Human Cathepsin V Protease Participates in Production of Enkephalin and NPY Neuropeptide Neurotransmitters

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Background: Proteases are required to generate peptide neurotransmitters.

Results: Human cathepsin V participates in the production of enkephalin and NPY peptide neurotransmitters illustrated by gene expression and silencing.

Conclusion: Human cathepsin V participates in producing enkephalin and NPY neurotransmitters in secretory vesicles.

Significance: Elucidation of human-specific proteases participating in peptide neurotransmitter production is essential for understanding human health and disease.

Proteases are required for processing precursors into active neuropeptides that function as neurotransmitters for cell-cell communication. This study demonstrates the novel function of human cathepsin V protease for producing the neuropeptides enkephalin and neuropeptide Y (NPY). Cathepsin V is a human-specific cysteine protease gene. Findings here show that expression of cathepsin V in neuroendocrine PC12 cells and human neuronal SK-N-MC cells results in production of (Met)enkephalin from proenkephalin. Gene silencing of cathepsin V by siRNA in human SK-N-MC cells results in reduction of (Met)enkephalin by more than 80%, illustrating the prominent role of cathepsin V for neurotransmitter production. In vitro processing of proenkephalin by cathepsin V occurs at dibasic residue sites to generate enkephalin-containing peptides and an ∼24-kDa intermediate present in human brain. Cathepsin V is present in human brain cortex and hippocampus where enkephalin and NPY are produced and is present in purified human neuropeptide secretory vesicles. Colocalization of cathepsin V with enkephalin and NPY in secretory vesicles of human neuroblastoma cells was illustrated by confocal microscopy. Furthermore, expression of cathepsin V with proNPY results in NPY production. These findings indicate the unique function of human cathepsin V for producing enkephalin and NPY neuropeptides required for neurotransmission in health and neurological diseases.

Peptide neurotransmitters, known as “neuropeptides,” are essential for cell-cell communication in the nervous system for regulation of neurological functions in health and disease. The biosynthesis of neuropeptides requires proteolytic processing of proprotein precursors to generate active peptides that are secreted to mediate intercellular communication. Recent studies illustrate the role of the cysteine protease cathepsin L in secretory vesicles for production of enkephalin, neuropeptide Y (NPY), and related neuropeptides based largely on results from cathepsin L gene knock-out mice (1–7). Cathepsin L functions with the subtilisin-like prohormone convertases in the conversion of precursors into active neuropeptides (5).

The human genome includes two highly homologous cathepsin V (CTSV, also known as CTSL2) and cathepsin L (CTSL, also known as CTSL1) cysteine proteases that are clan CA/family C1 cysteine proteases consisting of 11 human gene members (8–11). However, the mouse genome possesses only the cathepsin L gene (mouse Ctsl); no orthologues of human cathepsin V in mouse and other mammalian species exist (8–11). The mouse cathepsin L possesses greater homology with human cathepsin V than with human cathepsin L (8–12), predicting that human cathepsin V may participate in proprotein processing to generate active neuropeptides such as enkephalin and NPY.

We, therefore, investigated the role of human cathepsin V in the proteolytic conversion of proenkephalin to generate the enkephalin neuropeptide that regulates analgesia, stress, and brain behaviors (13, 14). Cellular coexpression of cathepsin V with the human proenkephalin (PE) precursor results in production of (Met)enkephalin. Importantly, transfection of cathepsin V siRNA into human SK-N-MC neuroblastoma cells resulted in substantial reduction of (Met)enkephalin levels by more than 80%, indicating the role of endogenous cathepsin V for enkephalin production. In vitro processing of recombinant

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The abbreviations used are: NPY, neuropeptide Y; PE, proenkephalin; AMC, 7-amino-4-methyl-coumarin; MCA, methylcoumarinamide; IP, immunoprecipitation; ME, (Met)enkephalin; LE, (Leu)enkephalin; PC, prohormone convertases; Z-, benzoyloxycarbonyl.
human PE by cathepsin V generates enkephalin peptides and a ∼24-kDa PE-derived intermediate that is present in human brain hippocampus and cortex where enkephalin peptides are produced. Cathepsin V possesses cleavage specificity for the N-terminal side of dibasic processing sites (Lys-Arg, as well as between dibasic residues, which differs from the prohormone convertases (PC1/3 and PC2) that preferentially cleave at the C-terminal side of paired basic residues within prohormones (5, 15, 16). Cathepsin V resides with enkephalin and NPY in secretory vesicles where neuropeptides are produced. Furthermore, cathepsin V expression with proNPY results in production of cellular NPY, a brain peptide responsible for feeding behavior and obesity (17, 18), and in the peripheral sympathetic nervous system NPY participates in stress and blood pressure regulation (18, 19). These significant findings demonstrate participation of human cathepsin V in the production of enkephalin and NPY neuropeptides that are essential for peptide neurotransmission in health and disease.

**EXPERIMENTAL PROCEDURES**

**Cathepsin V (CTSV), Proenkephalin, and ProNPY cDNA Plasmids for Cell Expression**—The human cathepsin V cDNA (encoding preprocathepsin V) in pCMV6-XL5 plasmid expression vector was obtained from OriGene (Rockville, MD). The human PE cDNA (encoding preproPE) was obtained by RT-PCR of total RNA from human striatum (Stratagene/Agilent Technologies, Santa Clara, CA) using RT-PCR methods as we have described previously (20). RT-PCR was performed with Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. PCR with the first strand cDNA as template utilized TaqDNA polymerase (Qiagen, Valencia, CA) and the primers 5’-AAAAACATATGGAATGCAGCCAGG-ATTGCCGCAAGC-3’ (the Ndel site is underlined) and 5’-AAA-AGGATCCTTAAAATCTCATAAATCCTCCGTATCTT- TTTTCC-3’ (the BamH1 site is underlined). The PE cDNA was ligated into the pcDNA3.1 vector at Ndel and BamH1 sites and amplified in XL-1 Blue Competent Cells (Strategene). Plasmid DNA purification (using the Plasmid Maxi Kit from Qiagen) was followed by DNA sequencing to confirm the sequence (Davis Sequencing, Inc., Davis, CA). ProNPY cDNA (encoding rat preproNPY) in the pcDNA3.1 plasmid expression vector was prepared as previously described (2, 3).

**Cathepsin V Production of Enkephalin and NPY Neuropeptides in PC12 Cells**—Cathepsin V production of (Met)enkephalin was assessed by coexpression of PE and cathepsin V cDNAs in PC12 cells (rat), cultured as we have described (2). PC12 cells were plated at 1.2 × 10^6 cells/well (70% confluency) in 6-well plates, and PE cDNA in pcDNA3.1 vector was transfected with the Geneporter 2 reagent (Genlantis, San Diego, CA); 3 days later cells were transfected with cathepsin V cDNA in pCMV6-XL5. Cells were harvested 2 days later for analyses of (Met)enkephalin cell content by radioimmunoassay and for Western blots of PE and cathepsin V, conducted as we have described (2).

For analyses of NPY production by cathepsin V, the proNPY cDNA in pcDNA3.1 vector was cotransfected with cathepsin V cDNA in pCMV6-XL5, and 3 days later cells were harvested for analyses of NPY content (by radioimmunoassay (RIA), as we have described (3).

**Human Neuroblastoma SK-N-MC Cells and (Met)enkephalin Production**—Cathepsin V was expressed in these cells to assess its role in producing endogenous (Met)enkephalin. SK-N-MC cells (American Type Culture Collection, Manassas, VA) were cultured in 5% CO2 with MEM medium (Invitrogen) containing 10% FBS (Invitrogen). Transfection of the cathepsin V cDNA in pCMV6-XL5 vector was conducted with Lipofectamine 2000 (Invitrogen). Two days later cells were harvested for RIA of (Met)enkephalin and Western blot of cathepsin V.

**Cathepsin V siRNA Treatment of Human Neuroblastoma Cells Reduces Enkephalin Production**—Human neuroblastoma SK-N-MC cells (1 × 10^6 cell/well) were transfected with siRNA to cathepsin V, consisting of AUUCGAAUUUGCUCUUCAAAGCGG and the complimentary sequence CGGCUUUGAGGAGGCACAUUGCAA (Invitrogen). Scrambled siRNA (of the same base composition) was utilized as control. Cells were transfected with siRNA to cathepsin V or transfected with scrambled siRNA using 25 or 50 nM siRNA in media with Lipofectamine RNAiMAX transfection reagent (Invitrogen). Transfection with no siRNA was included. Reduced expression of cathepsin V was confirmed by anti-cathepsin V Western blot. Cellular siRNA treatments were conducted in replicate experiments (n = 4). One day after transfection cells were harvested, and cell levels of (Met)enkephalin were measured by radioimmunoassay as we have described previously (2).

**In Vitro Processing of Recombinant PE by Cathepsin V**—Recombinant human PE with N-His tag was expressed in *Escherichia coli* and purified by nickel affinity chromatography as we have described (21). PE (4.8 µg/assay) and recombinant human cathepsin V (3 ng/assay, from R & D Systems, Inc., Minneapolis, MN) were incubated in 25 mM Tris-HCl, pH 7.1, and 1 mM DTT at 37 °C for 30 min. Formic acid was then added to 5%, and samples were heated to 80 °C for 2 min for Western blots with anti-(Met)enkephalin and anti-(Leu)enkephalin (Chemicon/Millipore, Temecula, CA) conducted as we have described (2).

For analysis of peptide products by nano-LC-MS/MS tandem mass spectrometry, samples in 1% formic acid were analyzed by the nano-LC-MS/MS XCT Ultra ion trap mass spectrometer instrument (Agilent Technologies, Inc., Wilmington, DE) as we have described previously (22). Peptides were identified using Spectrum Mill or InsPecT software with the search of both the human RefSeq data base (NCBI) and a data base of the human PE sequence.

**Cathepsin V Activity Assays with Fluorogenic Peptide Substrates**—Proteolytic activity of cathepsin V was assessed with peptide-MCA substrates consisting of Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, and Z-butoxy carbonyl-Gly-Arg-MCA (Bachem Americas, Inc. Torrance, CA). When peptide-MCA substrates consisting of Z-Phe-Arg-MCA, Z-Arg-Arg-MCA were incubated at 100 µm with cathepsin V (4 ng, R&D Systems, Minneapolis, MN) at 37 °C for 30 min in a total volume of 100 µl in buffer containing 25 mM sodium acetate, pH 5.5, 0.1 M NaCl, and 5 mM DTT. Formation of AMC was detected with a PerkinElmer Life Sciences fluorimeter with excitation and emission wavelengths of 365 and 456 nm.
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450 nm, respectively. Standard AMC concentrations were measured for calculating activity as nmol of AMC released/h/mg of cathepsin V. All proteolytic assays were performed in quadruplicate with each substrate; replicate values varied by less than 10%.

Western Blot Analyses of Cathepsin V in Human Brain Regions and Human Neuroblastoma Cells—The presence of cathepsin V in isolated secretory vesicles from human adrenal pheochromocytoma was examined by Western blot with anti-cathepsin V. The human pheochromocytoma tissue (from surgical specimen, with pathology report of benign tumor) was obtained according to an approved protocol approved by the University of California–San Diego human research protections program. The fresh pheochromocytoma tissue was used for purification of secretory vesicles (also known as chromaffin granules, CG) by differential sucrose density gradient centrifugation, as we have previously described (22). The high purity of these secretory vesicles, achieved by sucrose density gradient centrifugation, has been documented by enzyme markers showing the lack of other organelle markers in the purified secretory vesicle preparation and by electron microscopy showing the purity, homogeneity, and integrity of the purified secretory vesicles (23–26).

Human tissues from brain cortex and hippocampus were obtained from the Harvard Brain Tissue Resource Center consisting of brain tissues from three normal male patients of 69–72 years old. For human brain tissues as well as human SK-N-MC and SH-SY-5Y neuroblastoma cells, immunoprecipitation with anti-cathepsin V was conducted followed by Western blot of cathepsin V (Western blots performed as we have described (2–4)). Immunoprecipitation was conducted by preparation of cellular homogenates with a mixture of protease inhibitors (as we have described (27)) followed by immunoprecipitation (IP) with mouse anti-cathepsin V (R & D Systems). IP was first conducted (of homogenate) with normal mouse serum to remove nonspecific proteins followed secondly by IP with anti-cathepsin V. The first IP step utilized 0.5 mg of homogenate with 50 μl of normal mouse serum in 200 μl of IP buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.2% Tween 20, 3 mM CHAPS, 0.2% Triton X-100, 1 mM EDTA with protease inhibitors [0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μM chymostatin, 10 μM E64c (Bachem, Torrance, CA), 10 μM pepstatin A, 10 μM leupeptin]) and incubated for 2 h at 4 °C followed by incubation with Affi-Gel-agarose (50 μl) for 2 h. Affi-Gel beads were pelleted by centrifugation, and the supernatant was subjected to the second IP with anti-cathepsin V (15 μl) overnight at 4 °C and then incubated with Affi-Gel beads for 2 h at 4 °C. Protein bound to the beads was collected by centrifugation (after washing), and bound proteins were collected by heating (70 °C for 10 min) in sample buffer. These samples were subjected to SDS-PAGE gels (as described previously; Refs. 2–4) and Western blots with anti-cathepsin V. It is noted that different cellular samples required different optimal film exposure times for clarity of the bands. Control studies showed that the anti-cathepsin V did not detect human cathepsins B, L, and H (from Athens Research and Technology, Athens, GA) in Western blots (shown in supplemental Fig. S1).

RESULTS

Cellular Cathepsin V Expression Generates (Met)enkephalin from Proenkephalin—To assess the ability of human cathepsin V to produce active enkephalin from the human proenkephalin precursor, cathepsin V and PE cDNAs were coexpressed in PC12 neuroendocrine cells, and cellular (Met)enkephalin levels were measured by RIA. PC12 cells (derived from rat adrenal medulla) were selected for these studies because these cells express transfected proenkephalin and proteases for enkephalin production (2). Importantly, results show that cathepsin V expression results in increased levels of (Met)enkephalin that were elevated by 2-fold (Fig. 1a). The RIA specifically measures only (Met)enkephalin and does not recognize the PE precursor (1). Control experiments show effective expression of PE (Fig. 1b) and cathepsin V (a mature form of ~24 kDa) (Fig. 1c), illustrated by Western blots.

Expression of cathepsin V in human neuroblastoma SK-N-MC cells was also assessed to examine the role of cathepsin V in production of endogenous (Met)enkephalin expressed in SK-N-MC cells (29, 30). Expression of cathepsin V resulted in increased (Met)enkephalin by ~2-fold in SK-N-MC cells (Fig. 2a). Control Western blots show effective expression of

Immunofluorescent Confocal Microscopy of Cathepsin V, Enkephalin, and NPY in Human Neuroblastoma Cells—Colocalization of cathepsin V with enkephalin and NPY in human SK-N-MC and SH-SY-5Y neuroblastoma cells, respectively, was examined by immunofluorescent confocal deconvolution microscopy as we have described previously (3, 4). Neuronal cells (human SK-N-MC and SH-SY-5Y neuroblastoma cells) were fixed with formaldehyde 3.7% and permeabilized with 0.1% Triton X-100, incubated with 3% BSA (bovine serum albumin) in PBS (phosphate buffered saline) for 1 h at room temperature, and incubated with mouse anti-cathepsin V IgG antibody (25 μg/ml, R & D Systems) and then with rabbit anti-Met-enkephalin (1:50), rabbit anti-NPY (1:100) (Millipore, Billerica, MA) or rabbit anti-Lamp-1 (1:100, Abcam, Cambridge, MA) for 2 h at room temperature in PBS, 3%BSA. Cells were washed in PBS and incubated with goat anti-mouse Alexa 594 and goat anti-rabbit Alexa 488 (Invitrogen) for 45 min at room temperature. Immunofluorescent images were examined with the Delta Vision Spectris Image Deconvolution System on an Olympus IX70 microscope using Softworx Explorer software from Applied Precision. As control, incubation with only secondary antibodies (no primary antibodies) was performed, resulting in a lack of immunofluorescence. Another control for mouse anti-cathepsin V was conducted with normal mouse IgG (Calbiochem), resulting in markedly decreased immunofluorescence compared with anti-cathepsin V.

Quantitation of the colocalization of cellular cathepsin V with (Met)enkephalin, NPY, or Lamp-1 was conducted by measuring the Pearson correlation coefficient, Rr, using the Velocity software (Version 5.5.2 from PerkinElmer Life Sciences) (28). An Rr value of 1 indicates complete colocalization, and an Rr value of 0 indicates no specific colocalization. Rr values obtained indicate partial colocalization of cathepsin V with enkephalin and NPY.
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Cathepsin V as the mature form of ~24 kDa as well as proenzyme and preproenzyme forms of 38–40 and 40–45 kDa, respectively (Fig. 2b). These data indicate that cathepsin V can participate in the cellular production of (Met)enkephalin from its PE precursor.

Cathepsin V siRNA Treatment of Human Neuroblastoma Cells Decreases (Met)enkephalin—Human SK-N-MC neuroblastoma cells express endogenous cathepsin V (Fig. 2b) and (Met)enkephalin. Therefore, gene silencing of endogenous cathepsin V was assessed for reduction of enkephalin. Notably, the cathepsin V siRNA resulted in significant reduction of (Met)enkephalin by more than 80% compared with the controls of scrambled siRNA or no siRNA (Fig. 3a). These data indicate that endogenous cathepsin V participates in enkephalin production. The siRNA reduction of cathepsin V protein expression was verified by Western blot; cathepsin V was nearly absent in cells treated with 50 nm siRNA (Fig. 3b), which was used in the cathepsin V gene silencing experiment of Fig. 3a. Effective siRNA gene silencing of cathepsin V illustrates the endogenous role of cathepsin V for enkephalin production.

In Vitro Cathepsin V Cleavage of PE at Dibasic Residue Sites—To evaluate direct processing of recombinant human PE by cathepsin V, in vitro proneuropeptide processing was investigated. Incubation of PE with PE (for 30 min) resulted in production of an ~24-kDa band that was detected by antisera to (Met)enkephalin (ME) and (Leu)enkephalin (LE) in Western blots (Fig. 4a). The ~24-kDa PE-derived intermediate was observed in vivo in human cortex and hippocampus by Western blots with anti-ME and anti-LE (Fig. 4b). The presence of LE and ME is consistent with the 24-kDa fragment representing an intermediate product of PE (Fig. 5b). These findings indicate that cathepsin V generates an ~24-kDa intermediate from PE that parallels PE processing in vivo in human brain.

Peptide products generated from PE by cathepsin V were analyzed by nano-LC-MS/MS tandem mass spectrometry, and results are shown in Fig. 5b (mass spectrometry data are shown in supplemental Table S1 and Fig. S2). Results show that the Lys-Arg-ME peptide was generated, indicating cleavage of PE at the N-terminal side of dibasic Lys-Arg processing sites. Extended peptides of (Met)enkephalin-Arg-Phe (H) were generated by cathepsin V by processing at the dibasic K↓↓↓R site flanking LE. These peptide products indicate cathepsin V cleavage of dibasic residue sites (Lys-Arg) of PE.

Cathepsin V was also characterized with the peptide-MCA substrates t-butoxycarbonyl-Gly-Lys-Arg-MCA, Z-Arg-Arg-MCA, and Z-Phe-Arg-MCA (Table 1). Results confirmed cleavages by cathepsin V at dibasic as well as monobasic residues.

Cathepsin V in Human Neuronal Secretory Vesicles and Tissues That Produce Neuropeptides—The presence of cathepsin V in human neuronal tissues and secretory vesicles that produce enkephalin and neuropeptides was examined, as the presence of cathepsin V is necessary for its cellular role in neuropeptide production. The presence of cathepsin V in purified human sympathoadrenal pheochromocytoma secretory vesicles was examined (by Western blot, Fig. 6a), indicating cathepsin V as an ~24-kDa band, representing mature cathepsin V (calculated molecular mass of 23,999 daltons (31)). Cathepsin V was present mainly in the soluble fraction compared with the membrane fraction of the secretory vesicles.
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FIGURE 3. Gene silencing of cathepsin V reduces (Met)enkephalin in human neuroblastoma SK-N-MC cells. a, gene silencing of cathepsin V by siRNA reduces (Met)enkephalin production. Human neuroblastoma SK-N-MC cells were transfected with siRNA to cathepsin V as described under “Experimental Procedures.” As controls, transfection of scrambled sequences of siRNA was conducted as well as no siRNA. One day after transfection cells were harvested, and cell levels of (Met)enkephalin were measured by radioimmunoassay. A significant decrease in (Met)enkephalin occurred after transfection of cells with siRNA to cathepsin V (p < 0.05 by Student’s t test) compared with the controls of scrambled siRNA or no siRNA. b, cathepsin V protein expression is substantially reduced by siRNA gene silencing. Silencing of cathepsin V expression was assessed by Western blots after transfection of cathepsin V siRNA into SK-N-MC cells. Cathepsin V was substantially reduced with 50 nm siRNA (lane 2) and partially reduced with 25 nm siRNA (lane 3) compared with control transfection with scrambled siRNA (lane 1). These data show decreased cathepsin V expression when cathepsin V siRNA is transfected in SK-N-MC cells.

FIGURE 4. In vitro processing of PE by cathepsin V generates an intermediate present in human brain. a, in vitro processing of PE by cathepsin V is shown. PE was incubated with or without human cathepsin V for 30 min and subjected to SDS-PAGE and Western blot using anti-ME (panel i) or LE (panel ii). Results show the presence of an ~24-kDa PE-derived band recognized by both anti-ME and anti-LE antisera. b, shown is the PE-derived ~24-kDa intermediate in human brain cortex and hippocampus. Western blots of human brain cortex (panel i) and hippocampus (panel ii) with anti-ME and anti-LE are shown. Results show the presence of an ~24-kDa band that is recognized by both antisera, indicating the presence of a PE-derived ~24-kDa intermediate.

Cathepsin V was present in human brain cortex and hippocampus tissue regions that contain enkephalin, NPY, and numerous neuropeptides (Fig. 6b). Western blots show that these brain regions contain mature cathepsin V (~24 kDa) as well as bands of 40–45-kDa consistent with procathepsin V (8, 10, 31). Human neuroblastoma cells, SH-SY-5Y and SK-N-MC, contain primarily mature cathepsin V (~24 kDa) and preprocathepsin V (Fig. 6, c and d); these neuronal cells contain NPY and enkephalin, respectively (29, 30, 33, 34).

Cellular Colocalization of Cathepsin V with Enkephalin and NPY in Secretory Vesicles—Proteolytic processing of proenkephalin and other proneuropeptides occurs within secretory vesicles where enkephalin and NPY are produced (5). Therefore, involvement of cathepsin V in neuropeptide production predicts its localization in secretory vesicles with enkephalin and NPY. Colocalization of ME with cathepsin V was assessed by immunofluorescence confocal deconvolution microscopy in human SK-N-MC neuronal cells (Fig. 7). Results show the discrete, punctate localization of cathepsin V (red immunofluorescence), similar to the pattern of ME localization (green immunofluorescence). Colocalization of cathepsin V and ME were observed by the merged yellow fluorescence. Quantitation of the relative cellular cathepsin V colocalized with ME was assessed by measurement of the Pearson correlation coefficient (Rr value) of 0.45, indicating specific partial colocalization of cathepsin V with enkephalin (Table 2).

Because the overlap of cathepsin V and ME localization in the SK-N-MC cells was partial, experiments also assessed the subcellular localization of cathepsin V with Lamp-1, a marker for lysosomes. Cathepsin V and Lamp-1 immunofluorescence were partially colocalized (Fig. 8). Quantitation yielded the Rr value of 0.535, which indicates partial colocalization of cathepsin V and Lamp-1 (Table 2). Thus, cathepsin V is present in both secretory vesicles and lysosome organelles.

Cathepsin V is also colocalized with NPY in human SH-SY-5Y neuronal cells in a discrete pattern of localization that is consistent with that of NPY-containing secretory vesicles (Fig. 9a). Quantitation of the colocalization of cathepsin V with NPY by the Pearson correlation coefficient indicated an Rr value of 0.74, indicating a high degree of colocalization of cellular cathepsin V with NPY (Table 2). It is also noted, however, that NPY shows more widespread subcellular distribution than cathepsin V in SH-SY-5Y cells; therefore, some NPY secretory vesicles did not overlap with cathepsin V. It is possible that lower levels of cathepsin V may be present than that detected immunocytochemically. But most secretory vesicles with cathepsin V contain NPY, indicating their colocalization.

Control immunohistochemistry with normal mouse IgG showed markedly reduced immunofluorescence compared with anti-cathepsin V immunofluorescence observed in SH-SY-5Y and SK-N-MC cells, thus, supporting the specificity of the cathepsin V immunofluorescence (supplemental Fig. S3). These immunofluorescent microscopy findings combined with that showing cathepsin V in purified secretory vesicles...
indicate the novel subcellular location of cathepsin V in secretory vesicles that produce neuropeptides.

**Cellular Production of NPY by Cathepsin V**—The presence of cathepsin V in human neuronal SH-SY-5Y cells that contain NPY led to the prediction that cathepsin V may participate in producing NPY. Indeed, expression of cathepsin V and proNPY in PC12 cells resulted in elevated NPY (Fig. 9b), indicating a role for cathepsin V in the production of the NPY neuropeptide. These results demonstrate the functional role of cathepsin V in the production of enkephalin and NPY neuropeptides, mediators of cell-cell communication in the nervous and endocrine systems.

**DISCUSSION**

This study demonstrates the novel cellular function of human cathepsin V for producing enkephalin and NPY peptide neurotransmitters in secretory vesicles. Cellular expression of cathepsin V resulted in production of enkephalin and NPY. Importantly, cathepsin V siRNA treatment of human SK-N-MC neuroblastoma cells resulted in substantial reduction of enkephalin by more than 80%, indicating the endogenous role of cathepsin V for enkephalin production. Cathepsin V cleaves the enkephalin precursor proenkephalin at dibasic residues (i.e. Lys-Arg), resulting in production of Lys-Arg-(Met)enkephalin, which then requires removal of N-terminal basic residues by aminopeptidase B, as well as by carboxypeptidase E, that are present with neuropeptides in secretory vesicles (35). The colocalization of cathepsin V with enkephalin and NPY in secretory vesicles supports the demonstrated function of cathepsin V in producing neurotransmitters. Furthermore, human brain cortex and hippocam-
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![Image](https://example.com/image.png)

**FIGURE 7. Neuronal localization of cathepsin V with enkephalin in secretory vesicles assessed by immunofluorescent confocal microscopy in human SK-N-MC neuroblastoma cells.**

(a) localization of cathepsin V with enkephalin in secretory vesicles is shown. Cathepsin V was observed (red fluorescence) as a punctate, discrete pattern of localization like that of (Met)enkephalin (green fluorescence). The colocalization of the protease with enkephalin was illustrated by the merged yellow fluorescence. Quantitation of the relative amount of cellular cathepsin V that is colocalized with enkephalin was assessed with the Pearson correlation coefficient (Rr value) (Table 2). Measurement of the Rr value as 0.45 indicates partial colocalization of cathepsin V with enkephalin. It is noted that cathepsin V is also observed in nuclei; this is consistent with other reports demonstrating nuclear functions of cathepsin V (52, 53).

(b) shown is an enlarged view of secretory vesicles containing cathepsin V and enkephalin. An enlarged image more clearly shows the secretory vesicle colocalization of cathepsin V (red immunofluorescence) with enkephalin (green immunofluorescence) as indicated by the merged yellow immunofluorescence; arrows indicate the secretory vesicles containing both cathepsin V and enkephalin. Controls using normal mouse IgG instead of mouse anti-cathepsin V resulted in markedly reduced immunofluorescence (supplemental Fig. 3), illustrating the specificity of the antibody to cathepsin V.

**TABLE 2**

| Protease | Neuropeptide | Protease/Neuropeptide colocalization, Rr value |
|----------|--------------|---------------------------------------------|
| Cathepsin V | (Met)enkephalin | 0.448 ± 0.018 |
| Cathepsin V | NPY | 0.736 ± 0.030 |

Neuronal localization of cathepsin V with enkephalin in secretory vesicles assessed by immunofluorescent confocal microscopy in human SK-N-MC neuroblastoma cells.

Quantitative analyses of their partial colocalization was conducted with the Pearson correlation coefficient, indicated as 0.535 ± 0.036 (see Table 2, legend).

![Image](https://example.com/image.png)

**FIGURE 8. Localization of cathepsin V with Lamp-1 marker of lysosomes in human SK-N-MC cells.**

(a) shown is cathepsin V localization assessed with Lamp-1. Cathepsin V subcellular distribution was evaluated with Lamp-1, a marker for lysosomes (32). Cathepsin V (red) and Lamp-1 (green) display partial colocalization (merged yellow immunofluorescence), shown by arrows. Quantitative analyses of their partial colocalization was conducted with the Pearson correlation coefficient, indicated as 0.335 ± 0.036 (see Table 2, legend).

(b) shown is an enlarged view of cathepsin V localization with Lamp-1. An enlarged image shows the distinct colocalization of cathepsin V (red) with Lamp-1 (green) shown by the merged yellow immunofluorescence.

The unique nature of cathepsin V in the human genome raises the issue of the biological function of human cathepsin V with that of the highly homologous human cathepsin L. Notably, results of this study with others (7, 36) in the field indicate the joint roles of human cathepsin V and human cathepsin L for proneneuropeptide processing. However, in contrast to the human genome, the genomes of mouse and other mammals (bovine, sheep, and others) encode only cathepsin L but not orthologues of cathepsin V (8–11). Thus, proneneuropeptide processing in human is hypothesized to utilize both cathepsin V and cathepsin L, but mouse and other mammalian species possess only cathepsin L for neuropeptide production (1–7).

Studies of transgenic mice with knock-out of the cathepsin L gene indicated the important role of this protease in the production of numerous peptide neurotransmitters and hormones, including enkephalin, β-endorphin, dynorphins, NPY, ACTH, α-MSH (α-melanocyte stimulating hormone), cholecystokinin, and catestatin (1–7, 36). Expression of cathepsin V in the cathepsin L knock-out mouse rescues defects in T cell function (37) and keratinocyte proliferation (38, 39). These findings indicate that human cathepsin V and mouse cathepsin L share similar functions, consistent with their high homology (74.6% protein sequence identity for cathepsin V and cathepsin L) (31). It is of interest that human cathepsin V shares greater homology with mouse cathepsin L (74.6% identity in protein sequences) compared with its homology with human cathepsin L (71.5% identity in protein sequences of human cathepsin V and human cathepsin L). Importantly, in contrast to the single mouse cathepsin L gene, the human genome utilizes both cathepsin V and cathepsin L genes for cathepsin L-like functions.

Human cathepsin V expression and gene silencing of this study demonstrates the novel functional role of cathepsin V in...
secretory vesicles for production of enkephalin and NPY neuropeptides in model human neuroblastoma cells as well as in neuroendocrine PC12 cells. Cathepsin V is present in human brain regions and adrenal medulla of the neuroendocrine system as well as in human neuronal cell lines (SK-N-MC and SH-SY-5Y cells) that produce neuropeptides. Previous studies found cathepsin V to be present in thymus, testes, epidermis, and cornea, possessing a more restricted tissue localization than cathepsin L (8–10, 12, 40–43). Results here indicate the presence of cathepsin V in neuronal tissue regions that is compatible with its unique role in the production of enkephalin and NPY peptide neurotransmitters.

The findings from this study of human cathepsin V and from prior studies of cathepsin L (mouse, bovine, and human) indicate their role in processing dibasic residue sites of proprotein precursors in secretory vesicles for the production of neuropeptides (1–7, 36). Both cathepsin V and cathepsin L show cleavage of proneuropeptides at the N-terminal side of dibasic processing sites (i.e. Lys-Arg) as well as cleavage between the dibasic residues. Resultant peptide intermediates will then require removal of N-terminal basic residues by Lys-Arg aminopeptidase B and removal of C-terminal basic residues by carboxypeptidase E (5).

In addition to these cysteine proteases, the subtilisin-like prohormone convertases known as PC1/3 and PC2 participate in proneuropeptide and prohormone processing with somewhat different cleavage specificity for the C-terminal side of dibasic residue sites within prohormones (5, 15, 16). The dual cysteine protease pathway, consisting of cathepsin V and cathepsin L, combined with the subtilisin-like prohormone convertase pathway together function in the conversion of proproteins into active neuropeptides.

More specifically, PC2 also participates in producing (Met)enkephalin, illustrated by PC2 knock-out mice that possess brain (Met)enkephalin levels of 50% compared with wild-type controls (44). Thus, (Met)enkephalin production utilizes PC2 with cathepsin V (shown here) and cathepsin L (1, 2, 5). With respect to NPY, PC2 knock-out mice show no significant changes in brain levels of NPY, but PC2 knock-out results in a modest decrease in ileum NPY (44). These data show that NPY production in brain involves primarily cathepsin V (this study) and cathepsin L (3).

The findings of this study indicate the novel role of cathepsin V in secretory vesicles for producing active neuropeptides, which contrasts with the well known function of cathepsin V in lysosomes (11, 45). This study shows that human neuroblastoma cells contain cathepsin V in secretory vesicles as well as lysosomes. Cathepsin V displays activity in the acidic pH range of 5–7, consistent with the acidic internal milieu of both secretory vesicles and lysosomes (10, 46).

FIGURE 9. Cellular production of NPY by cathepsin V. a, localization of cathepsin V and NPY in human SH-SY-5Y neuroblastoma cells is shown. Cathepsin V localization in human SH-SY-5Y neuroblastoma cells was observed by immunofluorescent confocal microscopy. Cathepsin V (red fluorescence) and NPY (green fluorescence) were discretely localized, with colocalization among many secretory vesicles (yellow immunofluorescence, indicated by arrows). Quantitation of the relative cathepsin V colocalization with NPY was assessed by the Pearson correlation coefficient (Rr value) (Table 2). The measured Rr value of 0.74 indicates that cathepsin V is partially colocalized with NPY. Controls using normal mouse IgG instead of mouse anti-cathepsin V resulted in markedly reduced immunofluorescence (supplemental Fig. S3), illustrating the specificity of the antibody to cathepsin V. b, NPY production by cathepsin V in PC12 cells is shown. PC12 cells were cotransfected with proNPY cDNA (in pcDNA3.1 vector) and cathepsin V cDNA (in pCMV6-XL5 vector), and cells were harvested 3 days later for analyses of cellular levels of NPY (by RIA). Results are shown as x ± S.E., *, statistically significant (n = 6, p < 0.001, Student’s t test).
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It will be important for future studies to further define the relative roles of cathepsin V with cathepsin L for the production of diverse neuropeptides. Deficits and abnormalities in neuropeptide systems are the basis for normal function in the nervous system and participate in human neurological diseases with changes in particular neuropeptides. For example, endogenous opioid neuropeptides consisting of enkephalin, endorphin, and dynorphins are important for mediating stress and analgesia in disease as well as in healthy conditions (14, 47). NPY regulates feeding behavior and obesity (17, 18, 48, 49); cholecystokinin regulates anxiety and related behaviors (50). Galanin participates in regulating cognition and is involved in Alzheimer disease that results in severe memory loss (51). The prominent functions of neuropeptides indicate the high significance of this study's finding for participation of human cathepsin V in producing enkephalin and NPY neuropeptides for cell-cell communication in the nervous system.

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