HHS Public Access

Author manuscript

Cell Chem Biol. Author manuscript; available in PMC 2022 January 21.

Published in final edited form as:

Cell Chem Biol. 2021 January 21; 28(1): 105–112.e4. doi:10.1016/j.chembiol.2020.11.003.

Neuropeptidomic analysis of a genetically-defined cell-type in mouse brain and pituitary

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Summary

Neuropeptides and peptide hormones are important cell-cell signaling molecules that mediate many physiological processes. Unlike classical neurotransmitters, peptides undergo cell-type-specific post-translational modifications that affect their biological activity. To enable the

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Author Contributions

UH and WCW developed the strategy for the creation of the *CpeFlx* mice.

LDF, LAD, AKF, UH, and WCW contributed to the creation of the *CpeFlx* mice.

LDF and AKT performed the peptidomics analyses.

LDF analyzed the data and prepared the figures and supplemental files.

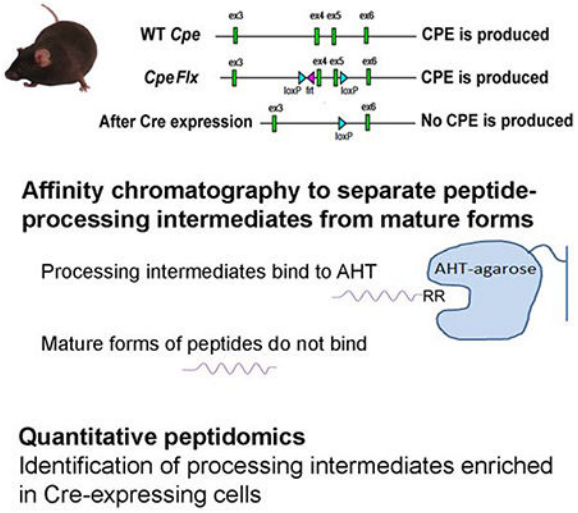
All authors contributed to experimental design and the writing of the manuscript.

Competing Interests

The authors declare that they have no competing interest.

identification of the peptide repertoire of a genetically-defined cell-type, we generated mice with a conditional disruption of the gene for carboxypeptidase E (*Cpe*), an essential neuropeptide-processing enzyme. The loss of *Cpe* leads to accumulation of neuropeptide precursors containing C-terminal basic residues which serve as tags for affinity-purification. The purified peptides are subsequently identified using quantitative peptidomics, thereby revealing the specific forms of neuropeptides in cells with the disrupted *Cpe* gene. To validate the method, we used mice expressing Cre recombinase under the proopiomelanocortin (*Pomc*) promoter and analyzed hypothalamic and pituitary extracts, detecting peptides derived from proopiomelanocortin (as expected) and also proSAAS in POMC neurons. This technique enables the analyses of specific forms of peptides in any Cre-expressing cell-type.

Graphical Abstract



eTOC Blurb

We report a method to identify neuropeptides in specific cell-types, using mice with a conditional deletion of carboxypeptidase E. It was validated by deleting CPE in proopiomelanocortin cells. Surprisingly, these mice have greatly reduced levels of the anorexigenic peptide α-MSH but are not obese.

Keywords

Proteomics; peptidomics; neuropeptidomics; neuropeptide; carboxypeptidase

Introduction

Neuropeptides and peptide hormones play important roles in communication between cells as neurotransmitters and neuromodulators (Fricker, 2012, Strand, 2003). Unlike classical neurotransmitters, neuropeptides are often produced in distinct forms by the differential processing of the protein precursor, with each form having discrete biological activities through action on G protein-coupled receptors (Gomes et al., 2020, Fricker, 2012). Thus, it

is important to define the precise forms of neuropeptides in various cell-types. Mass spectrometry-based peptidomic techniques can identify and quantify the peptide contents in tissue extracts (Fricker, 2018, Romanova et al., 2013) and in single cells of organisms with large neurons such as *Aplysia* (Rubakhin et al., 2011). However, the smaller size of mammalian neurons limits single-cell analyses (Zimmerman et al., 2010, Bora et al., 2008). Techniques that involve physical separation of neurons or other cell types are not ideal because gene expression and/or neuropeptide release are likely to be altered by this procedure (Shapiro et al., 2013). Therefore, a method to study peptides in genetically-defined mammalian cell-types, without prior separation of the cells, would greatly enhance our knowledge of neuropeptides and peptide hormones.

It is possible to study the function of genetically-defined cell-types in mice using Cre recombinase under the control of selective promoters (Sauer, 1998, Nagy et al., 2009). Optogenetic techniques that induce or inhibit neuronal firing have been used to examine the function of genetically-defined neuronal cell-types (Deisseroth et al., 2015, Kim et al., 2017). Cre-recombinase expression has been used to inhibit protein synthesis, allowing for studies on the roles of genetically-defined cell-types in memory consolidation (Shrestha et al., 2020). Analysis of RNA in a single-cell-type is an established technique, but doesn't reveal the post-translational modifications of proteins and peptides (Ding et al., 2020, Li et al., 2020). Proteomic analysis of genetically-defined cell-types in mouse brain has been developed, but this approach requires labeling proteins with non-canonical amino acids so that they can be isolated, and this would not detect most neuropeptides (Krogager et al., 2018).

Our approach to detect peptides in specific cell-types involves carboxypeptidase E (CPE), which is the major neuropeptide-processing carboxypeptidase in the regulated secretory pathway (Fig. 1). Another related enzyme, carboxypeptidase D, is present in the trans Golgi network and immature secretory vesicles but this enzyme is removed and recycled to the Golgi prior to vesicle maturation (Varlamov et al., 1999). Mice lacking CPE activity due to a natural point mutation (i.e., $Cpe^{fat/fat}$ mice) or gene disruption have greatly decreased levels of the mature forms and elevated levels of neuropeptide-processing intermediates containing C-terminal basic amino acids (Naggert et al., 1995, Zhang et al., 2008, Che et al., 2001, Fricker et al., 1996). *Cpe* mutant mice show many behavioral and physiological phenotypes, including obesity, impaired fertility, elevated anxiety- and depressive-like behaviors, and impaired development of hippocampal CA3 neurons (Coleman and Eicher, 1990, Naggert et al., 1995, Nillni et al., 2002, Rodriguiz et al., 2013, Woronowicz et al., 2008, Cawley et al., 2004). A long-term goal of generating a conditional *Cpe* mouse line is to provide tools for studies to identify the cell-types that contribute to the various phenotypes of the mice with a global disruption of *Cpe*. In addition to identifying cell-types associated with each of these phenotypes, it is important to identify the peptides within these cell-types, as receptors for these peptides are potential therapeutic targets for obesity, depression, anxiety, and other disorders.

To develop a method to identify peptides in a genetically-defined cell-type, we made use of the accumulation of neuropeptide-processing intermediates containing C-terminal basic amino acids that occur in the absence of *Cpe* (Naggert et al., 1995, Zhang et al., 2008, Che

et al., 2001, Fricker et al., 1996). The additional basic residues on the C-terminus provides a simple tag for the affinity purification of these peptides on anhydrotrypsin, which binds peptides with C-terminal Lys or Arg residues (Bures et al., 2001, Kumazaki et al., 1987, Che et al., 2001). To validate our method, we targeted neurons expressing Cre recombinase under the *Pomc* promoter. Proopiomelanocortin (POMC) is a neuropeptide precursor that is expressed in ~7000-9000 neurons within mouse hypothalamus and other brain regions (Anderson et al., 2016). Although additional cell-types express *Pomc* during development, the total number of neurons expressing Cre under this promoter represent only a small fraction of all neurons (Padilla et al., 2012). POMC is proteolytically-processed into α-melanocyte-stimulating hormone (α-MSH), one of the key peptides involved in regulation of body weight (Anderson et al., 2016, Sobrino Crespo et al., 2014). Previous studies on $Cpe^{fat/fat}$ mice and *Cpe* knock-out mice detected greatly reduced levels of the mature form of α-MSH in hypothalamus (Zhang et al., 2008, Cawley et al., 2010), and therefore it was of interest to test if selective loss of *Cpe* from *Pomc* cells could recapitulate the obesity observed in $Cpe^{fat/fat}$ mice. Most importantly, these mice provided an ideal test system to validate our method by the identification of peptides specifically expressed in *Pomc* cells.

Results

We generated mice with homologous integration of a frt-flanked neomycin cassette and loxP sites flanking critical exons of the *Cpe* gene (Fig. 2A). The presence of the neomycin cassette interfered with the expression of CPE, resulting in mice with greatly reduced metallocarboxypeptidase activity, reduced levels of mature neuropeptides, and a phenotype comparable to the *Cpe* null mice (data not shown). Removal of the cassette with Flp recombinase resulted in *CpeFlx* mice that have normal levels of metallocarboxypeptidase activity (Fig. 2B) and neuropeptides (Tables S1, S2), indicating that the loxP sites did not interfere with expression of CPE. These mice were used for all subsequent studies.

The *CpeFlx* mice were crossed with mice expressing Cre recombinase under the *Pomc* promoter. The resulting *PomcCre::CpeFlx* mice were compared to *CpeFlx* mice using quantitative peptidomics to measure peptides in hypothalamus (Fig. 2C; Table S1) and pituitary (Table S2). The presence of *PomcCre* produced a dramatic decrease in the levels of α-MSH and other mature forms of POMC-derived peptides except for β-endorphin 1-31 which is located on the C-terminus of POMC and does not require CPE. The levels of α-MSH and other mature forms of POMC-derived peptides in *PomcCre::CpeFlx* mice were comparable to their levels in mice lacking CPE activity in all cells ($Cpe^{fat/fat}$; Fig. 2C "null"). Several POMC intermediates containing C-terminal basic residues were detected in the *PomcCre::CpeFlx* hypothalamus and pituitary extracts; these were not detected in tissue extracts from *CpeFlx* mice (Tables S1 and S2). In addition, several processing intermediates derived from the precursor named proSAAS (gene name *Pcsk1n*) were greatly elevated in the *PomcCre::CpeFlx* hypothalamus and pituitary extracts (Tables S1 and S2). However, the mature forms of these peptides were not significantly altered by expression of *PomcCre* (Tables S1, S2). Taken together, these results suggest that proSAAS is expressed in cells targeted by *PomcCre* but unlike POMC, proSAAS is not exclusively expressed in these cells.

The *PomcCre::CpeFlx* mice were indistinguishable from *CpeFlx* mice and wild-type (WT) controls with respect to general appearance and fertility. Despite the large decrease in the levels of α-MSH and other POMC-derived peptides, the *PomcCre::CpeFlx* showed essentially normal body weight (Fig. 2D), except for a slight increase in young female mice at 6 weeks. In contrast, *Cpe* null mice (i.e., $Cpe^{fat/fat}$ mice or *Cpe* knock-out mice) show a delayed onset in the elevation of body weight starting around 8 weeks of age, and reaching ~40-50 g by 16 weeks primarily due to elevated mass of adipose tissue (Cawley et al., 2004). The relatively normal body weight of the *PomcCre::CpeFlx* mice suggests that the large decrease in α-MSH and most other POMC-derived peptides are not sufficient to cause obesity in *Cpe* null mice.

The detection of neuropeptide processing intermediates containing C-terminal basic residues in the *PomcCre::CpeFlx* mice was limited due to the high background of other neuropeptides present in brain extracts. To provide a more sensitive method for detection of neuropeptides in cells expressing *PomcCre*, we purified hypothalamic and pituitary extracts on anhydrotrypsin agarose. Anhydrotrypsin binds peptides containing C-terminal basic residues Lys and/or Arg, but does not bind peptides containing these residues in internal positions (Che et al., 2001). Thus, this commercially-available resin was ideal for enrichment of the neuropeptide processing intermediates. *Cpe*-null mice were included as a positive control along with duplicate pools of the negative control (*CpeFlx* mice without *PomcCre*) and the test group (*PomcCre::CpeFlx*). Following purification, peptides were labeled with one of five different isotopic tags and analyzed by liquid chromatography-mass spectrometry (Fig. 3A, 3B). The affinity column enabled the detection of many Lys- and Arg-extended peptides (Tables S3 and S4), compared to the analyses without affinity purification (Tables S1 and S2). For the affinity-purified peptides, their relative levels in each of the three genotypes fit into one of four different profiles; representative spectra of these four profiles are shown in Figure 3 panels C-F. In one profile, peptides were present at comparable levels in all three genotypes (Fig. 3C). Peptides with this pattern included non-secretory pathway protein fragments as well as secretory pathway peptides that are not substrates of CPE. A second profile represented peptides greatly elevated in the *Cpe*-null mice relative to the other two genotypes (Fig. 3D). These are substrates of CPE that are not abundantly expressed in *Pomc* cells, and include peptides derived from proenkephalin, proSAAS, prothyrotropin-releasing hormone, protachykinins A and B, chromogranins A and B, secretogranin II, and eight other precursors (Tables S3, S4). The third profile was of peptides present at comparable levels in *PomcCre::CpeFlx* and *Cpe*-null mice but not detected in *CpeFlx* mice lacking Cre (Fig. 3E). All of the peptides with this profile of expression levels were POMC-derived peptide-processing intermediates (Tables S3 and S4). The comparable levels of these peptides in *PomcCre::CpeFlx* and *Cpe*-null mice suggests that the disruption of the *Cpe* gene is complete in *Pomc* cells. A fourth profile of expression levels was also observed; these peptides were high in *Cpe* null mice, low in the *PomcCre::CpeFlx* mice, and undetectable in *CpeFlx* mice lacking Cre (Fig. 3F). All of the peptides with this profile are fragments of proSAAS, which fits with the extensive analysis performed on non-affinity-purified peptides (Tables S1 and S2). Their accumulation in the *PomcCre::CpeFlx* mice, albeit at low levels, indicates that proSAAS is expressed in multiple cell-types including cells that express *Pomc* either in adult mice or during development

(Wardman et al., 2011). Taken together, these results demonstrate that conditional deletion of *Cpe* in a cell-type specific manner can be used for the identification of the neuropeptidome of that cell-type.

Due to the use of mass spectrometry-based peptidomics techniques for the analysis of the affinity-purified peptides, the specific forms of peptides are identified (Tables S3, S4). For the vast majority of secretory pathway peptides detected in the present study, the observed endoproteolytic cleavages matched known consensus sites for prohormone convertases (Tables S3, S4). Several peptides were detected that resulted from cleavage at single Arg residues in which there was no upstream basic residue in the −2, −4, or −6 position (e.g. peptides from chromogranin A and B, proSAAS, procholecystokinin, and neurosecretory protein VGF), although the prohormone convertases are known to cleave at single basic sites and the consensus site is not a rigid requirement. We also identified peptides with post-translational modifications that are upstream of CPE, or which do not require CPE cleavage. This excludes C-terminal amidation, which requires a Gly residue on the C-terminus, but in all cases where a peptide was found with a C-terminal Gly-Lys or Gly-Lys-Arg the peptide is known to be amidated (Tables S1–S4). Thus, this modification can reliably be predicted from the peptides identified using our technique. Other known post-translational modifications were identified in the analysis of affinity-purified peptides (Tables S3, S4). These modifications include phosphorylation of Ser (e.g CLIP), acetylation of the N-terminus (e.g. J-peptide), and disulfide bond formation (e.g. oxytocin, vasopressin). We also identified several peptides with oxidized Met, although this is commonly a post-extraction modification that occurs during sample preparation. In addition, we detected other modifications (Tables S3, S4). One of these was identified as J-peptide with C-terminal Gly-Lys-Arg but with the loss of 18 Da from Asp9. Further studies are needed to determine if this apparent loss of water occurred during sample extraction or if it represents a modification that occurred within the secretory pathway.

Discussion

Our technique allows for the analysis of peptides in genetically-defined cell-types, which expands the repertoire of analyses that can be performed on specific cell-types. Analysis of RNA in single cells is well established (Ding et al., 2020, Li et al., 2020) and while this can provide information on the neuropeptide precursors that are potentially produced in a neuron, it does not reveal the specific forms of peptides produced in that neuron. While proteomic analysis of genetically-defined cell-types in mouse brain has been developed, this approach requires labeling proteins with non-canonical amino acids so that they can be isolated, and this would not detect most neuropeptides (Krogager et al., 2018). Our technique provides a simple approach to identify the neuropeptide contents of a genetically-defined cell-type in mice, including determination of precise forms. Neuropeptide precursors are often cleaved into distinct products with different biological activities (Gomes et al., 2020). Our method provides information on the endopeptidase processing of the peptide precursors/intermediates. For example, some of the proSAAS peptides were detected in POMC neurons while others were not found (Tables S1–4), suggesting differences in proSAAS processing in the POMC cells. Affinity purification of processing intermediates provides enrichment of key peptides. Small changes in mature peptide forms are difficult to

detect, even with large numbers of replicates. For example, no changes were detected in any of the mature forms of proSAAS peptides when comparing multiple replicates of *PomcCre::CpeFlx* mice with control *CpeFlx* mice (Tables S1, S2). By combining a cell-type-specific deletion of *Cpe* with affinity purification and quantitive peptidomics, we readily detect neuropeptides expressed in that cell-type.

Because POMC is expressed in a very small subset of neurons, the use of *PomcCre* to validate the method is appropriate. The use of anhydrotrypsin affinity columns greatly reduced the background from the hundreds of peptides seen in a typical peptidomics liquid chromatography/mass spectrometry run and allowed the detection of dozens of peptide processing intermediates; only a few of these intermediates were detected in the analyses without the affinity purification step (Tables S3 and S4 vs Tables S1 and S2). The vast majority of peptides in brain or other tissues do not contain C-terminal basic residues, and neuropeptide processing intermediates only accumulate in the absence of CPE activity, thereby providing selectivity. By using quantitative peptidomics techniques to compare positive and negative controls along with the test samples, it is straightforward to detect peptides that accumulate in the Cre-expressing cells.

By using the *PomcCre* mice to validate our method to isolate and identify neuropeptides in a genetically-defined cell-type, we could test if altered processing of POMC peptides was responsible for the late-onset obesity seen in *Cpe* null mice. Like the *Cpe* null mice, *Pomc* knockout mice also show late-onset obesity (Yaswen et al., 1999), and therefore it was generally assumed that the >90% decrease in α-MSH seen in *Cpe* null mice accounts for their elevated body weight. For example, a recent case report on three obese siblings with a mutation in the *CPE* gene stated that CPE was known to play a role in obesity via its established role in the production of α-MSH (Durmaz et al., 2020). While our present data support the role of CPE in the biosynthesis of this and other POMC-derived peptides, our data question the assumed role for α-MSH in obesity. Specifically, the normal body weight of the *PomcCre::CpeFlx* mice despite their dramatically lower levels of α-MSH (Fig. 2C) argues that decreased levels of this peptide are not sufficient to account for obesity in mice or humans that lack CPE activity.

Due to the presence of carboxypeptidase D in the trans Golgi network and immature vesicles, the loss of CPE activity does not completely eliminate the levels of the mature forms of most peptides (Zhang et al., 2008, Varlamov et al., 1999). It is possible that the ~5-10% remaining α-MSH is sufficient to maintain body weight in the *PomcCre::CpeFlx* mice, and therefore, also in the *Cpe* null mice. Mice heterozygous for *Pomc* deletion, which have half the levels of POMC peptides as WT mice, show normal body weight (Anderson et al., 2016). Another possibility is that the α-MSH processing intermediate that contains C-terminal Gly-Lys-Arg-Arg is active at the melanocortin receptors. This processing intermediate is elevated in the *PomcCre::CpeFlx* mice and also in the *Cpe* null mice, compared to mice with normal levels of CPE activity. If biologically active, this could explain why the *PomcCre::CpeFlx* mice have normal body weight despite the importance of α-MSH in regulation of body weight. Because α-MSH and adrenal corticotropic-stimulating hormone have similar affinities for melanocortin receptors MC1R, MC3R, MC4R, and MC5R (Gantz and Fong, 2003), it is clear that C-terminally extended α-MSH is able to bind

to the receptors, and therefore the α-MSH processing intermediate is likely to be biologically active.

Regardless of the mechanism, the present results demonstrate that the large decrease in levels of the mature form of α-MSH and increased levels of the C-terminally-extended processing intermediate are not the primary cause of obesity in *Cpe* null mice, and other peptides must contribute to the obesity phenotype. Additional anorexigenic peptides reported in the literature include cocaine- and amphetamine-regulated transcript (CART), neuromedin U, nesfatin 1, neuronostatin, vasopressin, oxytocin, peptide YY 3-36, glucagon-like peptide-1 and -2, and peptides derived from neurosecretory protein VGF (Helfer and Stevenson, 2020, Schalla et al., 2020, Ding and Magkos, 2019, Smith, 2018, Klockars et al., 2019). Except for CART, which does not require CPE for production of the bioactive form, these peptides are known or predicted products of CPE and therefore candidates to account for the elevated body weight in mice with a global disruption of *Cpe.*

In addition to obesity, mice lacking CPE activity in all cells ($Cpe^{fat/fat}$ mice or *Cpe* knock-out mice) have a number of phenotypic differences from WT mice including impaired fertility, impaired cognition, and anxiety- and depressive-like behaviors (Naggert et al., 1995, Rodriguiz et al., 2013, Woronowicz et al., 2008, Nillni et al., 2002, Cawley et al., 2010, Woronowicz et al., 2010, Cawley et al., 2004). Crossing the conditional *CpeFlx* mice with mice expressing Cre in genetically-defined cells can be used to identify cell-types that contribute to the altered phenotypes of the *Cpe*-null mice. For example, this approach could test the physiological effects of other anorexigenic peptides described above. Another direction involves the role of CPE in hippocampal development; mice lacking CPE show severe degeneration of the pyramidal neurons in the hippocampal CA3 region between 3 and 4 weeks (Woronowicz et al., 2008). It would be interesting to see if conditional mice lacking *Cpe* expression in the hippocampal CA3 region recapitulate this phenotype of *Cpe* null mice, for example using G32-4 Cre mice that express Cre under the *Grik4* gene, and our peptidomics method to identify the peptides in these cells. Other directions include the creation of conditional *Cpe* knockout mice to explore the role of CPE and peptide hormones produced in peripheral tissues. Tamoxifen-inducible Cre mice could be used to test whether the global knockout of *Cpe* still caused obesity and behavioral changes if the knockout was delayed until the mice were mature adults, or if the absence of CPE during development is critical for driving these phenotypes.

Our approach to identify peptides present in Cre-expressing cell-types can provide information on the signaling molecules that contribute to the varied phenotypes of global *Cpe* knockout mice. Because some of these phenotypes are not commonly considered to be due to neuropeptides (e.g., anxiety- and depressive-like behavior), the identification of the cell-types and the peptides expressed in these cell types can potentially lead to new therapeutic approaches for these disorders.

Significance

We report a method to identify neuropeptides in genetically-defined cell-types, without the need to physically separate brain cells, a process that is likely to alter the neurochemistry of

the cell. Our method is sensitive, allowing for the detection of peptides present in <10,000 neurons in hypothalamus pools of two mice. In addition, the specific forms of peptides are identified, including proteolytic cleavages and other post-translational modifications that are upstream of CPE, or which do not require CPE cleavage. This excludes C-terminal amidation, which requires a Gly residue on the C-terminus, but includes phosphorylation, acetylation, methionine oxidation, disulfide bond formation, and other modifications found in the present study. The technique is not limited to neuropeptides; CPE is the major carboxypeptidase involved in the production of peptide hormones such as insulin (Naggert et al., 1995, Davidson and Hutton, 1987). In addition to endocrine pancreas, relatively high levels of CPE are present in adrenal medulla, heart, testis, prostate and other selected cell-types [see supplemental Tables in (Fricker et al., 2020)], but in most cases the substrates are not known. Our technique can be used to identify peptides in any cell type based on the availability of Cre recombinase-expressing mouse lines, and it can also be used with Cre-expressing viruses. Because of the important roles of neuropeptides and peptide hormones in physiology, many drugs in current use target peptide receptors. Therefore, identification of cell-type-specific peptides can lead to new therapeutic targets.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Lloyd Fricker (Lloyd.Fricker@einsteinmed.org).

Materials Availability—The *CpeFlx* mouse line is in the process of being deposited in a central repository. Until it is available, requests for the line will be fulfilled by the Lead Contact.

Data and Code Availability—Mass spectrometry data have been submitted to the PRIDE archive (Perez-Riverol et al., 2019) and are available via ProteomeXchange with identifier PXD020999 (Deutsch et al., 2020).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—The *Cpe* locus on mouse chromosome 8 was targeted by homologous recombination in (129X1/SvJ x 129S1/Sv)F1-*Kitl*$^{+}$-derived R1 embryonic stem (ES) cells (Nagy et al., 1993) to place the *loxP* sites 286 bp 5’ to exon 4 and 736 bp 3’ from exon 5. A neo cassette used for selection followed the 5’ loxP site and was flanked by frt-sites to allow excision by Flp recombinase. Correctly targeted R1 ES cells were injected into recipient blastocysts from superovulated C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). The resulting chimeric animals were crossed to C57BL/6J mice; heterozygotes were further bred to C57BL/6J for 4 generations. Mice were then rederived by *in vitro* fertilization of sperm from *Cpe*$^{floxNeo/+}$ mice with eggs from C57BL/6J mice, and maintained by crossing with C57BL/6J mice. The neomycin cassette was removed by crossing with mice expressing a germ-line FLP recombinase (B6.Cg-Tg(Pgk1-flpo)10Sykr/J) obtained from The Jackson Laboratory (Stock No: 011065) (Wu et al., 2009). The removal of the neomycin cassette was

confirmed by PCR, described below, and the resulting mice are referred to as *Cpe*^{flox} to differentiate them from the line with the neomycin cassette, referred to as *Cpe*^{floxNeo}. The presence of the FLP recombinase in the *Cpe*$^{flox/flox}$ mouse line was not required for maintenance of the line and was omitted. The *Cpe*$^{flox/flox}$ mouse line is being submitted to a public repository.

PCR was used to genotype the mice, using standard assay conditions 3 min at 94°C to start, then 35 cycles of 94°C for 30 sec, 53°C for 1 min, and 72°C for 1 min. After 35 cycles, 72°C for 2 min and hold at 4°C. The following oligonucleotides were used:

A: 5’-acataactcaggactccgtgc

B: 5’-ccctctataagttgagatgtatgg

D: 5’-cattatacgaagttatgatctcgagt

E: 5’-gtcatggtagacagaggaagg

F: 5’-catgatccagtgaatgacagc

FLPr: 5’-tggctcatcaccttcctctt

FLPf: 5’-atagcagctttgctccttcg

Pomc forward: 5’-atagcagctttgctccttcg

Pomc reverse: 5’-tggctcatcaccttcctctt

Heterozygous *Cpe*$^{flox/+}$ mice have amplicons from A/B of 267 bp and from A/D of 301 bp. WT mice have amplicons from A/B and are negative for A/D, while homozygous *Cpe*$^{flox/flox}$ mice are positive for A/D and negative for A/B. Primer pair E/F detects the WT gene near the neomycin cassette; WT mice show a 350 bp band and floxed mice with the neomycin cassette removed show a 400 bp band (if no band, the neomycin cassette is still present).

For *PomcCre::CpeFlx* mice, *Cpe*$^{flox/flox}$ mice were crossed with male hemizygous Tg(Pomc1-cre)16Lowl/J obtained from The Jackson Laboratory (Stock number 005965). The mice were propagated using male *PomcCre*-positive mice, as per the recommended breeding strategy.

All mice were housed 3-5/cage on a 14:10 h light:dark cycle in a humidity- and temperature-controlled environment with food and water provided *ad libitum*. Adult male and female mice (8-20 weeks old) were used for the experiments described below. All animal experiments were conducted with an approved protocol (IACUC 2014-0291) from the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai.

METHOD DETAILS

Measurement of soluble metallocarboxypeptidase activity—Mice were euthanized by decapitation and the frontal cortex was frozen in dry ice and the weight of the tissue was determined, which was typically 70 mg (range 35-140 mg). Carboxypeptidase E (CPE) is primarily a soluble protein, in addition to a form that is peripherally bound to membranes at low pH but not at neutral pH (Fricker et al., 1990, Mitra et al., 1994). In contrast, carboxypeptidase D (CPD) is primarily an integral membrane-bound protein, although some of the full-length CPD is cleaved and present in a soluble form in brain (Song and Fricker, 1996). Therefore, enzyme assays were performed with a soluble extract to enrich for CPE in the assay. Frozen tissue was homogenized in 0.5 mL of 100 mM NaAc buffer, pH 5.5 using a mechanical tissue disruptor (Polytron, Brinkman) and centrifuged at 15,000 x g in a microfuge for 20 minutes at 4°C. Supernatant was transferred to a new tube and aliquots assayed for enzyme activity using the fluorescent substrate dansyl-Phe-Ala-Arg as described (Fricker, 1995), with the following modifications to adapt the assay to a 96-well plate-reader fluorimeter. The enzyme reaction was performed in plastic 0.6 mL microfuge tubes by combining 25 μL tissue supernatant, 25 μL of either 10 mM $CoCl_2$ or 10 μM GEMSA, 150 μL 100 mM NaAc pH 5.5, and 50 μL 1 mM dansyl-Phe-Ala-Arg substrate (final volume 250 μL). Following incubation for 60 min at 37°C, the reactions were stopped by the addition of 50 μL of 1 M HCl and 250 μL of ethyl acetate. Tubes were mixed on a vortex mixer and centrifuged for 1 min at 300 x g at room temperature. After centrifugation, 150 μL of the upper organic phase was transferred to a glass tube and dried overnight at room temperature in a fume hood protected from light. Samples were resuspended in 200 μL of phosphate-buffered saline containing 0.1% of Triton X-100 and transferred to 96-well plates. Fluorescence was determined using a 96-well plate spectrofluorometer: excitation 355 nm; emission 538 nm, top read, filter cutoff 455 nm. Metallocarboxypeptidase activity was determined from the difference between activity in the presence of $CoCl_2$ (which activates CPE) and GEMSA (a CPE inhibitor), assayed in triplicate for each biological sample. Activity was divided by the mass of the tissue. The number of biological replicates is indicated in the figure legend.

Quantitative peptidomic analysis of brain and pituitary

Peptide extraction: For peptidomics analyses of brain, it is essential to heat-inactivate the tissue within 1 min of death (Fridjonsdottir et al., 2018, Svensson et al., 2003, Che et al., 2005). Mice were euthanized by decapitation and their heads were placed in a standard microwave oven and irradiated for 5 seconds to bring the internal temperature to ~80°C, as described (Che et al., 2005). After cooling, the brain was removed and the hypothalamus and pituitary were dissected, frozen in dry ice, weighed, and stored at −70°C until peptide extraction. Prior to extraction, the tissue was transferred to a −20°C freezer. Cold water (200 μL) was added to each hypothalamus or pituitary sample immediately prior to homogenization. Hypothalami were homogenized in a tissue disruptor (Polytron, Brinkman), while the pituitaries were sonicated using a probe sonicator. Homogenates were incubated in a 70°C water-bath for 20 min and cooled to room temperature. For the 'traditional' method of quantitative peptides (e.g., without affinity purification of Lys/Arg-extended peptides), homogenates were directly labeled with isotopic tags as described

below. For the studies described in Figure 3 and supplemental Tables 3–4, peptides were purified on an affinity column prior to isotopic labeling.

Affinity purification of peptides: Tissue extracts from pools of two animals were combined. Affinity column sample buffer was added (1 mL of 100 mM NaAc pH 5.0; 0.5% CHAPS; 20 mM $CaCl_2$), the tubes were mixed, centrifuged at 16,400 x *g* for 30 min at 4°C, and the supernatants were applied to an anhydrotrypsin agarose column (Panvera Corporation, Madison WI) which can alternatively be generated as described (Kumazaki et al., 1987). The column volume for the hypothalamic samples was ~0.3 mL; the column volume for the pituitary samples was ~0.1 mL. The flow-through was recycled 3x through the column. Columns were washed with 10 mL of column sample buffer and rinsed with 2 mL of 10 mM NaAc (without CHAPS or $CaCl_2$). Peptides were eluted first with 1 mL water, then 2 mL of 5 mM HCl. These two eluates were combined and lyophilized using a vacuum centrifuge, then resuspended in 200 μL of 200 mM NaAc pH 6.0. Labeling and mass spectrometry (MS) analysis were performed as described below.

Labeling with isotopic tags: The isotopic labeling was performed using reductive methylation, as described (Dasgupta et al., 2018, Tashima and Fricker, 2018). In brief, tissue homogenates or affinity purified extracts (200 μL) were combined separately with 50 μL of 1 M sodium acetate buffer pH 6.0 and mixed. In a fume hood, 64 μL of freshly-prepared 4% formaldehyde solution was added. One of three formaldehyde solutions was used: light (e.g., natural isotopic form), intermediate (D_2CO), or heavy ($D_2{}^{13}CO$) according to the chart below (D represents deuterium, ^2H). One of two different reducing agents was added: 64 μL of 0.6 M $NaBH_3CN$ or $NaBD_3CN$ (see chart) and samples were mixed. After incubation for 2 hours at room temperature, samples were incubated for 2 days at 4°C. The reaction was quenched with 200 μL of freshly made 4% ammonium bicarbonate. Tubes were mixed and incubated for 2 hours at room temperature. The mixture was acidified with 200 μL of 10% formic acid in a fume hood (the reaction may produce hydrogen cyanide) and incubated 10 min at room temperature. Samples for each set were combined (e.g., the five individual tissue samples labeled with different isotopic tags that were to be compared in the peptidomics analysis; see representative scheme in Figure 3B). Samples were centrifuged for 30 min at 16,400 x g at 4°C and the peptides were purified as described below.

Set	Formaldehyde	Reducing agent	Added mass per free primary amine
A	H_2CO	$NaBH_3CN$	28.0313
B	H_2CO	$NaBD_3CN$	30.0439
C	D_2CO	$NaBH_3CN$	32.0564
D	D_2CO	$NaBD_3CN$	34.0690
E	$D_2{}^{13}CO$	$NaBD_3CN$	36.0757

Purification of peptides: Peptides were separated from proteins using microfiltration with a 10 kDa cutoff dialysis membrane (Ultracel-10 K units, Amicon), then purified on C18 spin columns (Pierce). The general procedure has been recently described (Dasgupta et al., 2018, Tashima and Fricker, 2018). In brief, the samples were applied to the ultrafiltration devices

that had been rinsed three times with water to remove potential contaminants. Samples were centrifuged at 2,300 × g until very little liquid remained in the samples (typically 30 min). The flow-through contained peptides; this fraction was saved in a 2 mL low-retention microfuge tube. The sample remaining in the upper portion of the microfiltration device was washed with 0.5 mL of water, the samples were centrifuged again until most of the liquid had passed through, and the flow-through at the washing step was combined with the initial flow-through. This sample was frozen at −70°C and lyophilized in a vacuum centrifuge.

For further purification and desalting of the peptides on C18 spin columns, the lyophilized samples were reconstituted in 1.2 mL 1% trifluoroacetic acid (TFA). The pH was checked by spotting a small aliquot onto pH paper. If the pH exceeded 4, a small volume of 10% TFA was added and the pH was rechecked (typically, samples required ˜20 μL to bring the pH to a 3-4 range). Samples were applied to a C18 spin column (Pierce) that had been activated as described by the manufacturer, except that the equilibration solution was 0.5% TFA without acetonitrile (not with the recommended 5% TFA). The sample was applied to the spin column in aliquots of 400 μL (the maximum capacity of the spin columns), until the total sample was loaded onto the column. The columns were washed 3 times with 200 μL of equilibration buffer (0.5% TFA) and peptides were eluted with 200 μL of 0.5% TFA containing 70% acetonitrile. These eluates were dried in a vacuum centrifuge and stored at −70°C until MS analysis.

Mass spectrometry: The liquid chromatography (LC) MS/MS experiments were performed on a Synapt G2 mass spectrometer coupled to a nanoAcquity capillary nano-LC system (Waters, Milford, MA, USA). Dried peptides were dissolved in 20 μL of 0.1% formic acid and the peptide mixture (5 μL) was desalted online for 5 min at a flow rate of 8 μL/min of phase A (0.1% formic acid) in a trap column (Acquity UPLC M-Class Symmetry C18 Trap Column, 100 Å, 5-μm particles, 300 μm x 25 mm; Waters). The mixture of trapped peptides was subsequently separated by elution with a 7–50% gradient of phase B (0.1% formic acid in acetonitrile) through a capillary analytical column (Acquity UPLC M-Class HSS T3 Column, 1.8-μm particles, 300 μm x 150 mm; Waters) over 60 min. The data were acquired in the data-dependent mode and the MS spectra of multiple-charged protonated peptides generated by electrospray ionization were acquired for 1 s from m/z 400–1600. The three most intense ions exceeding a base peak intensity threshold of 2500 counts were automatically selected and dissociated in MS/MS by 15- to 60-eV collisions with argon for 1 s. The typical LC and electrospray ionization conditions consisted of a flow rate of 3.5 μL/min, a capillary voltage of 3.0 kV, a block temperature of 100°C, and a cone voltage of 40 V. The dynamic peak exclusion window was set to 45 sec.

Data analysis: MS spectra were analyzed using the MassLynx 4.1 software (Waters). Peak groups representing peptides labeled with different isotopic labels were identified by the regular mass difference profiles of consecutive peaks (see Figure 3). The relative intensity of each monoisotopic peak was entered into the spreadsheet and adjusted to correct for isotopic overlapping of labeled peaks as described (Dasgupta et al., 2018, Tashima and Fricker, 2018).

To identify peptides, MS/MS data were analyzed using the Mascot search engine (Matrix Science Ltd, UK) and the NCBIprot database, specifying *Mus musculus* (house mouse) which consisted of 172780 sequences. No cleavage site was specified. Modifications included the reductive methyl labels and N-terminal protein acetylation, methionine oxidation, and C-terminal amidation species. All search results were manually interpreted to eliminate false positives, as described (Morano et al., 2008, Wardman and Fricker, 2011). In brief, the criteria included (i) an observed mass within 40 ppm (preferably 20 ppm) of the theoretical mass; (ii) the number of Me tags observed matched with the number of free primary amines available (i.e., Lys residue and N-terminus if not acetylated); (iii) the observed charge state(s) of the peptide was consistent with the expected number of positive charges; (iv) >80% of the major fragments observed in the MS/MS matched b or y ions, with a minimum of five matches. In addition, due to the small 2 Da difference between isotopic tags, we used a wider mass range for the initial MASCOT search and then manually confirmed the monoisotopic assignments, as described (Hsieh et al., 2010). Mass spectrometry data have been submitted to the PRIDE archive (Perez-Riverol et al., 2019) and are available via ProteomeXchange with identifier PXD020999 (Deutsch et al., 2020).

QUANTIFICATION AND STATISTICAL ANALYSIS

The technique used for quantification of brain and pituitary peptides is described above in the STAR methods section under the heading ‘Quantitative peptidomic analysis of brain and pituitary.’ Statistical analyses were performed using GraphPad Prism version 7.02. For comparing the *CpeFlx* mice and the *PomcCre::CpeFlx* mice, Student’s t-test was used. The number of mice in each group is indicated in the experimental details, but because some peptides were not detected in every mass spectrometry run, the number of individual mice for some peptides was lower than the total used in the experiment. Therefore, the number of mice that gave a signal for each peptide is indicated in Supplemental Tables 1–2. Other details (average, SEM) are also listed for each peptide in Supplemental Tables 1–2 and defined in the general notes for these tables in the Supplemental Information. For comparing the metalloCP activity in frontal cortex among WT, *CpeFlx*, and *Cpe*-null mice, statistical calculations were performed using one-way ANOVA with Tukey *post-hoc* tests. The number of mice (n) and other details are indicated in the legend to Figure 2. For the comparison of the relative levels of representative hypothalamic peptides among *PomcCre::CpeFlx* mice, *CpeFlx* mice without *PomcCre*, and *Cpe*-null mice, statistical calculations were performed using one-way ANOVA with Tukey *post-hoc* tests. The number of mice (n) and other details are indicated in the legend to Figure 2. For the comparison of body weight of *CpeFlx* mice and *PomcCre::CpeFlx* mice, statistical calculations were performed using Student’s t-test comparing *PomcCre::CpeFlx* to *CpeFlx* of the same age group and sex. Details of the number of mice (n) and other parameters are indicated in the legend to Figure 2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants DA008863 and NS026880 (to LAD) and by Fundação de Amparo à Pesquisa do Estado de São Paulo FAPESP, 2017/20106-9 and Financiadora de Estudos e Projetos (to AKT). The design of the *CpeFlx* was initiated in the Neurotransgenic Laboratory at Duke University Medical Center and supported by unrestricted funds (WCW). The authors thank Adrei Jeltyi, Dylan Bitensky, and Dr. Lindsay Lueptow for assistance with the breeding of the mice, Erika S. Nishiduka for assistance with the LC-MS/MS experiments, and Matthew Engel for assistance with peptidomic analysis.

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Highlights

- A method was developed to identify neuropeptides in genetically-defined cell-types
- This method does not require physical separation of brain cells
- The method was validated using mice lacking *Cpe* expression in *Pomc* cells
- *PomcCre::CpeFlx* mice have low α-MSH but are not obese like global *Cpe* knockout mice

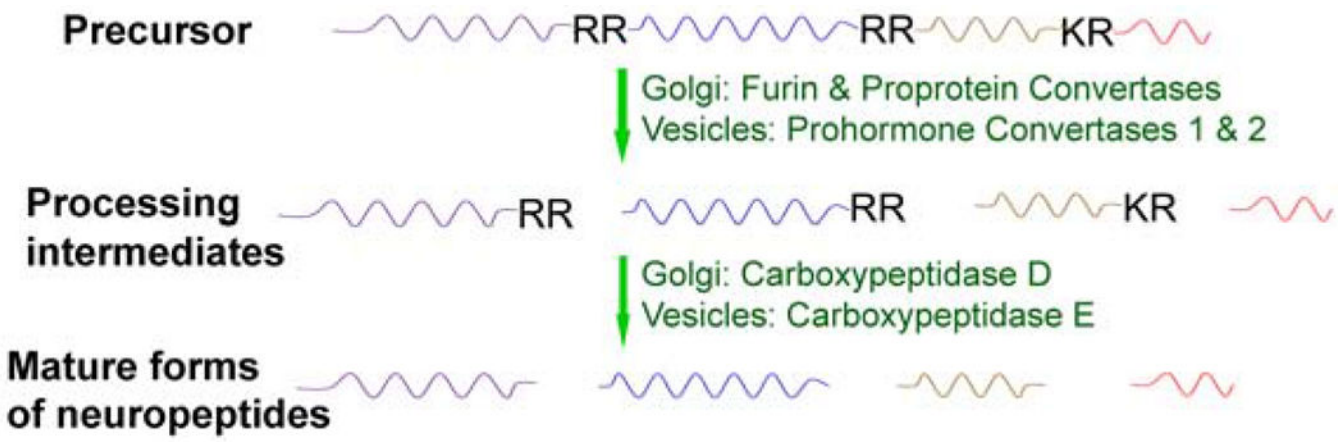

Figure 1. Overview of neuropeptide processing.

Neuropeptide precursors typically contain a number of distinct bioactive peptides that are inactive until they are processed into their mature forms by an endopeptidase followed by a carboxypeptidase. CPE is one of two carboxypeptidases that function within the secretory pathway of neuroendocrine cells, and the only carboxypeptidase found in mature secretory vesicles where the majority of peptide processing occurs (Varlamov et al., 1999). In the absence of CPE activity, there is an accumulation of processing intermediates with C-terminal basic residues (Naggert et al., 1995, Zhang et al., 2008, Che et al., 2001, Fricker et al., 1996, Cawley et al., 2010).

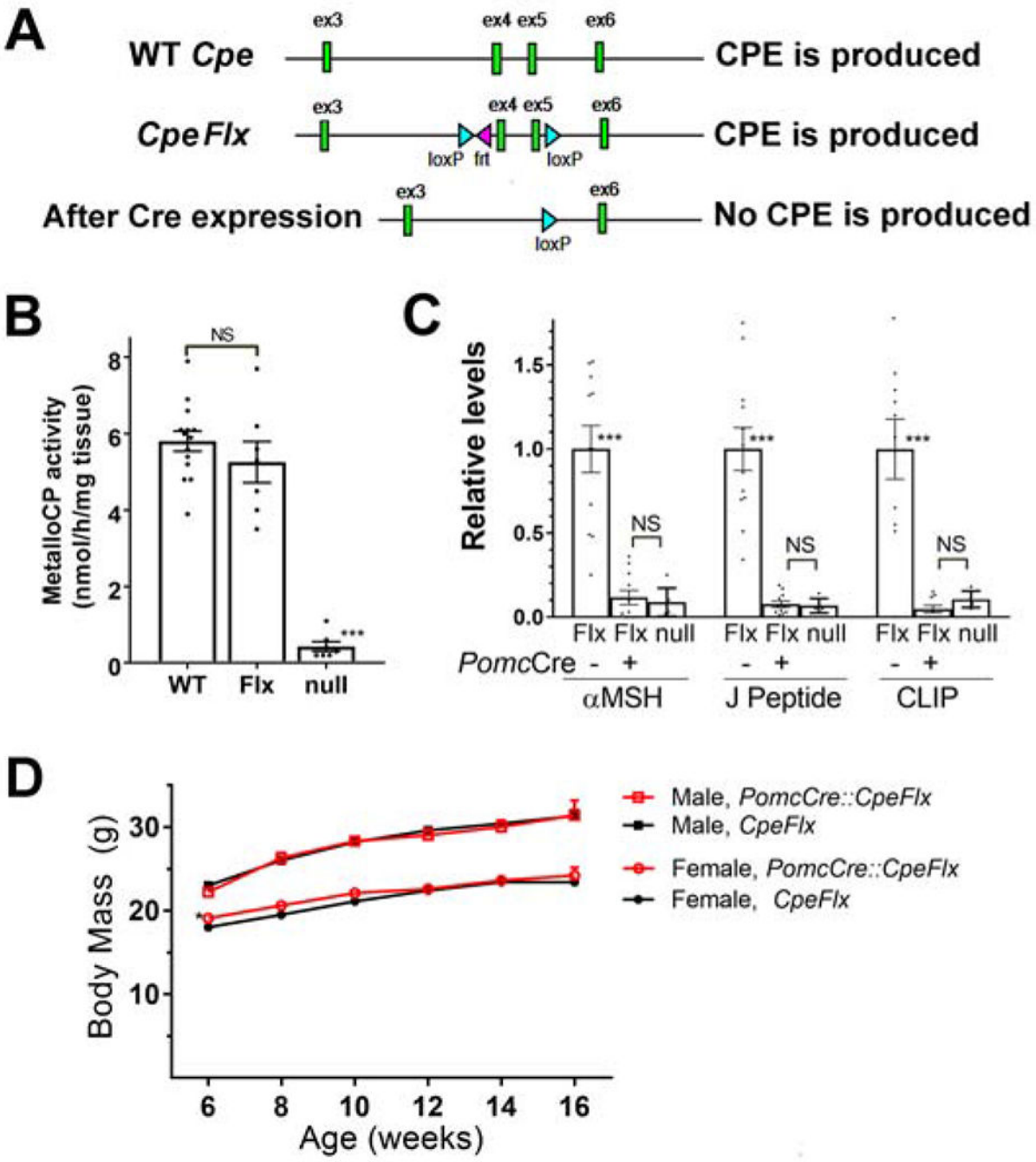

Figure 2. Validation of conditional knockout mice.

(A) Schematic of the wild-type (WT) *Cpe* gene and conditional *Cpe*$^{flox/flox}$ (*CpeFlx*) mice in which loxP sites flank exons 4 and 5. These exons contain essential amino acids for catalytic function. (B) MetalloCP activity in frontal cortex. Error bars show SEM (WT n=14; *CpeFlx* n=7; and *Cpe*-null mice n=7). The *Cpe*-null mice lack CPE activity in all cells. The residual activity in these mice is due to carboxypeptidase D, which cannot be distinguished from CPE in enzymatic assays. (C) Results from peptidomic analyses of hypothalamus from *PomcCre::CpeFlx* mice and two control groups: *CpeFlx* mice without *PomcCre*, and *Cpe*-

null mice (i.e., *Cpe*$^{fat/fat}$ mice). Error bars show SEM, n=9-12 mice of each *Flx* genotype, n=5-7 for *Cpe*-null. For panels B and C, statistical calculations were performed using one-way ANOVA with Tukey *post-hoc* tests: NS, not significant, ***, p<0.001 vs all other groups (D) Body weight of *CpeFlx* mice (black, filled symbols) and *PomcCre::CpeFlx* mice (red, open symbols) for male (squares) and female mice (circles). Error bars show SEM measured at 6, 8, 10, 12, 14, and 16 weeks for n=48, 57, 48, 35, 31, and 14 mice (*CpeFlx* males); n=14, 17, 15, 16, 16, and 8 (*PomcCre::CpeFlx* males); n=39, 38, 33, 24, 24, and 14 (*CpeFlx* females); and n=16, 17, 14, 13, 13, and 8 (*PomcCre::CpeFlx* females). For panel D, statistical calculations were performed using Student's t-test comparing *PomcCre::CpeFlx* to *CpeFlx* of the same age group and sex. *, p=0.035 between female mice at 6 weeks. All other time points for the females, and all time points for the males showed no statistical significance p<0.05.

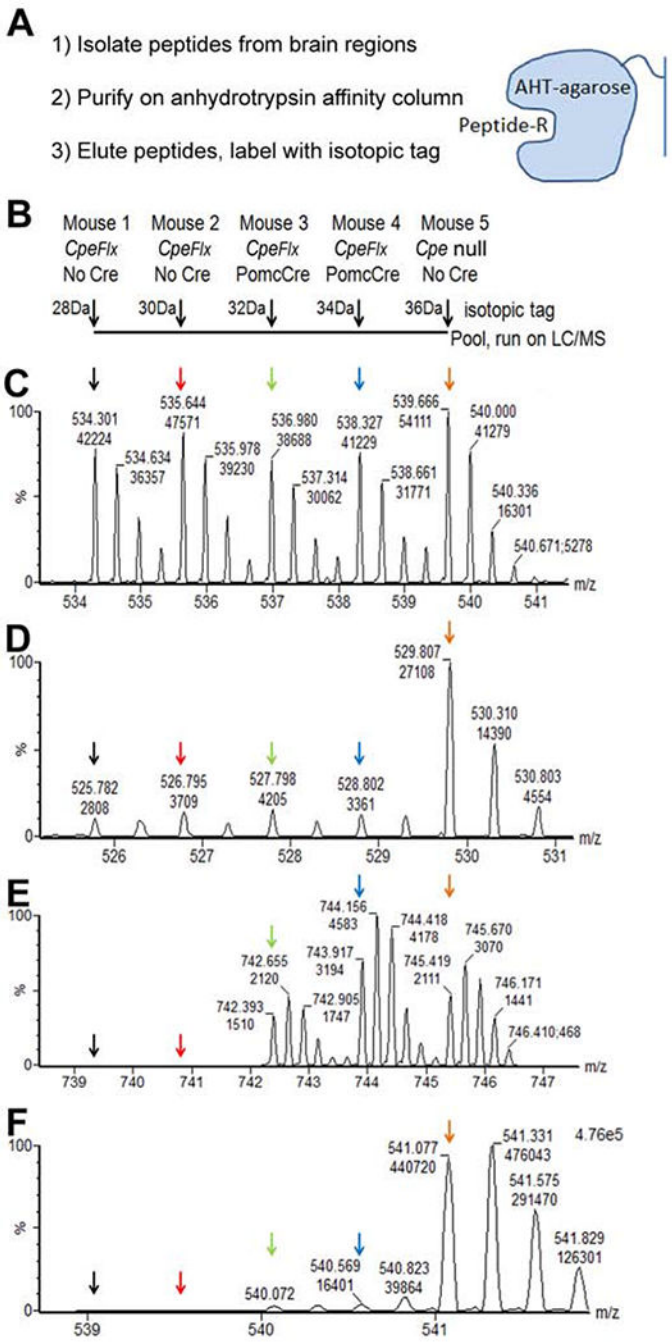

Figure 3. Overview of method and representative data.

(A) Basic steps of the method, highlighting the affinity resin which binds peptides with C-terminal basic residues. (B) Quantitative peptidomics strategy using 5-plex isotopic tags that add two methyl groups to each primary amine in a peptide. The mass of the addition of 2 methyl groups of each isotopic form is indicated. (C-F) Representative data for mouse hypothalamic samples. *Arrows* point to the peptide monoisotopic peaks if detected, or to the predicted m/z if not detected; *black* = light isotopic form (+28 Da); *red* = form with one deuterium per methyl group added (+30 Da for a pair of methyl groups); *green* = form with

two deuterium atoms per methyl group (+32 Da); *blue* = form with three deuterium atoms per methyl group (+34 Da); *purple* = form with three deuterium atoms and one ^{13}C atom per methyl group (+36 Da). (C) Peptide present at comparable levels in all five mice; Ac-ASSDIQVKELEKR, an N-terminal fragment of the cysotolic protein stathmin-1. (D) Peptide present at high levels in the *Cpe*-null mice and much lower levels in the *CpeFlx* mice (and not affected by Cre expression); ALNSVAYER, a fragment of protachykinin A. (E) Peptide present at high levels in *Cpe*-null mice and *PomcCre::CpeFlx* mice, but not detected in *CpeFlx* mice lacking *PomcCre*; RPVKVYPNVAENEpSAEAFPLEFKR, phospho-CLIP-KR. (F) Peptide present at high levels in *Cpe*-null mice, low levels in *PomcCre::CpeFlx* mice, and not detected in *CpeFlx* mice lacking *PomcCre*; SLSAASAPLVETSTPLRLRR, little SAAS-RR.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Anhydrotrypsin agarose column	Panvera Corporation, Madison WI; or generated as described (Kumazaki et al., 1987).	Cat# 7302
Deposited Data		
Mass spec data	ProteomeXchange	PXD020999
Experimental Models: Organisms/Strains		
CpeFlox mice, described in Methods	Described in methods; available from Lead Contact	*CpeFlox mice*
Hemizygous Tg(Pomc1-cre)16Lowl/J	The Jackson Laboratory	Stock number 005965
Oligonucleotides		
A: 5'-acataactcaggactccgtgc	Custom synthesized	N/A
B: 5'-ccctctataagttgagatgtatgg	Custom synthesized	N/A
D: 5'-cattatacgaagttatgatctcgagt	Custom synthesized	N/A
E: 5'-gtcatggtagacagaggaagg	Custom synthesized	N/A
F: 5'-catgatccagtgaatgacagc	Custom synthesized	N/A
FLPr: 5'-tggctcatcaccttcctctt	Custom synthesized	N/A
FLPf: 5'-atagcagctttgctccttcg	Custom synthesized	N/A
Pomc forward: 5'-atagcagctttgctccttcg	Custom synthesized	N/A
Pomc reverse: 5'-tggctcatcaccttcctctt	Custom synthesized	N/A
Software and Algorithms		
Graphpad Prism	Graphpad	https://www.graphpad.com/scientific-software/prism/
Mascot search engine	Matrix Science Ltd	https://www.matrixscience.com/