tissues.

Other posttranslational processing events include acetylation, sulfation, phosphorylation, glycosylation, and additional proteolytic cleavages.[51] There are also rare modifications such as *n*-octanoylation of a Ser residue within the peptide ghrelin.[52] Except for sulfation, the enzymes responsible for these modifications haven't been well characterized.[53,54] The additional proteolytic cleavages are intriguing, and a large number have been found in recent studies using mass spectrometry to characterize the precise form of peptides present in extracts of brain and pituitary.[9,13-16,55] In some cases the peptides may be processed after secretion by extracellular peptidases such as endopeptidase 25.11 (also known as neprilysin, enkephalinase, and other names), angiotensin-converting enzyme, and several amino peptidases.[56,57] The extracellular processing does not always lead to inactivation of the peptide; in some cases, the product of the processing reaction has a distinct profile of receptor binding compared with the original peptide.[58,59] In these cases, the extracellular reaction serves more of a modulatory role than an inhibitory one. While some of the nonbasic residue-directed cleavages are likely to occur outside the cell, others happen within the secretory pathway based on analyses of purified secretory vesicles[10,60] or on post-translational modifications. For example, fragments of α-melanocyte-stimulating hormone (α-MSH) are found in pituitary extracts that lack 1 or 2 N-terminal residues normally present on α-MSH, but containing an N-terminal acetyl group.[14] Because the acetylation reaction is only known to occur within the secretory pathway, and not following secretion, the presence of an acetyl group on the N terminus indicates that the cleavage occurred within the secretory pathway. The enzymes that cleave peptides within the secretory pathway at nonbasic sites are not known, but candidates include endothelin-converting enzyme-2 (ECE2) and the recently discovered carboxypeptidases A5 and A6. These latter 2 enzymes are detected in certain brain and/or pituitary cell types, and are predicted to be present within the secretory pathway and to cleave peptides at C-terminal aliphatic or aromatic residues.[61,62] ECE2 was originally described as a member of the endothelin-converting enzyme family, although ECE2 only has ~50% amino acid sequence identity to other members of this gene family and also has a neuroendocrine distribution.[63] Furthermore, ECE2 has been reported to be present in the secretory pathway and to be maximally active at pH 5 to 6, coinciding with the intravesicular pH range. Finally, ECE2 was tested with several peptides and found to cleave quite selectively.[64] Further studies are needed to examine the precise role of ECE2 and other enzymes in the intracellular processing of neuropeptides.

Peptide-Processing Enzymes and Drug Discovery

Typically, enzymes are considered to be potential drug targets if inhibition of the enzyme would produce a useful therapeutic effect. In this sense, most of the peptide-processing enzymes are poor targets for drugs for 2 reasons. First, many of these enzymes have too broad a role, and it is unlikely that inhibition would achieve a selective beneficial effect. For example, mice lacking PAM activity are not viable (John Pintar, oral communication, July 2001). While mice lacking CPE activity are viable, the phenotype is not considered to be desirable in a drug: obesity,[38] increased anxiety (Reeta Biswas and Lloyd Fricker, unpublished data, July 2001), and abnormal sexual behavior.[65] Secondly, the intracellular peptide-processing enzymes present a difficult target for drug development due to pharmacokinetic issues. Inhibitors of the enzymes that target basic amino acids (ie, the PCs and CPE) tend to contain multiple charges and

therefore do not readily penetrate the cell. However, to be effective as inhibitors in vivo, the compound would need to enter not only the cell but also the secretory vesicles. Although the extracellular peptide-processing enzymes do not have this problem with pharmacokinetic issues, they generally share the problem of nonspecificity.

An alternative approach to drug discovery is to use the peptide-processing enzymes to identify novel peptides, some of which are likely to bind to orphan receptors. Knowledge of the ligands for these orphan receptors is important for the development of drugs that target these receptors. This peptidomics approach was originally described as a method to isolate CPE substrates from mice lacking CPE activity (ie, *Cpe* $^{fat/fat}$ mice) although it should work for any organism that has a reduced level of a peptide-processing enzyme and which subsequently has elevated levels of the enzyme's substrates.[9] The basic approach is to use an affinity column that binds the peptide precursors to isolate these precursors from tissue extracts (Figure 2). To reduce the number of false positives, it is useful to compare the results with control organisms that have normal levels of the processing enzyme with the results from the organisms with reduced enzyme levels; those peptides present at different levels in the 2 extracts are likely to be substrates for the enzyme in vivo. Then, once the peptides have been affinity purified, they can be easily detected and identified using mass spectrometry (Figure 2).

In the case of the *Cpe* $^{fat/fat}$ mice, the Lys- and Arg-extended processing intermediates that accumulate in the absence of CPE activity can be readily purified with anhydrotrypsin agarose (Figure 2). This protein binds peptides with C-terminal basic residues (ie, trypsin products), but unexpectedly, doesn't bind peptides with internal basic residues (ie, trypsin substrates). It is not known why the conversion of trypsin into anhydrotrypsin eliminates its ability to bind substrates and enhances its ability to bind products. After purification of *Cpe* $^{fat/fat}$ mouse brain or pituitary extracts on the anhydrotrypsin column, hundreds of peptides were detected that were not present in the extracts from wild-type mice.[9] A large number of known neuropeptide-processing intermediates were identified, which both validates the approach and confirms the importance of CPE for the biosynthesis of numerous peptides. The known peptides or peptide-processing intermediates identified in *Cpe* $^{fat/fat}$ brain and pituitary include fragments of proopiomelanocortin, proenkephalin, prodynorphin, preprotachykinin A and B, provasopressin, prooxytocin, promelanin concentrate hormone, proneurotensin, chromogranin A and B, and secretogranin II.[9,12-16] Fragments of other proteins that are processed by CPE in the secretory pathway were also identified, such as N- and/or C-terminal pieces of the peptide-processing enzymes PC1, PC2, and PAM (above references,[9,12-16] and Fa-Yun Che and Lloyd Fricker, unpublished data, August 2005). Some of the identified peptides represent novel cleavages or posttranslational modifications such as acetylation, glycosylation, phosphorylation, and oxidation. In addition, novel peptides were identified that were subsequently found to be encoded by a novel protein, which was subsequently named proSAAS.[22] One function of proSAAS is to inhibit PC1 early in the secretory pathway. Because mice overexpressing proSAAS are slightly overweight even though PC1 activity does not appear to be affected, it is likely that the proSAAS-derived peptides serve other functions, possibly as neuropeptides.[22,66,67] Many more peptides

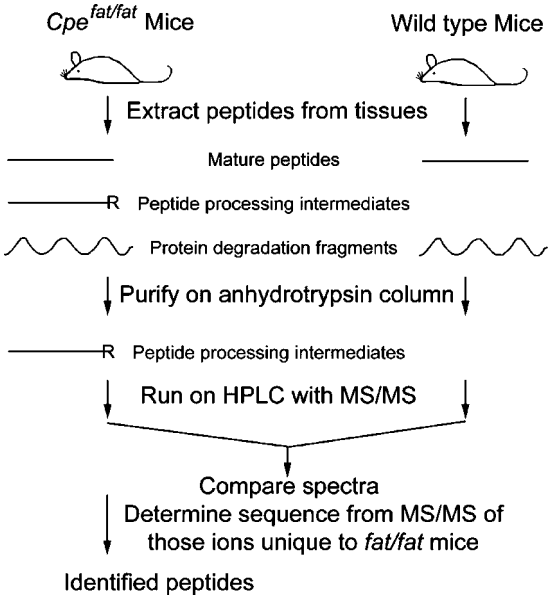

Figure 2. Purification of neuropeptide-processing intermediates from *Cpe* $^{fat/fat}$ mouse tissues. Peptides extracted from brain or other tissues of *Cpe* $^{fat/fat}$ mice fall into 1 of 3 categories. One group includes the mature forms of neuropeptides. These are generally present in *Cpe* $^{fat/fat}$ mice at lower levels than in wild-type mice. Another group of peptides is the peptide-processing intermediates that are C-terminally extended with basic residues (ie, the immediate substrates of CPE). These are greatly increased in the *Cpe* $^{fat/fat}$ mice, relative to wild-type mice, which show undetectable levels of most of these peptides. The third group of "peptides" represents protein degradation fragments that are either normally present in the tissue due to protein turnover or which may be induced by postmortem changes during the dissection and/or extraction. Some of these protein degradation fragments may contain C-terminal basic residues. The anhydrotrypsin column is used to purify peptides containing C-terminal basic residues from the other peptides. Then, the peptides are analyzed on HPLC with on-line tandem mass spectrometry (MS/MS) so that peptide sequence information can be obtained. The spectra from analysis of *Cpe* $^{fat/fat}$ and wild-type mice is compared; those peptides common to the 2 spectra typically represent protein breakdown fragments that contain C-terminal basic residues while those peptides unique to the *Cpe* $^{fat/fat}$ mice represent CPE substrates (ie, neuropeptide processing intermediates or other proteins that are normally processed by PCs and CPE within the secretory pathway).

have been found with partial amino acid sequences that do not match the database of known peptide precursors; these may represent additional novel peptides.

Future Directions

The overall goal of the peptidomic studies is to identify the biological activity of each peptide, and this knowledge could be then used for drug discovery. Because peptides are involved in so many physiological processes, practically any disorder, from addiction to anxiety, would be a potential area that could benefit from drugs based on neuropeptide receptors. The experimentally difficult part of this concept is the determination of the function of each peptide. The peptidomics approach described in Figure 2 provides a simple and elegant method to isolate a large number of peptide-processing intermediates, and mass spectrometry provides an efficient and sensitive approach to identify these peptides. However, the determination of function remains an extremely low-throughput venture. For example, a typical approach to determine the function of a protein is to generate transgenic and/or knockout mice, which can take several years to produce and characterize. Another approach is to determine which physiological states regulate the levels of each peptide, and then to use this knowledge to predict the function of the peptide. Although one would still need to directly test the peptide(s) for the proposed function, this approach would reduce the number of tests. For example, the peptides present in hypothalamus that are regulated by food deprivation are potentially involved in the control of body weight/energy balance. Similarly, peptides upregulated or downregulated by the chronic administration of drugs of abuse may be important mediators of the reward mechanisms and/or may contribute to the underlying neurochemical changes that lead to addiction. While this approach is not without false positives, it can at least reduce the number of candidate functions for a particular peptide. For example, the peptide hypocretin/orexin was found in one study to be upregulated by food deprivation, although subsequent studies suggested that this peptide was more involved in arousal states than in food intake.[68-70] However, it makes sense that in times of food deprivation an animal would spend more time awake and foraging for food, so the regulation of hypocretin/orexin by food deprivation is consistent with a function in both arousal and feeding.

It is possible to obtain accurate quantitative data from mass spectrometry if differential isotopic labels are incorporated into 2 samples that are then combined and analyzed.[71,72] The relative abundance of the material in the 2 samples can be readily determined by comparing the peak height of the heavy and light isotopic labels.[12,13,73] Using this approach, a large number of hypothalamic peptides were found to be altered by food deprivation.[16] Further refinement of the technique, both by improved extraction methods and by analysis on capillary columns with nanospray mass spectrometry will allow for the detection of even more peptides in each sample. Subsequent studies need to be performed to test the proposed function for each peptide, and to identify the receptors through which these peptides function. The peptidomics approach to identify and quantify neuropeptides provides a powerful method to rapidly screen for peptides, both known and novel, that contribute to a variety of physiological processes and it is likely that this information will be useful for drug development.

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