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Protease Mechanisms for the Biosynthesis of Peptide Neurotransmitters

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Janneca Chelsea Garcia Ames

Committee in charge:

Professor Vivian Hook, Chair
Professor Matthew Banghart, Co-Chair
Professor Matthew Lovett-Barron

2021

The thesis of Janneca Chelsea Garcia Ames is approved,
and it is acceptable in quality and form for publication on microfilm
and electronically.

University of California San Diego

2021

DEDICATION

I am wholeheartedly dedicating this thesis to my family and friends for all their support and effort they have given me. For my mother and father, Cora Garcia Ames and Reynaldo Velasco Ames, thank you for all of your sacrifices and your willingness to give me the world. For my brother, Julian Shane Garcia Ames, thank you for being someone I will always look up to and being the only person in my life who could truly get me through my struggles. For my lolo and lola, Priscilla Escano Garcia and Ramon Artacho Garcia, thank you for raising and taking care of me. I hope I bring honor and pride to our family. Thank you to Tina Vu, Christopher Su, Hermes Castillo, Marie Manipud, Brian Gutierrez, Bryant Thea, Chau Quach, Samantha Dadat, Sabrina Trinh, and Michael Anderson for all the love, guidance, and friendship throughout the years. I would not be who I am today without all of you. My success will always be shared with you all.

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I would also like to acknowledge William Poon, Gloria Shen, and Von Phan for pushing me to be more confident and helping me develop professionally. You all inspire me to become a great pharmacist and mentor to other students one day.

In Chapter 2, a section of my analysis is currently being prepared for submission for publication of the material. I would like to acknowledge Michael Yoon, PharmD/PhD candidate, for this material and his MSP-MS work in proteases that support my thesis.

Lastly, I would like to acknowledge Dr. Matthew Banghart and Dr. Matthew Lovett-Barron for being a part of my thesis committee. Your expertise is greatly valued for my research.

ABSTRACT OF THE THESIS

Protease Mechanisms for Biosynthesis of Peptide Neurotransmitters

by

Janneca Chelsea Garcia Ames

Master of Science in Biology

University of California San Diego, 2021

Professor Vivian Hook, Chair
Professor Matthew Banghart, Co-Chair

Neuropeptides are peptide neurotransmitters and hormones that play an integral role in mediating neurotransmission, cell-cell communication amongst neurons, and modulate functions in the neuroendocrine system. In particular, this thesis focuses on opioid neuropeptides which are involved in addiction, chronic pain, analgesia, cognitive function as well as the proteases that are associated with their biosynthesis. Cathepsin L, Cathepsin V, PC1, and PC2 are proteases found to process these endogenous opioid proneuropeptides at dibasic residues. Elucidating the properties of these proteases and how they influence the cleavages of these proneuropeptides may hold potential for developing future inhibitors that regulate these proteases and neuropeptides. These findings can have clinical therapeutic

application for diseases and impairments related to these proteases and peptides such as neurological and mental disorders, chronic pain, and more.

Hypothesis: Pro-neuropeptide processing enzymes consisting of the cysteine proteases Cathepsin L and Cathepsin V, and serine proteases PC1 and PC2, possess different cleavage specificities for processing dibasic residues for biosynthesis of neuropeptides.

1) Cathepsin L and Cathepsin V prefer to cleave between and at the NH_2-terminal side of dibasic residues with proneuropeptides
2) PC1 and PC2 prefer to cleave at the C-terminal side of dibasic residues of proneuropeptides.

Specific Aims

1) Assess cleavage specificities of the four processing proteases by unbiased, global multiplex substrate profiling by mass spectrometry (MSP-MS).
2) Evaluate processing protease cleavages of proenkephalin, prodynorphin, and POMC.
3) Evaluate processing protease cleavage specificities for variant dibasic residue peptide-AMC substrates.

Chapter One: Introduction and Background

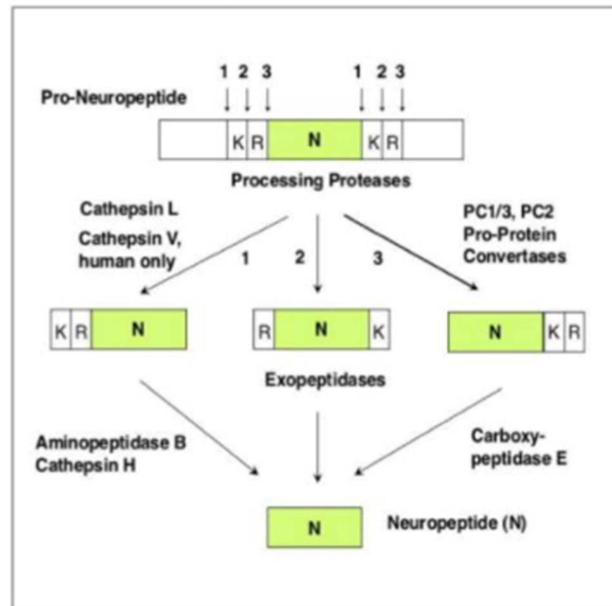


Figure 1 Protease pathways for neuropeptide biosynthesis. Diagram demonstrates the cleavage activity of the Cysteine proteases, cathepsin L and cathepsin V, and Serine proteases, PC1/3 and PC2 proprotein convertases, for processing proneuropeptides into mature neuropeptides. The exopeptidases Aminopeptidase B, Cathepsin H, and Carboxypeptidase E are additional proteases used to cleave intermediate products to generate active neuropeptides (Hook et. al 2018).

Neuropeptides: Importance in cell-cell signaling in neuroendocrine systems

Structure

Peptide neurotransmitters and hormones, otherwise known as neuropeptides, play an integral role in mediating neurotransmission, cell-cell communication among neurons and maintaining the integrity of nervous and endocrine system functions (Hook et al., 2008). In contrast to classical neurotransmitters that are produced from a single modified amino acid, neuropeptides are distinguished by their diverse amino acid sequences of peptides ranging from 3-40 amino acid residues (Hook & Bandeira, 2015). One or multiple active neuropeptides can be found within the peptide sequence of precursor proneuropeptides (Figure 2) (Hook et al., 2008, 2018).

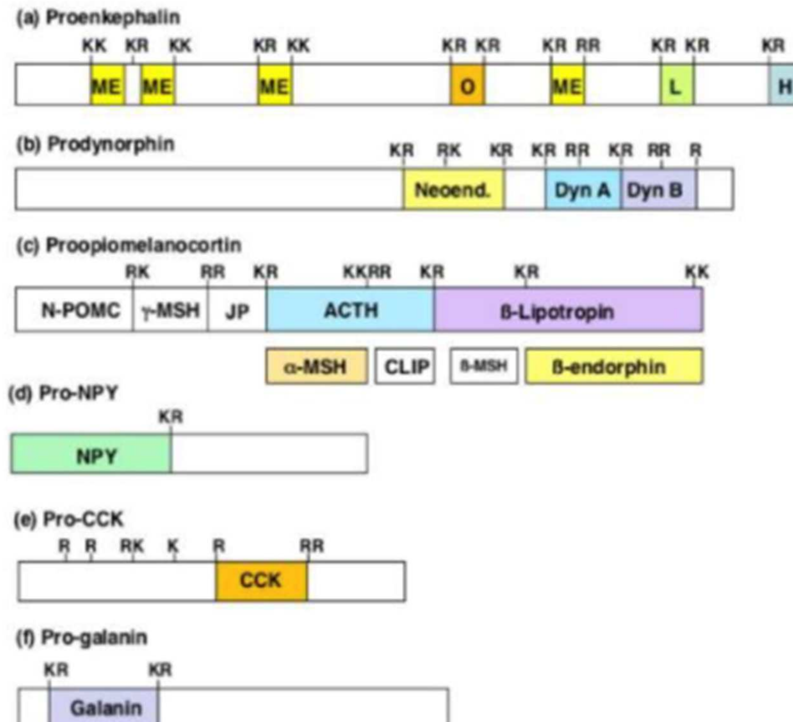


Figure 2. Neuropeptides and peptide Hormones derived from proneuropeptides. Neuropeptides/Hormones are derived from peptide precursors called proneuropeptides. These proneuropeptides may contain one or more active neuropeptides within their peptide sequence. These precursors are cleaved by proteases at dibasic residues to create mature active neuropeptides that are necessary for several physiological functions (Hook et al., 2018).

Locations in Human Physiological Systems

Neuropeptides and peptide hormones contribute to modulating diverse functions in the nervous and endocrine systems. Modulating cardiovascular control, motor control, hormone regulation, pain regulation, anxiety, metabolism, and cognitive health are just a few of many essential functions of neuropeptides (Table 1) (Hook et al., 2008). A single neuropeptide may have multiple functions, for example, enkephalin neuropeptides serve dual functions by acting as neurotransmitters regulating transmission in the nervous system, as well as acting as endocrine hormones that regulate intestinal motility and immune cell functions. The vast range of functions of these neuropeptides emphasize their significance and invite the exploration of their potential in therapeutics and drug research.

Table 1. Neuropeptide functions in the Nervous and Endocrine Systems This table lists a few examples of the physiological functions endogenous neuropeptides and peptide hormones possess (Hook et al., 2008)

Neuropeptides	Physiological functions
Enkephalins	Analgesia, pain relief
β-Endorphin	Analgesia, pain relief
Dynorphin	Analgesia, pain relief
CRH	Stress, glucocorticoid production
ACTH	Steroid production
α-MSH	Skin pigmentation, appetite
Insulin	Glucose metabolism
Glucagon	Glucose metabolism
Galanin	Cognition
NPY	Obesity, blood pressure
Somatostatin	Growth regulation
Vasopressin	Water balance
Calcitonin	Calcium regulation, migraine
Cholecystokinin	Learning, memory, appetite

Neuropeptides function as peptide neurotransmitters and peptide hormones. Examples of several neuropeptides and their biological functions are shown in this table.
ACTH = adrencorticotropin hormone; α-MSH = α-melanocyte stimulating hormone; NPY = neuropeptide Y; CRH = corticotropin releasing hormone.

Cell-Cell Signaling: Brain

In neural cells, neuropeptides function as neurotransmitters to mediate communication between neurons through chemical signaling. Neuropeptides are synthesized through proteolytic maturation within secretory vesicles in neural cells. This process occurs during axonal transport, from the neural body to the nerve terminal (fig.2) (Hook et al., 2008). Once matured, the neuropeptides undergo activity-dependent secretion into the synapse where they can bind to neuropeptide receptors on neighboring neural cells and mediate neurotransmission. Receptors of neuropeptides primarily consist of seven transmembrane protein receptors, G-protein-coupled receptors (GPCRs) (Hoyer & Bartfai, 2012).

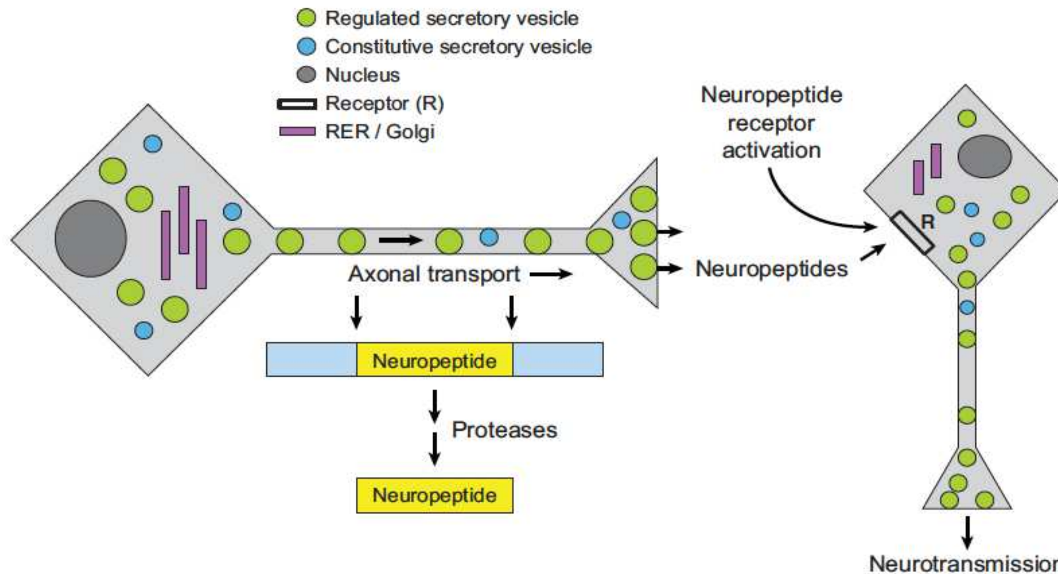


Figure 3. Synthesis of neuropeptides within neural cell secretory vesicles. Proteolytic processing and maturation of neuropeptides occur as secretory vesicles travel through the neural cell's axon. They are eventually transmitted through the synapse, accounting for cell-to-cell communication (Hook et al., 2008).

A diverse array of brain neuropeptides exists, and they serve multiple roles in the central nervous system (CNS). Neuropeptide S (NPS) is a peptide-ligand that targets the NPS receptor (NPS), a GPCR protein previously named GPR154. NPS is currently being investigated for its possible anxiolytic-like and fear extinction effects (Grund & Neumann, 2019). Another example includes Galanin, a peptide-ligand targeting three GPCR subtypes, GalR1-3 and is involved in cognitive function (Webling et al., 2012). Studies have also shown that Galanin can produce anti-seizure activity in epilepsy and epileptogenic animal models (Hoyer & Bartfai, 2012). As significant regulators within the brain, dysfunction of the processes that regulate neuropeptides may lead to neurological diseases and mental disorders.

Cell-Cell Signaling: Endocrine System

In the endocrine system, neuropeptides (hormones) are co-regulated in a feedback system to maintain physiological homeostasis. These peptide hormones mediate intercellular signaling amongst neuroendocrine and target physiological and cellular systems (fig.4) (Hook et al., 2008). As an example, vasopressin is a neuropeptide (hormone) that is produced within the

hypothalamus and released by the pituitary gland to regulate water balance. Additionally, adrenocorticotropic hormone (ACTH), a neuropeptide derived from proopiomelanocortin, binds to five subtypes of melanocortin receptors and is involved in steroid production (Duque-Díaz et al., 2019).

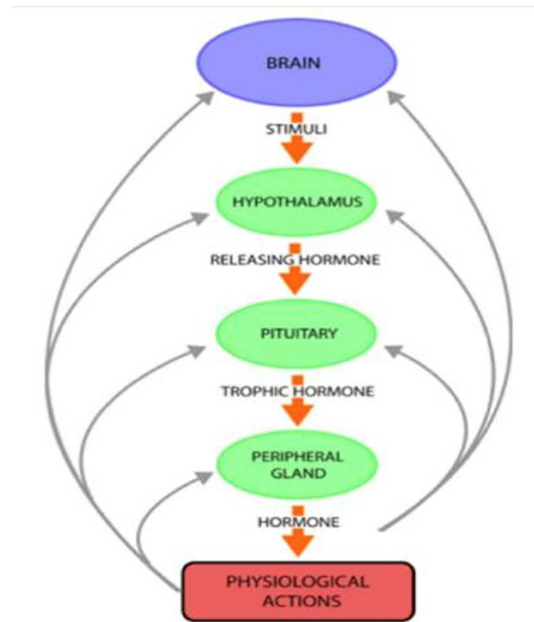


Figure 4. Neuropeptide (Hormone) in the endocrine system. Neurohormones participate in stimulating physiological functions and the feedback loop in the neuroendocrinal system and target tissues to regulate homeostasis (Hook et. al 2018).

Opioid Neuropeptides

Opioid proneuropeptides consisting of proenkephalin, proopiomelanocortin, and prodynorphin have physiological function in reward processing, mood control, addiction, analgesia, and endocrinal functions. Key targets of these opioid neuropeptides include mu, delta, and kappa receptors of the opioid pathway. Activity between mu receptors and the opioid neuropeptide, B-endorphin, has demonstrated a role in regulating the reward properties of non-opioid drugs of abuse such as cocaine and nicotine. Interaction between enkephalin neuropeptides and delta receptors modulate the processes controlling anxiogenic and depressive behaviors. Lastly, the activity between kappa receptors and dynorphin

neuropeptides facilitate the processes that limit drug reward and mediate dysphoric effects of cannabinoids and nicotine (Charbogne et al., 2014).

While opiates may be beneficial for promoting analgesia, the reward pathway involved in the opiate system mediates addiction caused by chronic use of opiate narcotic drugs. Therefore, targeting opioid neuropeptides and their receptors can elucidate potential therapeutic conventions for treating pain without addictive components that is problematic with current opiates being used.

Proenkephalin (PENK)

Proenkephalin is a 243 amino acid peptide that possesses four types of active neuropeptides. It contains four copies of Met-enkephalin, one copy of Met-enkephalin-Arg-Gly-Leu, one copy of Leu-enkephalin, and one copy of Met-enkephalin-Arg-Phe (Figure 5A). Enkephalin opioid neuropeptides/hormones have been found to have active analgesia effects as well as having a role in modulating immune-cell functions (V. Y. H. Hook et al., 1996). When bound to opioid receptors near the terminals of sensory pain fibers, enkephalins can inhibit the release of other neurotransmitters and neuropeptides such as substance P, vasopressin, and dopamine (McLaughlin, 2013). In general, enkephalins are widely studied neuropeptides due to their diverse effects.

Prodynorphin (PDYN)

Prodynorphin is a 234 amino acid peptide that contains seven types of active peptides within its sequence. It contains Alpha-neoendorphin, Beta-endorphin, Big Dynorphin, Dynorphin A (1-17), Leumorphin, Rimorphin, and three copies of Leu (Figure 5B). Dynorphin peptides are known to be involved in the reward pathway and addiction, cognitive processes, and analgesia (Yakovleva et al., 2006).

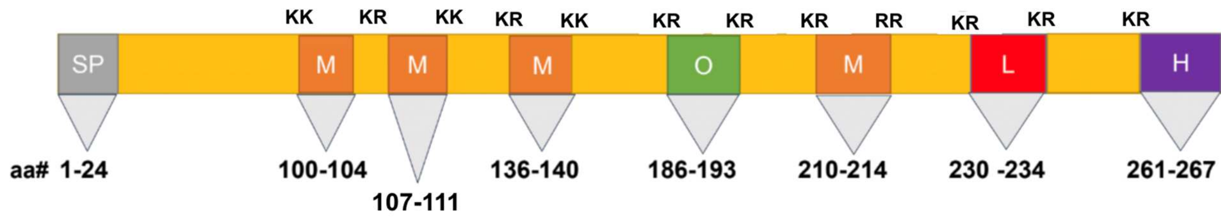
Pro-opiomelanocortin (POMC)

Pro-opiomelanocortin is a 241 amino acid peptide that contains seven types of active peptides within its sequence. It contains the peptides Adrenocorticotropic hormone,

Melanotropin gamma, Melanocyte-stimulating hormone alpha, corticotropin-like intermediary peptide, melanocyte-stimulating hormone beta, beta-endorphin, and met-enkephalin (Figure 5C) Besides acting as peptides involved in analgesia, peptide hormones derived from the precursor POMC are also known to be involved in pigmentation, adrenal function, essential for regulating appetite, energy homeostasis, body composition, and more (Bicknell, 2008; Harno et al., 2018).

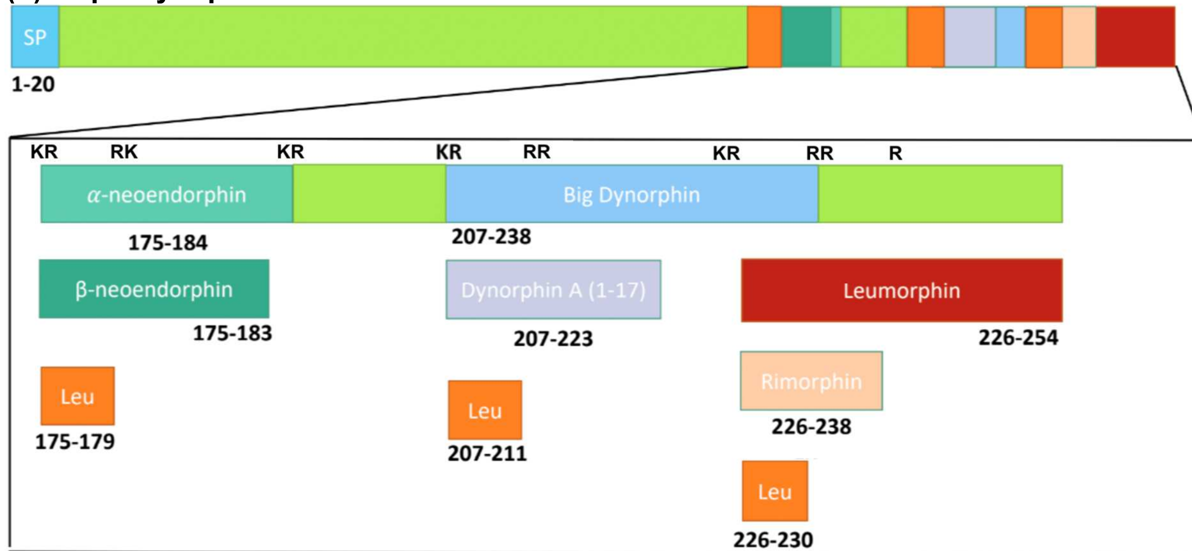
Figure 5a-c. Processing of proneuropeptides PENK, POMC, PDYN. Schematic of active neuropeptides found within opioid peptide precursors. Amino acid length and location of cleavage indicated within each figure. Specific cleavage sites at dibasic residues indicated by amino acid letter annotation (**a)** preproenkphalin**,** 267 amino acid peptide **(b)** preprodynophin, 254 amino acid peptide **(c)** Preproopiomelanocortin, 267 amino acid peptide.

(a)Preproenkphalin

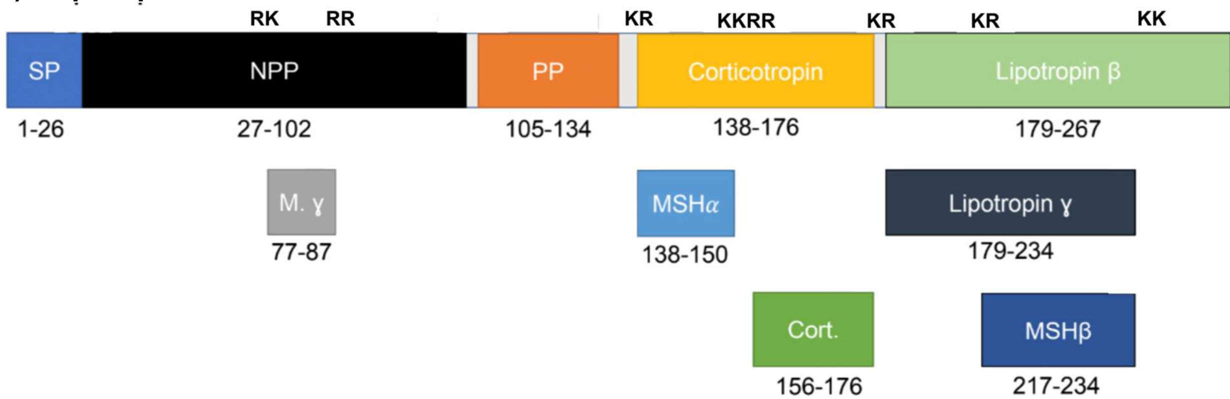


SP – Signal Peptide
M – Met-enkephalin
O – Met-enkephalin-Arg-Gly-Leu
L – Leu-enkephalin
H – Met-enkephalin-Arg-Phe

(b)Preprodynophin



(c)Preproopiomelanocortin



SP: Signal Peptide
M. γ: Melanotropin gamma:
PP: Potential peptide
MSHα: Melanocyte-stimulating hormone alpha
Cort.: Corticotropin-like intermediary peptide
MSHβ: Melanocyte-stimulating hormone beta
β-end.: Beta-endorphin
Met: Met-enkephalin

Biosynthesis from proneuropeptides by proteolytic processing

The biosynthesis and proteolytic processing of proneuropeptides into mature neuropeptides first begins cotranslationally at the rough endoplasmic reticulum (RER) of neural cells (Hook et al., 2008)(Figure 6). The translation of mRNA at the RER produces the primary precursor called the preproneuropeptide. Simultaneously with translation, the signal peptide found at the N-terminus of the preproneuropeptide is cleaved by the signal peptidase enzyme, creating the precursor peptide termed, proneuropeptide. These proneuropeptides are then routed to the Golgi apparatus where they are packaged within secretory vesicles along with protease enzymes, where they will be converted into mature and active neuropeptides.

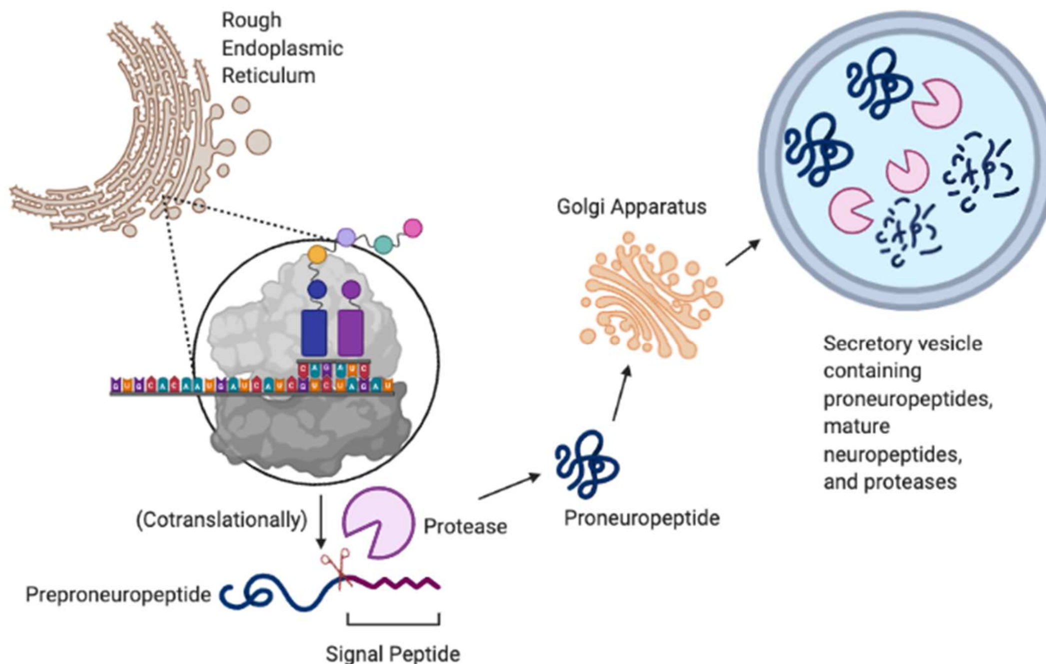


Figure 6 Biosynthetic Pathway of Proneuropeptides in Neural Cells. A diagram of the initial steps of neuropeptide biosynthesis. Within neural cells biosynthesis begins at the cell body at the rough endoplasmic reticulum. At the RER, mRNA is translated to its primary peptide structure called the preproneuropeptide. Cotranslationally, the signal peptide is cleaved off of the preproneuropeptide, producing the proneuropeptide. The proneuropeptide is sent to the Golgi apparatus where it is packaged within a secretory vesicle with proteases that generate mature neuropeptides. Image created using BioRender.

Proneuropeptides, properties of dibasic cleavage sites for proteolytic processing

Neuropeptides are biosynthesized and stored within secretory vesicles. The initial proneuropeptides packaged within these secretory vesicles are cleaved by proteases at monobasic, dibasic, and multibasic proteolytic cleavage sites that flank the NH_2 and COOH termini. Monobasic, dibasic, and multibasic cleavage sites are amino acid regions within a proprotein structure that endoprotease enzymes target for cleavage in order to produce active peptides. A monobasic site contains one basic amino-acid residue, a dibasic site contains a connected pair of basic amino-acid residues, and so on. The dibasic site, Lys-Arg (KR) is the most common dibasic cleavage site that flanks the NH_2 and COOH termini.

Discovery of proteases through biochemical and molecular homology approaches

Proteases are required for the biosynthesis of neuropeptides from their inactive precursors. In order to elucidate which proteases were responsible for neuropeptide production, a series of biochemical and molecular homology approaches were used (Hook et al., 2008). Chromaffin granules were used as model secretory vesicles to detect and identify proteases for neuropeptide biosynthesis. These vesicles contain proneuropeptide precursors and can produce, store, and secrete active enkephalin and related neuropeptides.

Within chromaffin granules, the precursor proenkephalin was found to undergo proteolytic processing. Purification of this processing activity led to the isolation and identification of the 'prohormone thiol protease' complex (PTP) (Sukkid Yasothornsrikul et al., 1999). Based on the complex's molecular weight and sensitivity to cysteine protease inhibitors, it was deduced that the PTP activity belonged to the cysteine protease family. Protein identification of the complex implicated the role of Cathepsin L, a cysteine protease, that represents PTP activity. Cathepsin L has previously been known to function as an enzyme involved in lysosomal degradation but was found to function in the biosynthesis of neuropeptides and peptide hormones in secretory vesicles. DCG-04, an activity probe that

inhibits cysteine proteases was used to target and label the purified cathepsin L enzyme. The labeled protein was identified as Cathepsin L through the utilization of mass spectrometry of tryptic peptides. Knockout of cathepsin L in mice resulted substantial reductions in brain levels of (Met)enkephalin and resulted in reductions in pituitary beta-endorphin and ACTH (Hook et al., 2008; Yasothornsrikul et al., 2003; Miller et al., 2003). Through the use of immunoelectron microscopy and fluorescence immunochemistry, Cathepsin L was found to colocalize with enkephalin within chromaffin granules (Figure 7).

Through molecular biological approaches, several serine proteases, proprotein convertase (PC) family of Subtilisin-like proteases such as PC1/3 and PC2 were found to participate in the biosynthesis of neuropeptides and hormones. These proteins were identified by molecular homology cloning based on its similarities to the yeast *KEX2* gene. The *KEX2* gene produces a subtilisin-like serine protease that is responsible for cleaving precursor peptides at dibasic residues in yeast bacteria, similar to how PC proteases cleave at dibasic sites that flank precursor peptides (Rockwell et al., 2002). In the past literature it has been elucidated that proprotein convertase family proteases, PC1/3, PC2, furin, PACE4, PC3, PC5/6, and PC7 have all been found to be involved in the processing of peptides at basic residues. In terms of neuropeptide biosynthesis in humans, PC1/3 and PC2 activity and expression have been prominently recognized in neuroendocrine tissues. The physiological roles of the active neuropeptides synthesized by PC1/3 and PC2 are consistent with the proteases' localization. PC1/3 and PC2 have been found in secretory vesicles localized in pancreas, pituitary, and adrenal medulla tissues, where they are involved in biosynthesis of neuropeptides and neurohormones derived from proinsulin, POMC, and proenkephalin within these secretory vesicles. These findings show how these proteases play specific roles in neuroendocrine function, which contrasts with other proprotein convertase family proteases such as furin, PACE4, and PC5/6, whose tissue localization pattern is not as narrow and distinct.

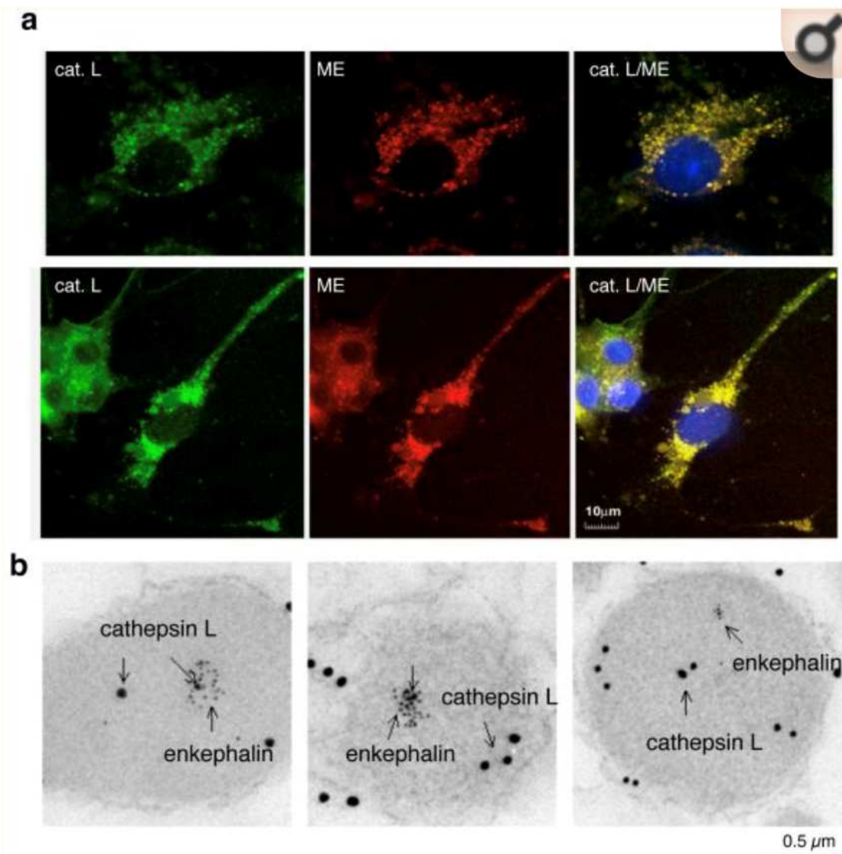


Figure 7 Colocalization of Cathepsin L and Enkephalin Utilizing immunoelectron microscopy and fluorescence immunochemistry, it was found that Cathepsin L and Enkephalin colocalized within chromaffin granules (Hook et al., 2008)

<u>Cysteine protease and serine protease pathways for neuropeptide production</u>

Endoprotease activity occurs at both the NH_2-terminus and COOH-terminus of proneuropeptides. The two pathways that encompass the proteolytic activities of neuropeptide production are the cysteine protease pathway and the serine protease pathway (Figure 1). Cathepsin L and Cathepsin V are two cysteine proteases involved in processing proneuropeptides and prohormones. Evidence shows that these cysteine proteases prefer cleaving dibasic sites at the NH_2-terminal side and between the dibasic residues. The intermediates produced after cysteine protease cleavage undergo additional cleavage activity by Arg/Lys aminopeptidase in order for the mature neuropeptide to form. When processing occurs between the dibasic residues or at their COOH-termini, both carboxypeptidase and

aminopeptidase will be involved to cleave the intermediate products to remove basic residues from NH_2- and COOH-termini.

In addition, serine proteases such as proprotein convertase (PC) family of Subtilisin-like proteases, PC1/3 and PC2, prefer to cleave dibasic sites at the COOH-termini side. The intermediates produced after serine protease activity undergo additional cleavage activity by Lys/Arg carboxypeptidase in order to produce the mature neuropeptide.

After proteolytic processing, additional posttranslational modifications including phosphorylation, amidation, acetylation, and others can occur to create the final active and mature neuropeptide.

Proteases identified for opioid pro-neuropeptide processing

Proenkephalin (PENK) processing proteases

The prohormone thiol protease (PTP) was an early cysteine protease complex found to process proenkephalin into its mature enkephalin neuropeptides such as met-enkephalin (Schiller et al., 1995). Within chromaffin granules of bovine adrenal medulla, which contain enkephalin precursors and peptides, PTP activity was detected through recombinant proenkephalin substrate and fluorogenic peptide-MCA assays. With the use of fluorogenic peptide-MCA substrate assays, it was seen that this cysteine protease preferred to cleave at dibasic residues (Lys-Arg, Arg-Arg, Lys-Lys, Arg-Lys) at the NH_2-terminal side of dibasic residues as well as between the dibasic residues (Table 2) (Hook et al., 1996). Alongside PC1/3 and PC2, which make up 20% of protease activity within enkephalin containing chromaffin granules, PTP made up approximately 60% of protease activity. Endogenous PC1/3 and PC2 showed preference to cleave at dibasic and monobasic residue sites within the peptide-MCA substrates (Table 3). Aspartic Proteinase was also found to be involved in processing between the Lys-Arg dibasic pair flanking the COOH-terminus.

Table 2 PTP Cleavage of Peptide-MCA Substrates Using fluorogenic Peptide-MCA substrate assays, PTP cleavage activity was measured with presence and absence of aminopeptidase M (APM) (V. Y. H. Hook et al., 1996)

TABLE 1. PTP Cleavage of Peptide-MCA Substrates[a]

Substrate	Proteolytic Activity μmol AMC[b]/h/mg −APM	+APM
Z-Phe-Arg-MCA	1,176	1,915
Bz-Arg-MCA	7	24
Boc-Gln-Gly-Arg-MCA	6	84
Bz-Val-Leu-Lys-MCA	695	885
Z-Arg-Arg-MCA	16	49
Boc-Gln-Arg-Arg-MCA	15	118
Boc-Gly-Arg-Arg-MCA	7	42
Z-Arg-Val-Arg-Arg-MCA	19	50
Boc-Gly-Lys-Arg-MCA	21	73
Boc-Glu-Lys-Lys-MCA	7	26

Table 3 PC1/3 and PC2 Cleavage of Peptide-MCA Substrates Using fluorogenic Peptide-MCA substrate assays, PC1/3 and PC2 Cleavage of Peptide-MCA substrates was used to measure cleavage activity with the absence and presence of APM (V. Y. H. Hook et al., 1996)

TABLE 2. PC1/3 and PC2 Cleavage of Peptide-MCA Substrates[a]

Peptide-MCA Substrate	Activity, nmol AMC/h/m PC1/3 −APM	+APM	PC2 −APM	+APM
Boc-Arg-Val-Arg-Arg-MCA	351	345	762	747
pGlu-Arg-Thr-Lys-Arg-MCA	342	329	683	658
Boc-Gly-Lys-Arg-MCA	208	300	515	552
Boc-Phe-Val-Arg-MCA	144	144	416	416
Boc-Gln-Arg-Arg-MCA	125	192	345	364
Boc-Gly-Arg-Arg-MCA	116	118	336	360
Boc-Val-Leu-Lys-MCA	144	188	272	333
H-Arg-Gln-Arg-Arg-MCA	25	461	123	577
Boc-Glu-Lys-Lys-MCA	32	51	81	104
Boc-Gln-Gly-Arg-MCA	2	29	6	36

PTP was identified as Cathepsin L by probe labeling and mass spectrometry. As explained earlier, Cathepsin L is a cysteine protease found to colocalize with enkephalin substrates within chromaffin granule secretory vesicles. Cathepsin V, the human analog of Cathepsin L, was also found to participate in the synthesis of enkephalin neuropeptides (Funkelstein et al., 2012). Since Cathepsin L and Cathepsin V cleave dibasic residues at the NH_2-terminal side or between the dibasic residues, an aminopeptidase activity represented by

Cathepsin H is utilized to remove NH_2-terminal basic residues, assessed through nano-LC-MS/MS tandem mass spectrometry, immunoelectron microscopy, and confocal immunofluorescence microscopy (Lu et al., 2012).

Pro-opiomelanocortin (POMC) Processing Proteases

Within pituitary secretory vesicles, 70 kDa aspartic proteinases have been found to participate in processing POMC into mature peptide products such as neurohormone ACTH and B-endorphin-related peptides (Hook et al., 1996). Extensive data showed the participation of the serine proteases PC1/3 and PC2 as the prohormone convertases mostly associated with the hypothalamus, skin, and pituitary to produce adrenocorticotropic hormone (ACTH) (Harno et al., 2018). Cleavage of POMC by the PCs occurs at the COOH terminal at dibasic residue sites. Peptides derived from ACTH such as alpha-MSH are synthesized from PC2 enzymes. Studies have also showed that PC4, PACE4, PC5/6. PC7, S1P/SKI-1, and PCSK9 are also involved in the processing of POMC peptides into their active mature neuropeptides/hormones.

Prodynorphin Processing Proteases

PC1/3 and PC2, proteolytic enzymes mainly expressed in neural and endocrine cells, were found to be colocalize with the precursor prodynorphin and cleave the precursor at monobasic and dibasic residue sites (Day et al., 1998). Cathepsin L also participates in dynorphin production in brain, shown by protease gene knockout and expression (Minokadeth et al., 2010). Thus, Cathepsin L and PC2 process prodynorphin into their final neuropeptide products.

Chapter 2: Cleavage Profiling of Proteases Involved in Neuropeptide Biosynthesis: Cysteine Proteases Cathepsin L and Cathepsin V, and Serine Proteases PC1/3 and PC2

Multiplex Substrate Profiling Mass Spectrometry (MSP-MS)

How will protease cleavage profiling by MSP-MS test the hypothesis?

The hypothesis of this project proposes that the specific cleavage properties and preferences of cysteine proteases Cathepsin L and Cathepsin V compared to the serine proteases PC1 and PC2 differ for processing dibasic residues of proneuropeptides. Utilizing MSP-MS (multiplex substrate profiling by mass spectrometry) assays will allow the unbiased profiling of the specific cleavage preferences of Cathepsin L, Cathepsin V, PC1, and PC2 by using a library of 228 14-mer peptides curated to possess all possible cleavage sites. Additionally, uncovering this data will allow a further understanding of whether these proteases favor cleaving activity towards the N-terminal side or C-terminal side of dibasic residues within peptide substrates.

Background

MSP-MS is a direct cleavage assay used to reveal the cleavage properties and specificity of endo- or exo-peptidases by utilizing liquid chromatography-tandem mass spectrometry sequencing of peptide products derived from a peptide library containing all known protease cleavage sites (O’Donoghue et al., 2012). MSP-MS utilizes a diverse peptide library of 228 14-mer peptides flanked by unique combinations of dipeptides at each terminus. By generating all combinations of neighbor and near-neighbor amino acid pairs, it is an unbiased technique that can detect cleavage preferences and overcome the limitations of libraries that only contain endogenous peptides. To create these randomized tetradecapeptides, the central decapeptide region contained two copies of every amino acid pair (XY) and one copy of every X*Y and X**Y pair (where X and Y represents defined amino acids and “*” indicates a random

amino acid). In order to prepare peptide diversity, a unique dipeptide sequence was placed at the N terminus and C terminus flanking the decapeptide. The randomization of these 14-mer peptides resulted in 1,612 potential cleavage sites (Figure 8a). After the library has been introduced to a peptidase, the assay is quenched at different time intervals and injected into an LC-MS/MS to show intensities of cleaved peptide products (Figure 8b).

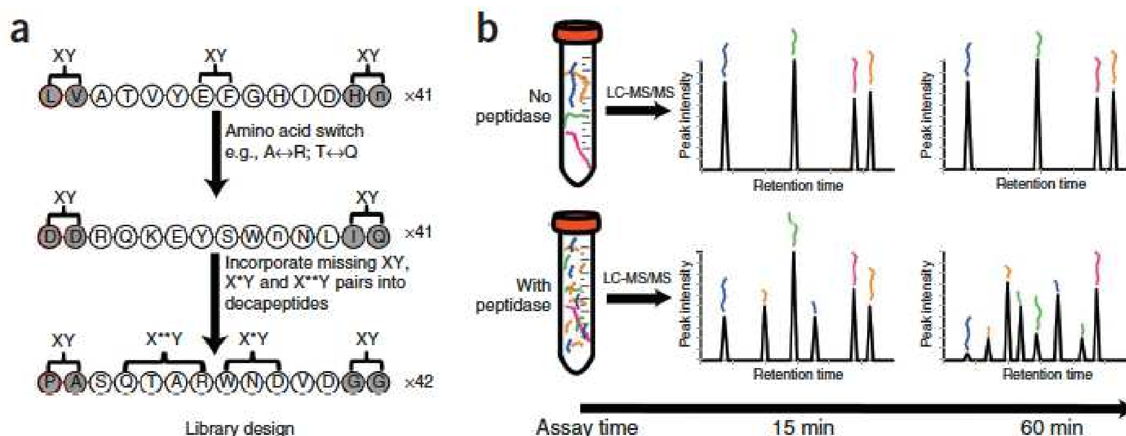


Figure 8a-b. MSP-MS Library Design and LC-MS/MS (a) The design of the 14-mer peptide library was generated by randomization of amino acid sequences. (b) Displays the multi substrate-profiling assay. At different time intervals samples of the assay are injected into an LC-MS/MS. As shown, without the peptidase added, proteolytic activity does not occur, however, with the addition of the peptidase, proteolytic fragments are shown to appear as evidenced by the additional intensities shown. (O'Donoghue et al., 2012)

<u>Distinct Dibasic Cleavage Specificities of Neuropeptide-Producing Cysteine and Serine Proteases Revealed by Global Multiplex Substrate Profiling by Mass Spectrometry (Hook lab, in progress)</u>

This project focused on elucidating the dibasic cleavage preferences of Cathepsin L and Cathepsin V in comparison to PC1/3 and PC2 by utilizing MSP-MS. MSP-MS was used to create unbiased assays to analyze all possible cleavage specificities of the proteases mentioned. It was found that Cathepsin L and V preferred to cleave at the N-terminal side of dibasic residues KR, RK, and KK. These enzymes also showed preference for hydrophobic

residues at the P2 position. Additionally, it was elucidated that PC1/3 and PC2 preferred to cleave at the C-terminal sides of dibasic resides, RR and KR.

Methods

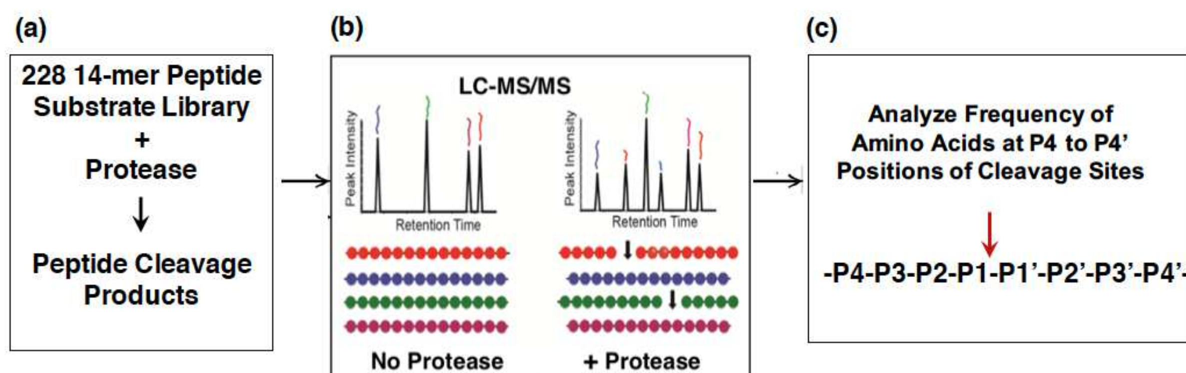


Figure 9. Workflow of MSP-MS Assay A library of 228 14-mer peptide substrates was subjected to incubation with proteases PC1, PC2, Cathepsin L, and Cathepsin V. Cleavage products underwent LC-MS/MS. Frequency of cleavage sites between positions P4 to P4' were calculated (Hook lab in progress 2021).

The 228 15-mer peptide substrate library was incubated at pH 5.5 and was subjected to cleavage activity by proteases of interest (figure 9). The MSP-MS assay was utilized to test which substrates the proteases of interest preferred to cleave and examine cleavage properties. LC-MS/MS was then utilized to identify and quantitate cleavage products. PEAKS (v 8.5) bioinformatics software was used to identify the peptides indicated by LC intensity peaks. Lastly the frequency of amino acid residues at P4 to P4' positions of the cleavage sites were analyzed.

Results

Cathepsin L and Cathepsin V prefer to cleave the dibasic residue at its N-terminal side, whereas PC1/3 and PC2 prefer to cleave dibasic residues at their C-terminal side (Table 4). Cathepsin L and Cathepsin V showed preferences to cleave at basic residues Lys and Arg at the P1 and P1' positions (figure 10). Proteolytic activity of Cathepsin L at dibasic sites consisted of cleavages at K*R, R*K, and at the N-terminal side of dibasic sites *KR and *RK. Meanwhile Cathepsin V preferred to cleave between the dibasic residues K*R, R*K, and K*K. At the P2 position, both Cathepsin L and Cathepsin V showed strong preferences for hydrophobic

residues of Leu, Val, Phe, Trp, and Tyr. These findings are consistent with past studies that found Cathepsin L's and Cathepsin V's preference for a hydrophobic residue at the P2 position for cleaving proNPY (Figure 11) (Funkelstein et al., 2008).

In terms of analysis for the serine proteases PC1/3 and PC2, MSP-MS analysis (Table 4) was consistent with past literature that found that these proteases preferred to cleave on the C-terminal side of dibasic or tetrabasic residues.

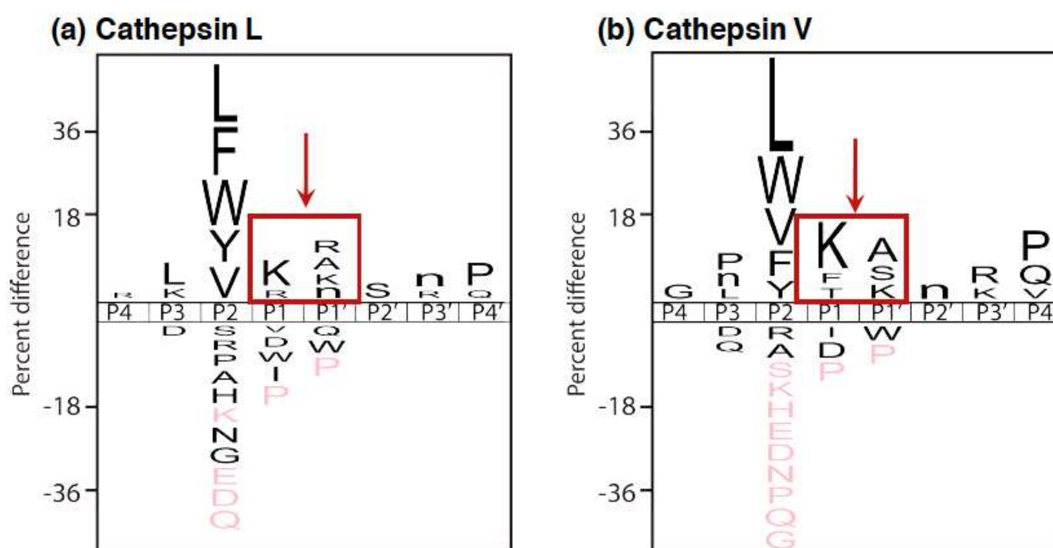


Figure 10a-b Cleavage Preferences Shown by IceLogo of Cathepsin L and Cathepsin V
Different heights of each amino acid (indicated by single letter) represent the 'percent difference' which is the frequency for the amino acid appearing in the experimental data minus the frequency for an amino acid appearing in the reference peptide library MSP-MS data. Amino acid letters displayed showed a significant difference ($p<0.05$) in comparison to reference library. Positive differences are represented above the midline while negative differences are represented below the midline. Pink letters indicate amino acids that never occurred at the indicated position. (Hook lab in progress 2021)

Table 4 Dibasic residue cleavages of peptide substrates by the cathepsin L and cathepsin V cysteine proteases, and the PC1/3 and PC2 serine proteases Cleavage preference of each protease characterized by identification and quantification of cleaved peptide products . Cleavage site is indicated by bolded downward arrow. (Hook lab, in progress 2021)

Protease	**Peptide**	**Fold-Change** (60 min.)
Cathepsin L	GnYY**K↓R**FnAHWVGI	142
	TPHHVNWY**K↓R**APNQ	46
	TPHHVNWY**↓KR**APNQ	5
	EGADIWY**R↓K**HSHQL	5
	LGWHAnF**↓RK**YPInA	124
Cathepsin V	GnYY**K↓R**FnAHWVGI	14
	TPHHVNWY**K↓R**APNQ	6
	LGWHAnF**R↓K**YPInA	12
	DAWAPnVI**K↓K**ESSI	32
PC1/3	GnYY**KR↓**FnAHWVGI	395
	IEPPWVDSHA**KR↓**Nn	23
	VDYIEHKDQV**RR↓**nN	14
PC2	YWnSTHLAG**KR↓R**DW	34

<u>Future Implications in Relation to Protease Mechanisms for Biosynthesis of Peptide Neurotransmitters</u>

From this study, results show that Cathepsin L and Cathepsin V have cleavage preferences at the N-terminal side of dibasic residues KR, RK, and KK. These enzymes also showed to have preference at hydrophobic residues at the P2 position. Additionally, it was found that PC1/3 and PC2 preferred to cleave at the C-terminal sides of dibasic resides, RR and KR. These results play an important role in identifying, quantifying, and elucidating more information about the role of proteases and how they specifically cleave proneuropeptides.

The significance of this study is that it reveals new information about these proteases, which can be used to further progress in therapeutic research. In fact, in relation to the current project at hand, with evidence of Cathepsin L, Cathepsin V, PC1/3, and PC2 cleavage specify, these results can be used to predict the outcomes of the proteases of interest on opioid proneuropeptides PENK, PDYN, and POMC. It is expected to see that Cysteine proteases Cathepsin L and Cathepsin V, compared to serine proteases Proprotein Convertases 1 and 2,

will have different cleavage specificities for processing dibasic residue sites of opioid pro-neuropeptides because this multiplex substrate profiling by mass spectrometry (MSP-MS) of these proteases revealed that Cat. L and Cat. V cysteine proteases prefer to cleave at the N-terminal side of dibasic residues while PC1 and PC2 serine proteases prefer to cleave at the C-terminal side of dibasic residues.

Comparison of proneuropeptide (prohormone) cleavage sites of these processing proteases in the literature

In the literature it is known that proteases are involved in processing precursor substrates into mature neuropeptides that are significant for a diverse group of functions, yet there is a necessity to elucidate more information about the mechanisms behind the biosynthesis of these mature and active peptides. With the results previously exhibited above, we are better able to expand on the knowledge that is currently available or proneuropeptide (prohormone) cleavage site preferences of our proteases of interest.

In past literature findings, the cleavage preferences of PC1 and PC2 on different proneuropeptides and prohormones have been prevalently been studied. PC1/3 was shown to cleave proinsulin after the dibasic site RR, while PC2 cleaved this same proneuropeptide after LR towards the C-terminal of the peptide (Bailyes et al., 1992; Seidah et al., 2013). POMC is cleaved at the C-terminus of basic residue sites AQRR*, EGKR*, GKKR*, EFKR*, and KDKR* by PC2, and both PC1 and PC2 are able to cleave at GKR* or GKKR* (Benjannet et al., 1991). For the processing of proglucagon (proG), cleaved at dibasic pairs, KR, RR, and at a monobasic site R, which yielded GLP-1(Dhanvantari et al., 1996). Additionally, it was indicated that an R at the P4 position was a preference for PC2 (Johanning et al., 1998). Another study detected that PC1 has an inability to cleave sites containing a large aliphatic residue at the P1’ position, however this site can still be efficiently cleaved by PC2 (Peinado et al., 2003). These are just a few examples, but there is a clear pattern in the literature that these proteases prefer cleaving at the C-terminal of basic resides, especially at the residues dibasic RR, KR. The selectivity for

PC1/3 and PC2 for processing proenkephalin mainly consists of KK and KR (figure 12). As for POMC, PC1/3 preferred to cleave at the C-terminal side of RR and KR (figure 13). For PC2, the protease demonstrated a wider breadth of cleavage preferences including the C-terminal side of RR, KR, KKRR, KR, and KK.

As for the cysteine proteases Cathepsin V and Cathepsin L, the literature present is not as vast in comparison to the serine proteases discussed above. Cathepsin V, was found to process proenkephalin with its preferences for processing at the dibasic KR site (Funkelstein et al., 2012). Cathepsin L was found to cleave at several sites of met-enkephalin, including dibasic RR, monobasic R sites and could cleave Peptide F at dibasic LL and LR sites (S. Yasothornsrikul et al., 2003).

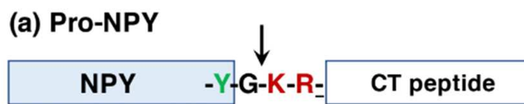


Figure 11 Cathepsin L cleavage of pro-NPY Mass spectrometry analysis of Cathepsin L cleavage products on recombinant pro-NPY. Arrow represents cleavage site at the N-terminal side of the dibasic residue KR (Funkelstein et al., 2008)

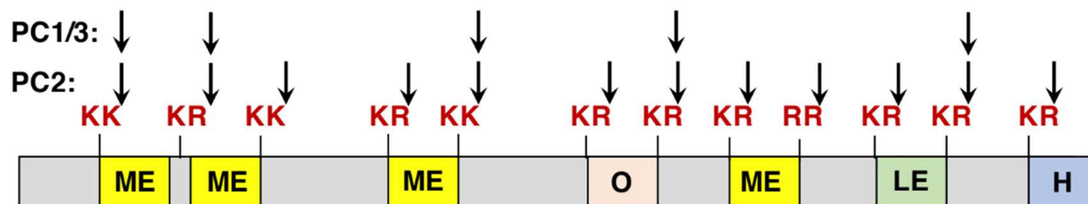


Figure 12 PC1/3 and PC2 cleavage of proenkephalin MALDI-TOF analyses of PC1/3 and PC2 cleavage products on proenkephalin. Arrows represent site of cleavage preferences (Peinado et al., 2003).

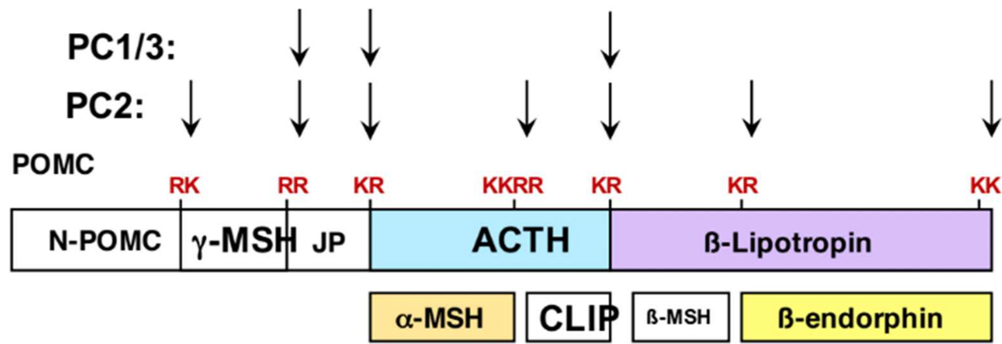


Figure 13 PC1/3 and PC2 cleavage of POMC POMC undergoes proteolytic processing by proteases PC1/3 and PC2 at dibasic and tetrabasic sites, resulting in the mature active peptides ACTH, alpha-MSH, B-endorphin. The cleavage preferences of this proteases were found using HPLC, gel electrophoresis, and immunoassays (Benjannet et al., 1991, Zhou et al., 1993, Loh 1994).

I would like to acknowledge Michael Yoon, PharmD/PhD candidate, and the rest of the Hook lab, for this material and the MSP-MS work in proteases that support my thesis.

Chapter 3: Analyses of protease cleavages at variant dibasic processing sites of peptide-AMC substrates, models for proneuropeptide processing sites

Peptide-AMC Fluorogenic Assays

How will Peptide-AMC fluorogenic assays test the hypothesis for selectivity in cleaving dibasic residue processing sites?

Building on the cleavage preference of the proteases of interest found by the Hook lab MSP-MS assays, the peptide-AMC fluorogenic assays will further investigate the distinct cleavage site preferences of these same proteases. These assays can help confirm the hypothesis that Cathepsin L and Cathepsin V prefer to cleave at the NH_2-terminal side of dibasic residues and that PC1 and PC2 prefer to cleave at the C-terminal side of dibasic residues. The peptide-AMC fluorogenic substrates possess different dibasic residue processing sites for the proteases to cleave. It is predicted that PC1/2 prefers to cleave at the C-terminal side of dibasic residues such as KR, and Cat.V and Cat. L prefer to cleave between or at the N-terminal side of dibasic residues such as KR (table 4). Based on fluorogenic activity, it can be elucidated whether a protease will cleave at a predicted site and which proteases are the most effective at cleaving certain substrates. The peptide-AMC data will allow the comparison of cleavage properties of the proteases and compare information to cleavage properties from prior studies by the Hook lab.

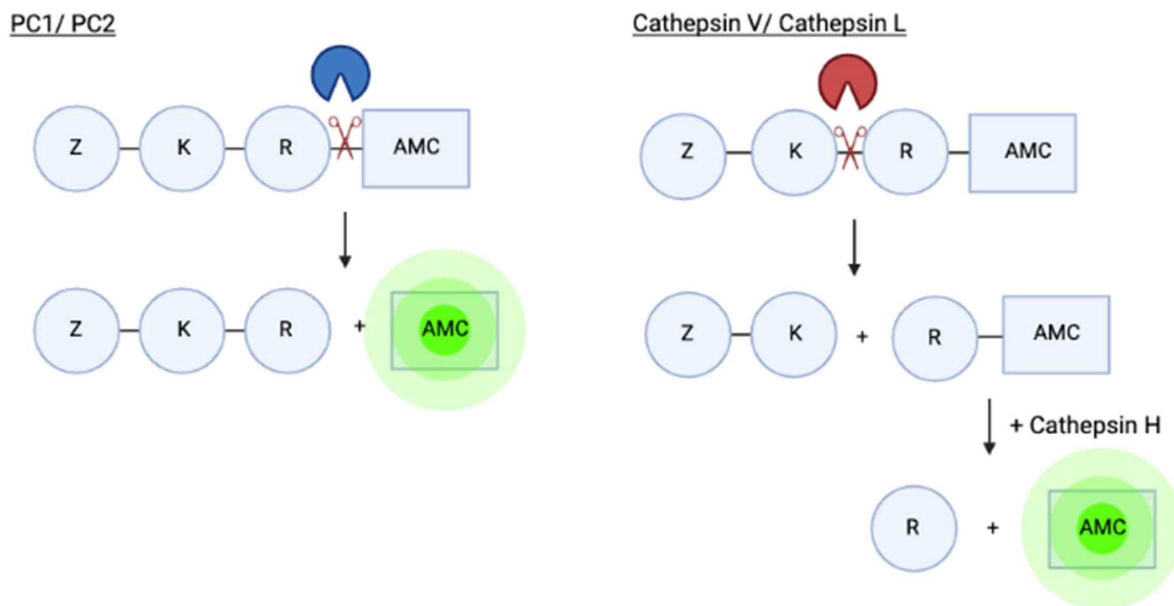


Figure 14 Expected Observations for PC1/PC2 and Cathepsin V/ Cathepsin L in Peptide-AMC Substrate Assays. PC1 and PC2 are expected to cleave the bond at R-AMC, producing two products, ZKR peptide and free AMC. The free AMC product will have detectable fluorescense. Cathepsin V and Cathepsin L are expected to cleave in between the dibasic residue KR. AMC is not expected to be detactable after this cleavage since free AMC is not released. The subsequent addition of Cathepsin H to remove N-terminal basic residues will produce free AMC fluorescence. Image created using BioRender.

Background

Peptide-AMC fluorogenic assays are a simple method used to label proteins of interest (Pickering & Davies, 2012). This method utilizes the fluorophore 7-amino-4-methycoumarin (AMC) attached to the peptide substrate in the form of Z-peptide-AMC. Upon cleavage to generate free AMC, AMC fluorescence is readily detected in a fluorimeter (figure 11). Short-peptide-AMC substrates can be subjected to incubation with proteases of interest, allowing cleavage of peptide bonds to occur. Depending on the dibasic sites the proteases cleave at, free AMC may be released from the substrate and fluoresce, which can be quantitatively measured. If AMC stays covalently bound to the peptide substrate after initial cleavage, additional proteases whose cleavage preferences are known can be added to the substrate to release AMC from the peptide.

Methods

Protease Peptide-AMC Substrate Assays

11 peptide-AMC substrates, Z-KR-AMC, Z-RK-AMC, Z-KK-AMC, Z-RR-AMC, Z-LKR, Z-WKR-AMC, Z-FKR-AMC, Z-YKR-AMC, Z-VKR-AMC, Z-GKR-AMC, Z-AKR-AMC were subjected to protease processing by Cathepsin L, Cathepsin V, PC1, or PC2 with or without the addition of Cathepsin H. Cathepsin H is an aminopeptidase that can remove N-terminal basic residues, Arg or Lys.

Cathepsin L followed by Cathepsin H with peptide-AMC substrates assay

Human recombinant protease Cathepsin L was obtained from R&D Systems (cat. No 952-CY-010, Lot EWZ1320021). The final assay conditions for Cathepsin L contained 5 mM Citrate-Phosphate pH 5.5, 4 mM DTT, 1 mM EDTA, 50 mM NaCl, 0.0001% BSA, 60 uM Z-FR-AMC, 1% DMSO (from substrate), and 0.040 ng/ul Cathepsin L. Using a 96-well plate (black, U-bottom), 100 ul assay, in each well 30 ul H_2O, 50 ul 2X Assay buffer, 10 ul 0.6 mM peptide-AMC in 10% DMSO, and 10 ul 0.40 ng/ul were added to each well. Assay plate was kept on ice while working. Assay plate was mixed by shaking for approximately five seconds on a thermomixer or plate reader. Assay plate was then incubated at room temperate (bottom of plate in water bath at 22 degrees Celsius) for 30 minutes. Fluorescence at 360/460 excitation/emission with a gain of 50.

After addition of Cathepsin L, RFU readings were taken after 30 minutes and 60 minutes. Then, Cathepsin H was added and RFU readings were taken after 30 minutes and 60 minutes incubation at RT. For addition of Cathepsin H, reagents consisted pf 10 ul 500 mM MES pH 6.5, 2 ul of activated Cathepsin H, 25ng/ul that were mixed by gentle shaking. AMC fluorescence was measured at 360/460, gain 50. Z-FR-AMC was used as a control substrate for this experiment.

Cathepsin V followed by Cathepsin H with peptide-AMC substrates Assay

Human recombinant protease Cathepsin V was obtained from R&D Systems (cat. No 1080-CY-010, Lot#FY10416051). The final assay conditions for Cathepsin V included 5 mM Citrate-Phosphate pH 5.5, 5 mM DTT, 1 mM EDTA, 50 mM NaCl, 60 uM Z-FR-AMC, 1% DMSO (vehicle for substrate), and 0.2 ng/ul Cathepsin. Using a 96-well plate (black, U-bottom), 100 ul assay, in each well 30 ul H_2O, 50 ul 2X Assay buffer, 10 ul 0.6 mM peptide-AMC in 10% DMSO, and 10 ul 2 ng/ul were added to each well. Assay plate was kept on ice while working. Assay plate was mixed by shaking for approximately five seconds on a thermomixer or plate reader. Assay plate was then incubated at room temperate (bottom of plate in water bath at 22 degrees Celsius) for 30 minutes. Fluorescence at 360/460 excitation/emission with a gain of 50.

After addition of Cathepsin V, RFU readings were taken after 30 minutes and 60 minutes. Then, Cathepsin H was added, and RFU readings were taken after 30 minutes and 60 minutes incubation at RT. The Cathepsin H reagents consisted pf 10 ul 500 mM MES pH 6.5, 2 ul of activated Cathepsin H, 25ng/ul that were mixed by gentle shaking. AMC fluorescence was measured at 360/460, gain 50 then read again at 60 min incubation with Cathepsin H. Z-FR-AMC was used as a control substrate for this experiment.

PC1 followed by Cathepsin H with peptide-AMC substrates assay

PC1 was obtained from R&D Systems. The final assay conditions for PC1 included 5 mM Sodium-acetate pH 5.5, 5 mM $CaCl_2$, 0.5% Brij-35, 60 uM pERTKR-AMC substrate, and 0.9 ng/ul PC1. Using a 96-well plate (black, U-bottom), 100 ul assay, in each well 30 ul H_2O, 50 ul 2X Assay buffer, 10 ul 0.6 mM peptide-AMC in 10% DMSO, and 10 ul 10 ng/ul PC1 were added to each well. Assay plate was kept on ice while working. Assay plate was mixed by shaking for approximately five seconds on a thermomixer or plate reader. Assay plate was then incubated at room temperate (bottom of plate in water bath at 22 degrees Celsius). RFU was read at 60 minutes and 120 minutes incubation, fluorescence at 360/460 excitation/emission with a gain of 50.

After addition of PC1, RFU readings were taken after 30 minutes and 60 minutes. Then, Cathepsin H was added, and RFU readings were taken after 30 minutes and 60 minutes incubation at RT. Cathepsin H reagents consisted pf 10 ul 500 mM MES pH 6.5, 2 ul of activated Cathepsin H, 25ng/ul that were mixed by gentle shaking. AMC fluorescence was measured at 360/460, gain 50 then read again at 60 min incubation with Cathepsin H. pERTKR-AMC was used as a control substrate.

PC2 followed by Cathepsin H with peptide-AMC substrates assay

PC2 was obtained from R&D Systems (cat no. 5018-SE-010, Lot TET031906) The final assay conditions for PC2 included 5 mM Sodium-acetate pH 5.5, 5 mM $CaCl_2$, 50 mM NaCl, 0.5% Brij-35, 60 uM pERTKR-AMC substrate, and 2.0 ng/ul PC1. Using a 96-well plate (black, U-bottom), 100 ul assay, in each well 30 ul H_2O, 50 ul 2X Assay buffer, 10 ul 0.6 mM peptide-AMC in 10% DMSO, and 10 ul 20 ng/ul PC1 were added to each well. Assay plate was kept on ice while working. Assay plate was mixed by shaking for approximately five seconds on a thermomixer or plate reader. Assay plate was then incubated at room temperate (bottom of plate in water bath at 22 degrees Celsius). RFU read at 60 minutes and 120 minutes incubation, fluorescence at 360/460 excitation/emission with a gain of 50.

After addition of PC1, RFU readings were taken after 30 minutes and 60 minutes. Then, Cathepsin H was added, and RFU readings were taken after 30 minutes and 60 minutes incubation at RT. Cathepsin H reagents consisted pf 10 ul 500 mM MES pH 6.5, 2 ul of activated Cathepsin H, 25ng/ul that were mixed by gentle shaking. AMC fluorescence was measured at 360/460, gain 50. Addition of pERTKR-AMC as a substrate was used as a positive control for this experiment.

Results

Table 5. Specific Activities of Proneuropeptide Processing Enzymes: Cathepsin L, Cathepsin V, Pro-protein convertase 1/3, Pro-protein convertase 2 Shows differences for high to low specific activity of the processing proteases.

Protease	Substrate	Specific Activity pmol AMC/ng/min
Cathepsin L	Z-Phe-Arg-AMC	8.5
Cathepsin V	Z-Phe-Arg-AMC	0.88
Pro-protein convertase 1/3	pGly-Arg-Thr-Lys-Arg-AMC	0.132
Pro-protein convertase 2	pGly-Arg-Thr-Lys-Arg-AMC	0.0067

Table 5 demonstrates the specific activity (pmol AMC/ng/min) of the proteases Cathepsin L, Cathepsin V, PC 1/3, and PC2. Cathepsin L demonstrated the highest specific activity (8.5 pmol AMC/ng/min) followed by Cathepsin V (0.88 pmol AMC/ng/min), PC1/3 (0.132 pmol AMC/ng/min), and lastly PC2 (0.0067 pmol AMC/ng/min), which demonstrated the lowest specific activity. Specific Activity was calculated by dividing the mean RFU by the concentration of enzyme used to process each substrate. This number was then divided by the mean slope of the standard curves for Cathepsin V, Cathepsin L, PC 1/3, and PC2

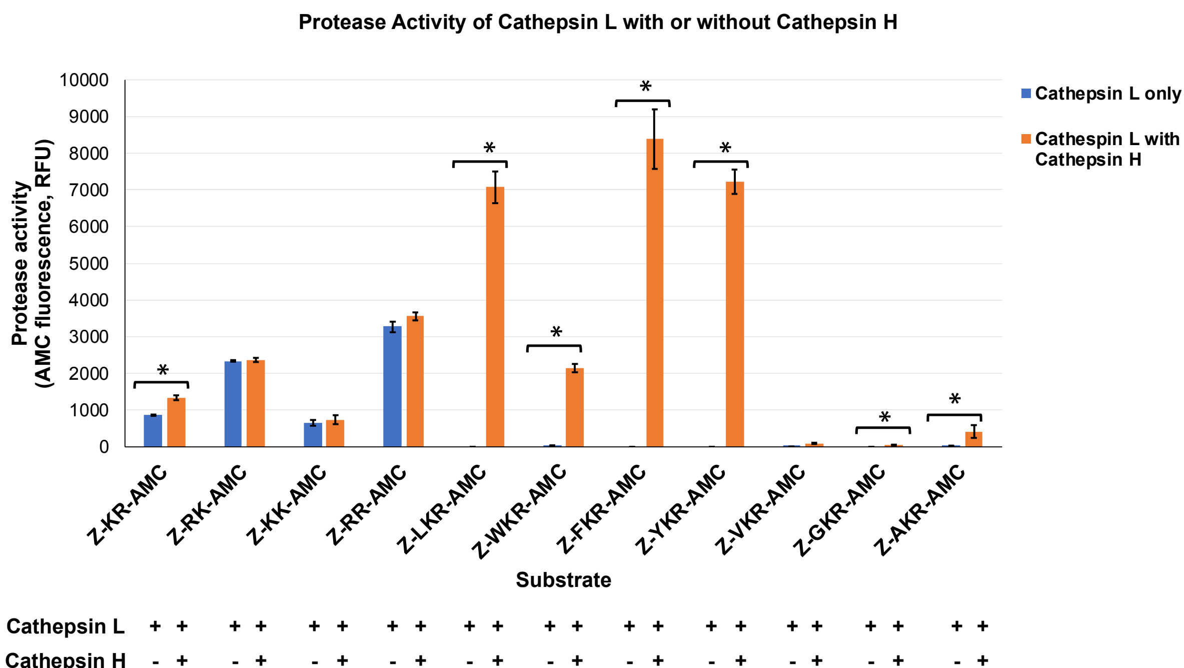


Figure 15 Protease Activity of Cathepsin L vs. Cathepsin L & Cathepsin H. Dibasic processing of various peptide-AMC substrates by either Cathepsin L or Cathepsin L with Cathepsin H. Significant difference between Cathepsin L activity and Cathepsin L+ Cathepsin H activity were found for substrates Z-KR-AMC, Z-LKR-AMC, Z-WKR-AMC, Z-FKR-AMC, Z-YKR-AMC, Z-VKR-AMC, Z-GKR-AMC with p-values 0.015, 0.0012, 0.0088, 0.0031, 0.00070, 0.0073, and 0.0038 respectively.

In figure 15, elevated levels of AMC fluorescence were detected for substrates Z-LKR-AMC, Z-FKR-AMC, and Z-YKR-AMC when incubated with both Cathepsin L and Cathepsin H. AMC fluorescence significantly increased with the addition of Cathepsin H with Cathepsin L in substrates Z-KR-AMC, Z-LKR-AMC, Z-WKR-AMC, Z-FKR-AMC, Z-YKR-AMC, Z-VKR-AMC, Z-GKR-AMC with p-values 0.015, 0.0012, 0.0088, 0.0031, 0.00070, 0.0073, and 0.0038 respectively.

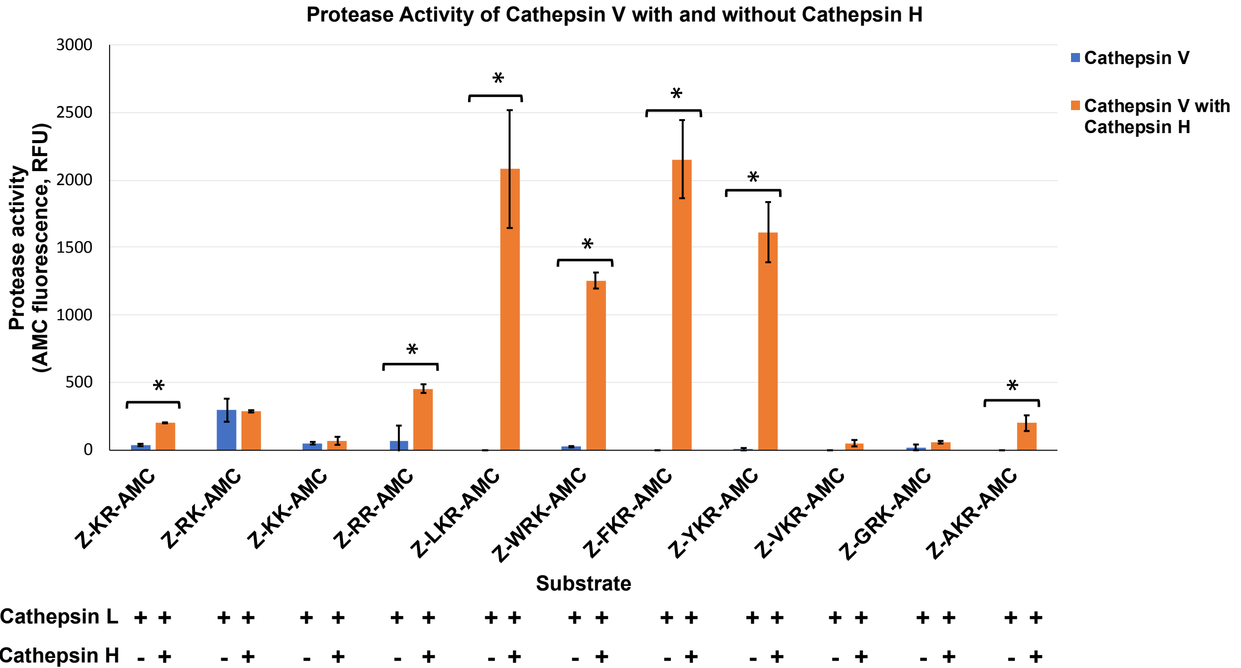


Figure 16 Protease Activity of Cathepsin V vs. Cathepsin V & Cathepsin H. Dibasic processing of various peptide-AMC substrates by either Cathepsin V or Cathepsin V in addition to Cathepsin H. Significant difference between Cathepsin V activity and Cathepsin V + Cathepsin H activity were found for substrates Z-KR-AMC, Z-RR-AMC, Z-LKR-AMC, Z-WKR-AMC, Z-FKR-AMC, Z-YKR-AMC, Z-AKR-AMC with p-values 0.00012, 0.022, 0.014, 0.0018, 0.0060, 0.0062, 0.027 respectively.

In reference to figure 16, elevated levels of AMC fluorescenes were detected for substrates Z-LKR-AMC, Z-WRK-AMC, Z-FKR-AMC, Z-YKR-AMC when incubated with both Cathepsin V and Cathepsin H. AMC fluorescence significantly increased when substrates Z-KR-AMC, Z-RR-AMC, Z-LKR-AMC, Z-WRK-AMC, Z-FKR-AMC, Z-AKR-AMC were incubated with Cathepsin V and Cathepsin H, with p-values 0.00012, 0.022, 0.014, 0.0018, 0.0060, 0.0062, 0.027 respectively.

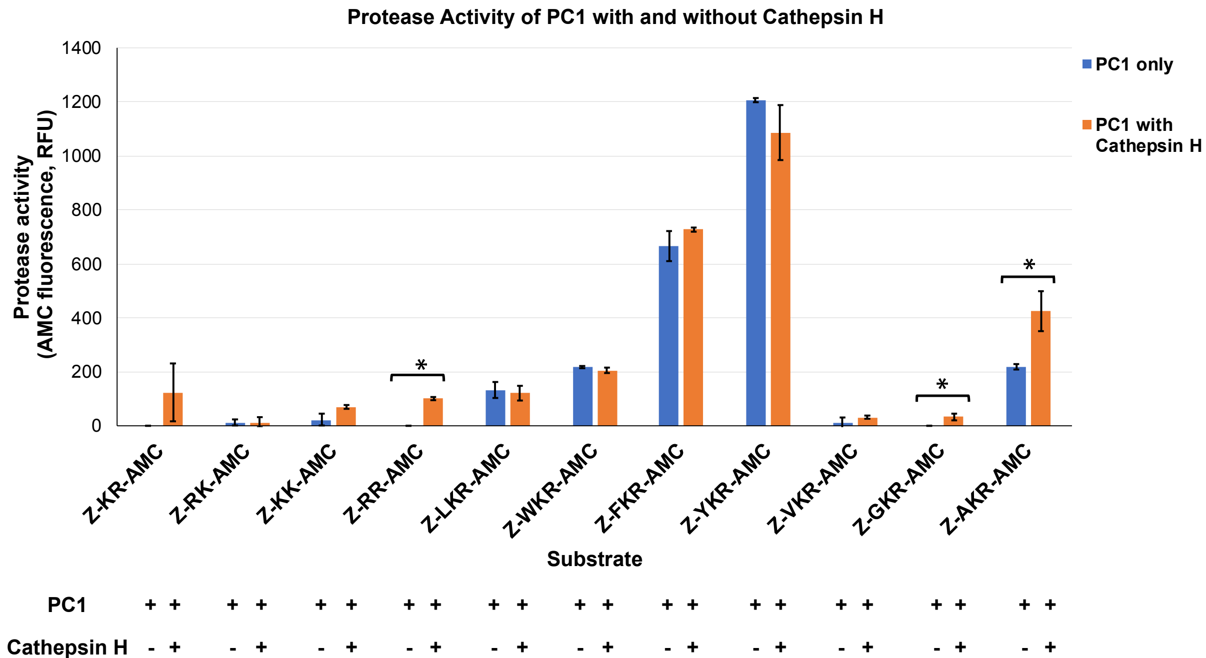


Figure 17 Protease Activity of PC1 vs. PC1 & Cathepsin H. Dibasic processing of various peptide-AMC substrates by either PC1 or PC1 in addition to Cathepsin H. Significant difference between PC1 activity and PC1 + Cathepsin H activity were found for substrates Z-RR-AMC, Z-GKR-AMC, and Z-AKR-AMC with p-values 0.0010, 0.040, and 0.038 respectively.

Figure 17 displays the differing AMC fluorescence levels detected between various substrates incubated with solely PC1 or PC1 in addition to Cathepsin H. Elevated levels of protease activity were detected for substrates Z-FKR-AMC Z-YKR-AMC, Z-AKR-AMC when they were incubated with both PC1 and Cathepsin H. There was a significant increase of AMC fluorescence when substrates Z-RR-AMC, Z-GKR-AMC, and Z-AKR-AMC were incubated with proteases PC1 and Cathepsin H in comparison to incubation with solely PC1. The p-values were 0.0010, 0.040, and 0.038 respectively.

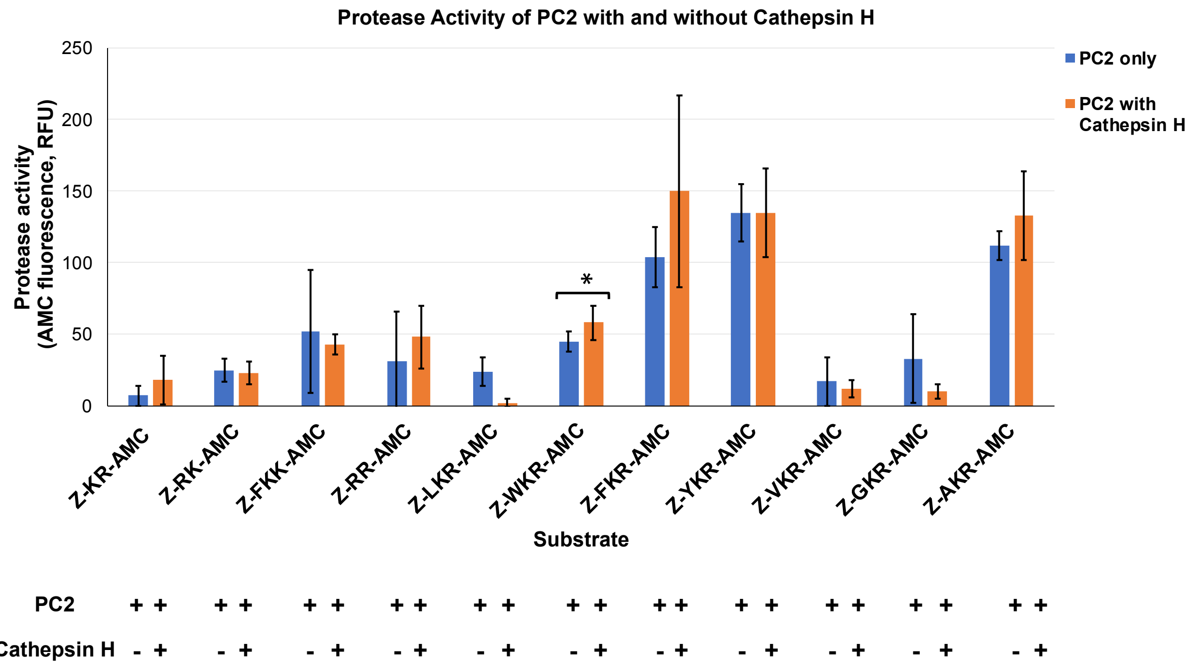


Figure 18 Protease Activity of PC2 vs. PC2 & Cathepsin H Dibasic processing of various peptide-AMC substrates by either PC2 or PC2 in addition to Cathepsin H. Significant difference between PC2 activity and PC2 + Cathepsin H activity was found for substrate Z-WRK-AMC with a p-value of 0.021

In figure 18, PC2 protease activity significantly increased for substrate Z-WKR-AMC when incubated with PC2 and Cathepsin H with a p-value of 0.021. Across the various substrates, protease activity was slightly higher with substrates Z-FKR, Z-YKR-AMC, and Z-AKR-AMC.

Table 6 Protease Activity of Cathepsin L and Cathepsin V with and without addition of Cathepsin H. The table shows mean protease activity in RFU of Cathepsin L and Cathepsin V with and without Cathepsin H (CH). The “Ratio +/- CH” is the mean RFU of the protease with Cathepsin H divided by the mean RFU of the protease without Cathepsin H.

	Cathepsin L (mean +/-SD)			Cathepsin V (mean +/- SD)		
Substrate	**- CH**	**+ CH**	**Ratio +/- CH**	**- CH**	**+ CH**	**Ratio +/- CH**
Z-K-R-AMC	862 ± 18	1335 ± 68	1.5	39 ± 9	202 ± 4	202.4
Z-R-K-AMC	2341 ± 22	2365 ± 55	1.0	296 ± 85	287 ± 8	1.0
Z-K-K-AMC	651 ± 81	735 ± 122	1.1	51 ± 9	70 ± 30	1.4
Z-R-R-AMC	3267 ± 144	3549 ± 109	1.1	67 ± 116	456 ± 32	6.8
Z-L-K-R-AMC	0 ± 0	7073 ± 430	**CH only**	0 ± 0	2081 ± 436	**CH only**
Z-W-K-R-AMC	29 ± 6	2144 ± 110	73.9	26 ± 4	1257 ± 59	48.3
Z-F-K-R-AMC	0 ± 0	8387 ± 814	**CH only**	0 ± 0	2155 ± 290	**CH only**
Z-Y-K-R-AMC	0 ± 0	7226 ± 331	**CH only**	6 ± 10	1614 ± 223	269.0
Z-V-K-R-AMC	3 ± 4	89 ± 16	29.7	0 ± 0	52 ± 23	**CH only**
Z-G-K-R-AMC	0 ± 0	46 ± 5	**CH only**	21 ± 21	59 ± 9	2.8
Z-A-K-R-AMC	16 ± 16	418 ± 174	26.1	0 ± 0	200 ± 58	**CH only**

Table 6 demonstrates the effects the addition of Cathepsin H to the assay containing either Cathepsin L or Cathepsin V with various peptide-AMC substrates. The incubation of Cathepsin H with Cathepsin L increased detection of protease activity by 73.9, 29.7, and 26.1-fold with substrates Z-WKR-AMC, Z-V-K-R-AMC, and Z-AKR-AMC respectively. Protease activity of Cathepsin L was only detectable when Cathepsin H was added to the assays containing Z-LKR-AMC, Z-FKR-AMC, Z-YKR-AMC, and Z-GKR-AMC. The addition of Cathepsin H to the assay measuring protease activity of Cathepsin V increased protease activity by 202.4, 48.3, and 269.0-fold when incubated with substrates Z-KR-AMC, Z-WKR-

AMC, and Z-YKR-AMC respectively. Protease activity of Cathepsin V was only detectable when Cathepsin H was added to the assays containing substrates Z-LKR-AMC, Z-FKR-AMC, Z-VKR-AMC, and Z-AKR-AMC. For Cathepsin L and Cathepsin V, 7 out of the 11 substrates showed greater fluorescence in the presence of Cathepsin H, indicating that 64% of the substrates were cleaved at dibasic residues between or at the N-terminal side.

Table 7 Protease Activity of PC1/3 and PC2 with and without addition of Cathepsin H. The table indicates mean protease activity in RFU of PC1/3 and PC2 with and without the presence of Cathepsin H (CH). The "Ratio +/- CH" is the mean RFU of the protease with Cathepsin H divided by the mean RFU of the protease without Cathepsin H.

	PC1/3 (mean +/- SD)			PC2 (mean +/- SD)		
Substrate	**- CH**	**+ CH**	**Ratio +/- CH**	**- CH**	**+ CH**	**Ratio +/- CH**
Z-K-R-AMC	0 ± 0	124 ± 107	**CH only**	7 ± 7	18 ± 17	2.6
Z-R-K-AMC	13 ± 11	13 ± 19	1.0	25 ± 8	23 ± 8	0.9
Z-K-K-AMC	23 ± 22	70 ± 7	3.0	52 ± 43	43 ± 7	0.8
Z-R-R-AMC	0 ± 0	101 ± 6	**CH only**	31 ± 35	48 ± 22	1.5
Z-L-K-R-AMC	133 ± 29	121 ± 27	0.9	24 ± 10	2 ± 3	0.1
Z-W-K-R-AMC	218 ± 4	206 ± 10	0.9	45 ± 7	58 ± 12	1.3
Z-F-K-R-AMC	666 ± 56	727 ± 8	1.1	104 ± 21	150 ± 67	1.4
Z-Y-K-R-AMC	1207 ± 8	1086 ± 102	0.9	135 ± 20	135 ± 31	1.0
Z-V-K-R-AMC	11 ± 20	32 ± 6	2.9	17 ± 17	12 ± 6	0.7
Z-G-K-R-AMC	0 ± 0	33 ± 12	**CH only**	33 ± 31	10 ± 5	0.3
Z-A-K-R-AMC	219 ± 10	425 ± 74	1.9	112 ± 10	133 ± 31	1.2

Table 7 demonstrates the effects of the addition of Cathepsin H to the assays containing either PC1/3 or PC2 with various peptide-AMC substrates. Protease activity of PC1/3 on substrates Z-KR-AMC, Z-RR-AMC, and Z-GKR-AMC when Cathepsin H was added to the assay. In regard to PC1/3, 2 of 11 substrates showed greater fluorescence in the presence of Cathepsin H representing 18% of the 11 substrates. For PC2, 1 out of 11 substrates showed

greater fluorescence with cathepsin H condition. Although the pvalue = 0.021, this is less significant than the other cathepsin H data since the other p-values were much lower.

Cathepsin L exhibited the highest levels of protease activity with the various peptide-AMC substrates, as evidenced by its elevated levels of AMC fluorescence in comparison to the other proteases Cathepsin V, PC1, and PC2. PC2 exhibited the lowest levels of protease activity with the tested substrates.

Chapter 4: Discussion of New Knowledge Gained About Cleavage Properties of Proteases Involved in Neuropeptide Production.

Overall, these results support the hypothesis that cysteine proteases, Cathepsin L and Cathepsin V, and serine proteases, PC1 and PC2, possess different cleavage specificities and prefer to cleave at different dibasic residues.

In regard to Cathepsin L, a significant increase in protease activity was found in seven out of eleven (64%) peptide-AMC substrates with the subsequent addition of Cathepsin H. Processing of substrates Z-KR-AMC, Z-LKR-AMC, Z-WKR-AMC, Z-FKR-AMC, Z-YKR-AMC, Z-VKR-AMC, Z-GKR-AMC had significantly greater detection of AMC fluorescence when incubated with Cathepsin L and Cathepsin H in comparison to incubation with solely Cathepsin L (Figure 15). Furthermore, with substrates Z-LKR-AMC, Z-FKR-AC, Z-YKR-AMC, and Z-GKR-AMC protease activity was only detectable when Cathepsin H was added to the assay (Table 6). As evidenced by the little to no activity seen with the incubation of the substrates with solely Cathepsin L, it can be inferred that Cathepsin L cleaved the substrates either in-between or at the N-terminal side of the dibasic residue, KR, leaving an arginine or lysine-arginine bound AMC substrate that is not able to fluoresce. With the addition of Cathepsin H, AMC fluorescence increases by several folds for several substrates (Table 6). In fact, AMC fluorescence detection was dependent on the addition of Cathepsin H with substrates Z-LKR-AMC, Z-FKR-AMC, Z-YKR-AMC and Z-GKR-AMC. The addition of Cathepsin H increases detection of protease activity because of Cathepsin H's ability to cleave the R-AMC bond, thus releasing quantifiable free AMC products. These finding are consistent with the findings of the Hook lab (Table 4) and supports the hypothesis that the cysteine protease, Cathepsin L, prefers to cleave between and at the NH2-terminal side of dibasic residues within a peptide.

Furthermore, Cathepsin L was found to have elevated protease activity for substrates Z-LKR-AMC, Z-FKR-AMC, and Z-YKR-AMC compared to the other eight peptides within the

assay. This is consistent with the Hook lab by MSP-MS which found that Cathepsin L preferred to cleave at sites where the P2 position contained hydrophobic residues such as Leu, Val, Phe, Trp, and Tyr (Table 4). While the experiment did not demonstrate elevated protease activity for a peptide substrate that contained Val or Trp, substrates that contained Leu, Phe, and Tyr produced the most AMC fluorescence. Compared to the other proteases that processed these same substrates, Cathepsin L had the highest overall specific protease activity on the substrates (Table 5).These findings indicate that Cathepsin L was the most efficient at cleaving these particular substrates at the dibasic residue KR, in comparison to the other proteases tested. With this information it can be inferred that Cathepsin L may have a broader specificity for its cleavage preferences, and due to this it can cleave substrates more efficiently.

Moving onto Cathepsin V, significant increases of protease activity were found when substrates Z-KR-AMC, Z-RR-AMC, Z-LKR-AMC, Z-WRK-AMC, Z-FKR-AMC, Z-AKR-AMC were incubated with Cathepsin V and Cathepsin H in comparison to substrates being incubated solely with Cathepsin V (figure 16). Additionally, protease activity was only detectable with the addition of Cathepsin H for substrates Z-LKR-AMC, Z-FKR-AMC, Z-VKR-AMC, and Z-AKR-AMC (Table 6). Similar to the findings for Cathepsin L, protease activity was detected significantly more with the addition of Cathepsin H to the assay. These findings indicate that like Cathepsin L, Cathepsin V shows preference for cleaving in between or towards the N-terminal side of dibasic residues such as KR or RR, thus supporting the hypothesis. The significant increase in protease activity with substrate Z-RR-AMC is a finding that distinguishes Cathepsin V from Cathepsin L. With this is mind, for future experiments it may be useful to test Cathepsin V's protease activity with varying substrates that contain the dibasic residue RR.

Amongst all of the substrates, elevated levels of AMC fluorescence were detected with substrates Z-LKR-AMC, Z-WRK-AMC, Z-FKR-AMC, Z-YKR-AMC when incubated with both Cathepsin V and Cathepsin H. These findings are consistent with the MSP-MS studies by the Hook lab finding that Cathepsin V preferred to cleave substrates that had hydrophobic residues

Leu, Val, Phe, Trp, or Tyr at the P2 position. While the assay did not demonstrate elevated activity for the substrate that contained Val, elevated levels of protease activity was demonstrates with substrates that contained Leu, Trp, Phe, and Tyr. However, protease activity was not as elevated with these substrates in comparison to the other cysteine protease, Cathepsin L.

Nonetheless, Cathepsin L and Cathepsin V do share similar patterns for their cleavage preferences. Cathepsin L and Cathepsin V show greater preference for cleaving dibasic residues at the N-terminal side or between the dibasic residues. These proteases also show some cleavage preferences at the C-terminal side but this is less prevalent than the other cleavage sites at the N-terminal side and between the dibasics.

Focusing next on the serine protease, PC1, there was a significant increase in protease activity with substrates Z-RR-AMC, Z-GKR-AMC, Z-AKR-AMC when Cathepsin H was subsequently added to the assay (Figure 17). It was also found that, protease activity detection was dependent on the subsequent addition of Cathepsin H with substrates Z-KR-AMC, Z-RR-AMC, and Z-GKR-AMC (Table 1). The eight other substrate assays did not show evidence for significant differences between the addition of solely PC1 or the subsequent addition of Cathepsin H with PC1. The significant increases found with the previously mentioned substrates lead to new findings that the serine protease cleaves at the C-terminal side of dibasic residues as well a between dibasic residues. With the expectation that PC1 protease activity would be suffice enough to produce free AMC, the increase in protease activity by subsequent addition of Cathepsin H does not support our hypothesis. Yet, overall, the findings do support the hypothesis somewhat because the majority of the substrates that were cleaved had no significant differences in protease activity when it came to the addition or withholding of Cathepsin H from the assay. The most elevated levels of PC1 protease activity in this assay were found when PC1 was incubated with the substrates Z-FKR-AMC and Z-YKR-AMC, demonstrating its preference for these two substrates over the others (Table 6). Additionally,

within the literature, cleavage after a GFK bond is expected by this protease, yet it was seen that there was no activity found with solely PC1 and Z-GKR-AMC (Benjannet et al., 1991). Even with addition of Cathepsin H, very little protease activity is detected.

In regard to PC2, only one substrate Z-WKR-AMC, showed a significant increase with the addition of Cathepsin H. While the significance of this finding is given a p-value of 0.021, it should be noted that this p-value is much higher in comparison to the other cathepsin H data with the other proteases, Being that only one of the eleven substrates showed a significant increase with a subsequent addition of Cathepsin H, this finding supports the hypothesis that PC2 prefers to cleave at the C-terminal side of dibasic residues. Additionally, unlike the other proteases, there was no evidence that showed that detection of protease activity was dependent on the subsequent addition of Cathepsin H (Table 6). These findings were expected due to the fact that AMC is attached to the carboxyl side of the dibasic residue, therefore protease activity by PC2 should produce detectable free AMC because it is proposed to prefer to cleave at the C-terminal side. Furthermore, the addition of Cathepsin H is not expected to have a significant effect on protease activity since PC2 is expected to cleave the same bond. This explains why there were very few significant differences found amongst the processing of the different substrates. Out of all the substrates, PC2 showed the most protease activity for substrates Z-FKR-AMC, Z-YKR-AMC, and Z-AKR-AMC with and without the subsequent addition of Cathepsin H (Table 6). However, in comparison to PC1, PC2 was less efficient at processing the substrates Z-FKR-AMC, Z-YKR-AMC, and Z-AKR-AMC since its detectable protease activity levels were generally lower.

It is important to note that the performance of the serine proteases PC1 and PC2 was very low compared to the abundant protease activity of cysteine proteases, Cathepsin L and Cathepsin V. Table 4 demonstrates the stark contrast in specific activity between the serine and cysteine proteases. Cathepsin L demonstrated the highest specific activity, 8.5 pmol AMC/ng/min, with the substrate Z-Phe-Arg-AMC, which is approximately 10 times more than the

specific activity of Cathepsin V, 64 times more than the specific activity of PC1/3, and almost 1,300 times more than the specific activity of PC2. Despite the relatively high concentrations of proteases used for the PC1 and PC2, 10 ng/ul and 20 ng/ul respectively, protease activity was not prevalent amongst the processing of the tested peptide-AMC substrates. To provide reference, abundant protease activity was found with Cathepsin L and Cathepsin V with the concentrations 0.4 ng/ul and 2 ng/ul respectively. These results reveal that the cysteine proteases Cathepsin L and Cathepsin H have higher activity with these substrates, suggesting that they share a more prominent role in proneuropeptide processing in comparison to the serine proteases, PC1/3 and PC2. These findings in regard to the serine proteases were unexpected based on the results of the Hook lab as well as many other studies found in the literature. In their study using unbiased MSP-MS assays, they found that the serine proteases preferred to cleave at the C-terminal side of KR and RR and in-between KR and R, however our substrates were relatively unprocessed in comparison to the cysteine proteases. It can be said that the serine proteases are able to cleave the dibasic residue substrates within our assay, however they were not as efficient at cleaving these types of substrates. For future experiments, it would be insightful to test if the proteases showed more protease activity with RR substrates.

While there is an abundance of literature that has elucidated the expected cleavage preferences for serine proteases PC1 and PC2, it is important to note that our observed results demonstrated inconsistencies with past findings. While it is useful to know the preferences these proteases may have, it is also important to disclose how efficient these proteases may be at processing various substrates, Therefore, more modernized assays should be conducted in order to verify past findings. However, on a better note, despite there not being an abundance of information found on the cleavage preferences of cysteine protease Cathepsin V and Cathepsin L on proneuropeptides, the findings from the peptide-AMC substrate assays support and elucidate novel information to this field.

Overall, this research has provided novel findings as well as revealed inconsistencies with past literature. The evidence provided by these experiments support the hypothesis that cysteine proteases Cathepsin L and Cathepsin V, and serine proteases PC1 and PC2 possess different cleavage specificities for processing dibasic residues. While Cathepsin L and Cathepsin V prefer to cleave between and at the NH_2-terminal side of dibasic residues, PC1 and PC2 prefer to cleave at the C-terminal side of dibasic residues.

Future Work

The biosynthesis of neuropeptides is a complex process and consists of several proteases that are all a part of an intricate synergistic system. The AMC-peptide substrate assay demonstrated within this thesis is one of many assays that have been conducted to understand proteolytic properties of individual proteases, however, it is also necessary to gain a broader understanding of how protease systems work to create a neuropeptidome, a spectrum of peptides generated from proteolytic processing (Jiang et al., 2021). Proteomics, peptidomics, and peptide cleavage profiling by mass spectrometry are all modes of research being used to try to understand neuropeptidome production.

The study by Jiang et al., 2021, demonstrated the utility of these modes of research in understanding neuropeptidome production. Their studies focus was to compare the neuropeptidomes formed in chromaffin granules at the extracellular pH 7.2 and intracellular pH 5.5. The cleavage profiles of the chromaffin granules were assessed by utilizing MSP-MS with products from proteomic, neuropeptidomics, and peptide substrate cleavage specificity assays (figure 19). Proteomics was demonstrated to be a useful technique to identify precursor neuropeptides as well as proteases. In terms of neuropeptidomics, this technique was used to define the spectrum of peptides biosynthesized from protease activity of the chromaffin granules at the different pH levels (5.5 and 7.2). Lastly, during peptide substrate cleavage specificity assays, class-specific protease inhibitors were added in order to isolate and assign roles of proteases during biosynthesis of the peptides.

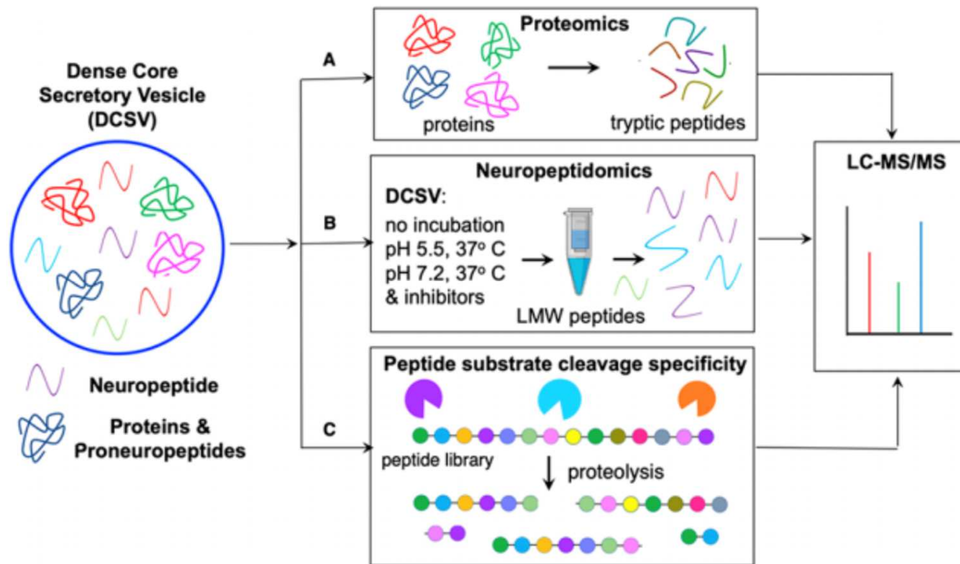


Figure 19 Proteomics, Neuropeptidomics, and Peptide substrate cleavage specificity on chromaffin granules. Purified chromaffin granules were subjected to (a) proteomics, (b) neuropeptidomics, and (c) peptide substrate cleavage specificity and later LC-MS/MS in order to gain an understanding of neuropeptidome production at the intracellular pH 5.5 versus the extracellular pH 7.2 (Jiang et al., 2021).

Figure 20 demonstrates the breadth of data that can be collected from utilizing the techniques that were previously mentioned. Utilizing proteomics, the Jiang et al., 2021, study was able to isolate the unique proteins found within the chromaffin granules, further isolating the proteases which made up about 2.53% of the total protein abundance. As it can be seen in figure 20, the proteases can be further categorized by their class (figure 20a-b) and compared by their abundance within the chromaffin granules. Furthermore, the identification of proneuropeptides present and their abundance were also achieved by using this proteomics technique (figure 20c). Moving onto peptidomics, this technique allowed the specific identification of neuropeptides formed after proteolytic processing within the chromaffin granules (figure 20d). By separating the peptides by molecular weight, they were analyzed by LC-MS/MS, allowing the identification and quantification of 1239 unique peptides derived from proneuropeptides. Figure 20d demonstrates that the majority of these peptides came from chromogranins proenkephalin (PENK), adrenomedullin (ADM), and secretogranin precursors.

Lastly peptide substrate cleavage specificity assays were used to uncover the cleavage specificity profiles of the peptides that were processed. Figure 20e demonstrates the alignment of peptide fragments to the precursor and the frequency of an amino acid that occurs at the N- and C-terminal of the peptide, indicating the specific amino acid and position the fragment was cleaved at. The breadth of data that can be curated by these techniques demonstrates great potential for future experiments. For example, these techniques can be used for the development of clinical inhibitors for particular proteases, comparing peptidomes of disease-models, and much more.

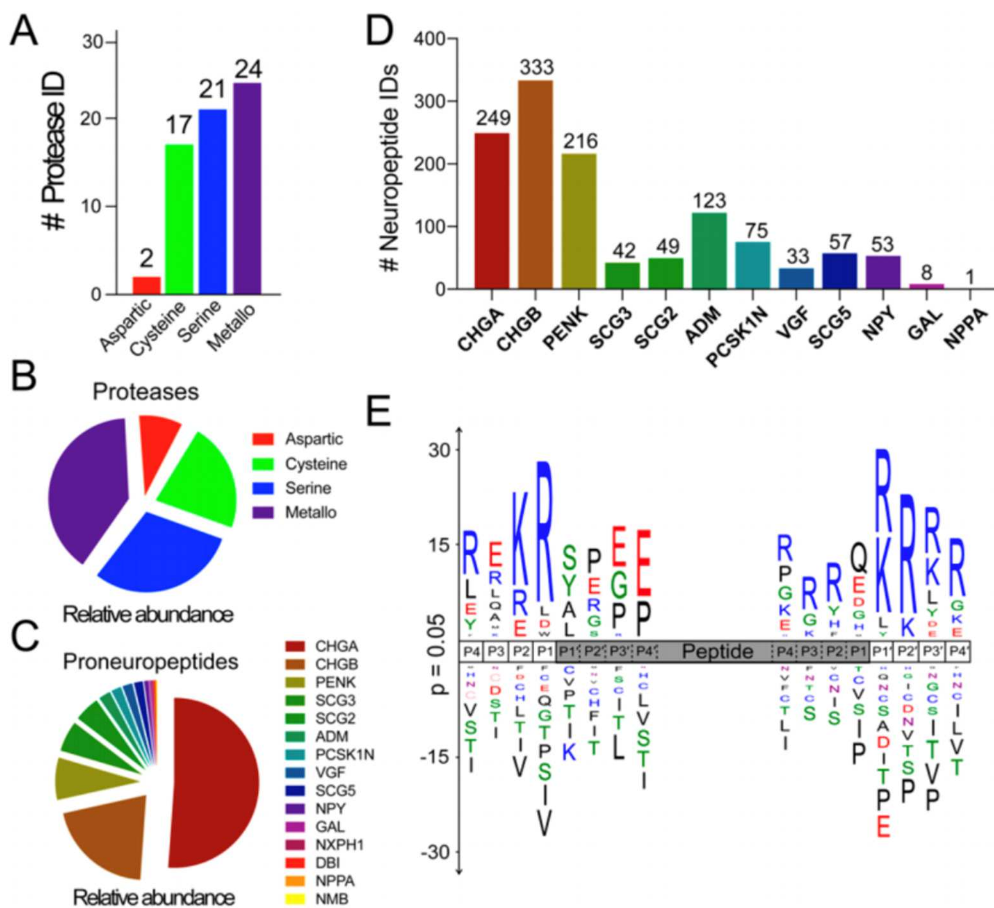


Figure 20 Breadth of Data from Proteomics, Peptidomics, and Peptide substrate cleavage specificity (a,b,c) Proteomics can be used to identify and quantify unique proteases and proneuropeptides with chromaffin granules (d) Peptidomics reveals the identification and quantification of unique peptide fragments derived from several proneuropeptides (d) peptide substrate cleavage specificity assays were used to determine amino acid preference and position of cleavage sites.

The use of class-specific protease inhibitors is a further step that can be used to dissect the complex protease system that is used in the biosynthesis of neuropeptides. In Jiang et al.'s study, the protease inhibitors pepstatin, E64c, AEBSF, and EDTA were used to block the activity aspartic, cysteine, serine, and metallo proteases respectfully. By comparing control groups with protease-inhibitor treated samples, it could be determined what proteases were involved in the biosynthesis of certain peptides. Again, using peptidomics, the unique peptides created within the chromaffin granule can be identified and quantified. Figure 21 is a heat map that demonstrates the level abundance of each peptide formed depending on which inhibitor was added to the sample. As seen in the heat map, one can determine the sensitivity of neuropeptide production to certain inhibitors, therefore revealing which classes of proteases are more significant for the formation of certain peptide fragments. The ability to obtain this data from the utilization of several proteases and a multitude of peptide fragments is revolutionary and will benefit the efficiency of future experiments within this field.

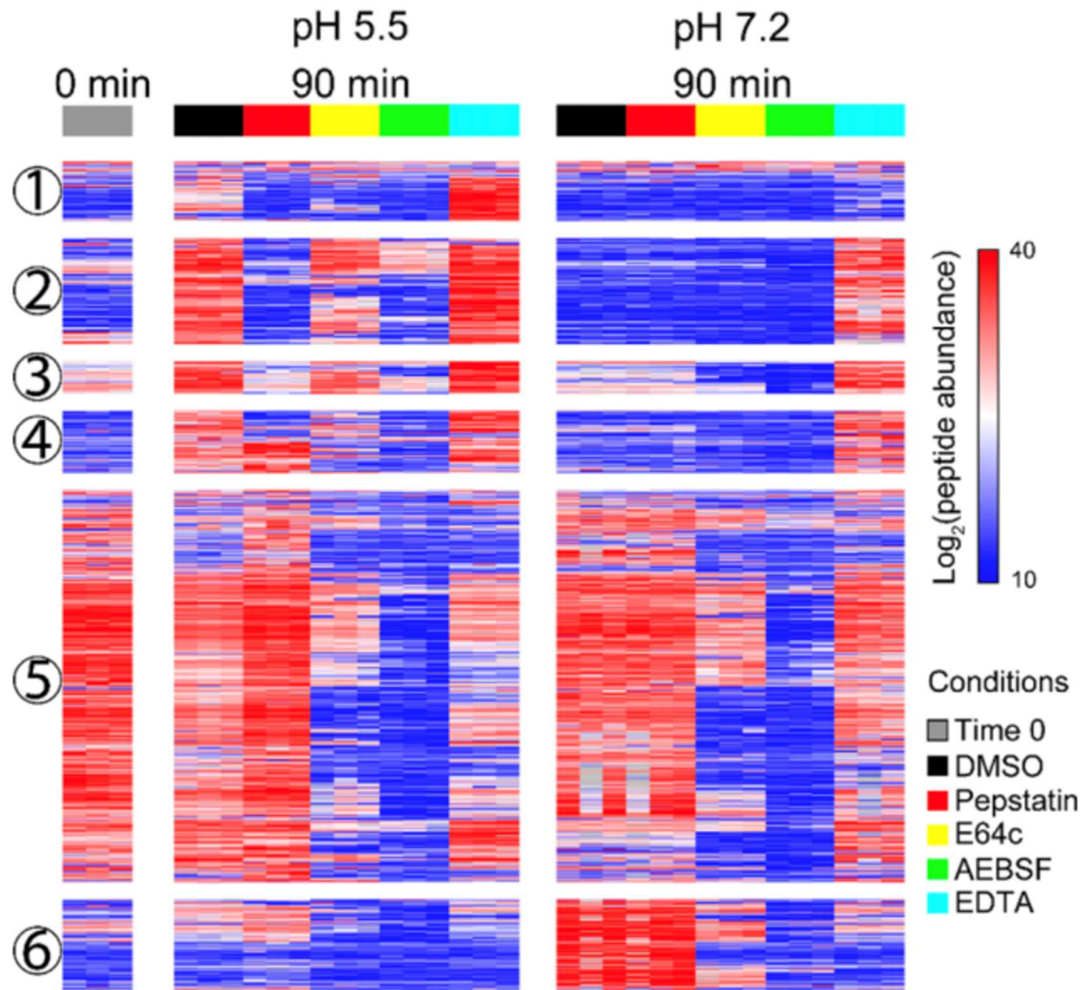


Figure 21 Heatmap of Peptide abundance in the presence of various protease inhibitors This heatmap indicates the abundance of neuropeptides formed after a 90 min incubation of the precursor peptides at either pH 5.5 or pH 7.2 with the aspartic, serine, cysteine, and metallo proteases with various protease inhibitors pepstatin, E64c, AEBSF, and EDTA. Each row indicates a single neuropeptide. Red indicates a higher abundance, while blue indicates a lower abundance (Jiang et al. 2021).

Lastly, protease inhibition profiles can be generated in order to analyze the fragments generated from precursor peptides such as chromogranin a (CHGA) and proenkephalin (PENK) (figure 22). By noting the regions of the peptide with the highest intensity and accounting for the peptide-inhibitor that was used and comparing to the control (DMSO), it can further determine which proteases are most involved in the processing of a certain neuropeptide as well as the general region this protease prefers to cleave.

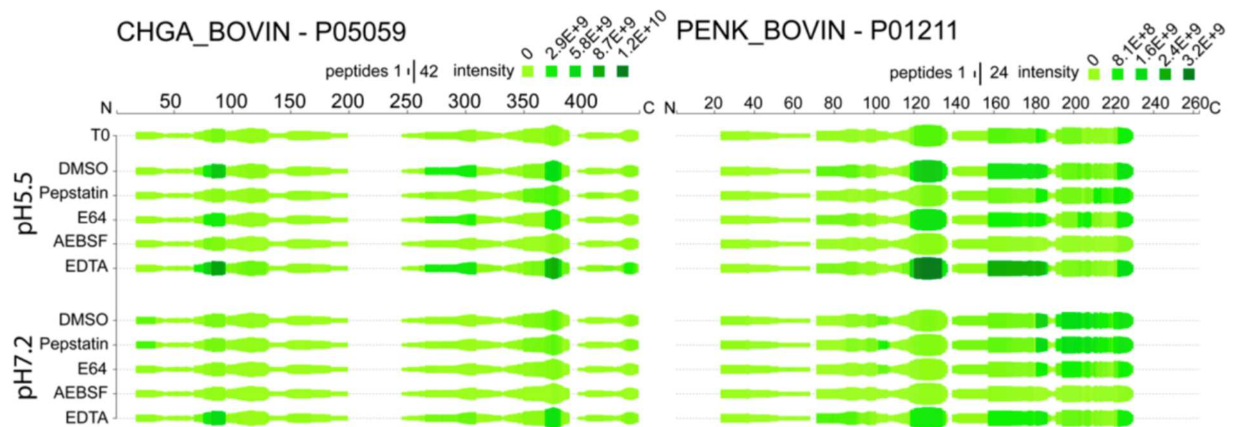


Figure 22 Neuropeptidomes in the presence of class-specific protease inhibitors CHGA and PENK were subjected to protease activity by serine, cysteine, metallo, and aspartic proteases in addition to class-specific protease inhibitors at pH 5.5 and pH 7.2. The height of the green bar is proportional to the number of amino acids overlapping the indicated region and the darkness of the color is proportional to the quantified peptide intensity (Jiang et al. 2021).

Overall it is confounding what can be elucidated using these peptidomics and proteomic techniques. These tools can improve efficiency as well as produce a vast breadth of data that can provide valuable information on the properties of protease systems and the neuropeptidomes they create. By studying the biosynthesis of peptides and the proteases that create them, a further understanding of the physiological functions associated with them can be obtained.

Chapter 5: Conclusions of Cysteine and Serine Protease Cleavage Specificities and Roles in Processing Neuropeptides

Ultimately, the field of studying neuropeptides is novel and uncharted, therefore it is significant to explore their diversity, their functions, and the proteases that biosynthesize them. Neuropeptides have demonstrated to be key regulators of cell-cell communication in the nervous system, as well as key players in modulating neural-endocrine physiological functions. With the new findings provided by these experiments, there is a further understanding achieved about how Cathepsin L, Cathepsin V, PC1, and PC2 process substrates similar to the residues found in endogenous proneuropeptides. While Cathepsin L and Cathepsin V prefer to cleave between and at the NH_2-terminal side of dibasic residues, PC1/3 and PC2 prefer to cleave at the C-terminal side of dibasic residues. In addition, Cathepsin L and Cathepsin V showed characteristics of efficient processing of peptide substrates at dibasic residues as demonstrated by their elevated specific activity, in contrast to PC1 and PC2, which exhibited significantly lower specific activity.

Elucidation of protease properties and how they influence cleavage of neuropeptide precursors will contribute to possible therapeutic application. Specifically, protease inhibitors may be developed in order to target diseases and impairments related to the neuropeptides that are biosynthesized from them. Currently, very few selective enzymes exist to this day. In particular, studying the protease production of opioid neuropeptides may benefit future studies that aim to enhance treatment of chronic pain, neurological and mental disorders, and other diseases or impairments associated with these peptides.

In conclusion, the central goal of this research was to define the cleavage properties of candidate pro-neuropeptide processing enzymes. Fortunately, a better insight has been gained on how these endogenous neuropeptides are generated. With the vast diversity of roles

neuropeptides possess, the possible impacts future research in this field will have on human health is endless.

Manuscripts

Yoon MC, Ames J, Mosier C, O'Donoghue AJ, Hook V. Differential Dibasic Cleavage Properties of Neuropeptide-Producing Cysteine and Serine Proteases Revealed by Peptide Cleavage Profiling Mass Spectrometry. manuscript to be submitted by Aug., 2021.

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