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Peptidomics of *Cpe^{fat/fat}* mouse brain regions: Implications for neuropeptide processing

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SUMMARY

Quantitative peptidomics was used to compare levels of peptides in wild type and *Cpe^{fat/fat}* mice, which lack carboxypeptidase E (CPE) activity due to a point mutation. Six different brain regions were analyzed: amygdala, hippocampus, hypothalamus, prefrontal cortex, striatum, and thalamus. Altogether, 111 neuropeptides or other peptides derived from secretory pathway proteins were identified in wild type mouse brain extracts by tandem mass spectrometry, and another 47 peptides were tentatively identified based on mass and other criteria. Most secretory pathway peptides were much lower in *Cpe^{fat/fat}* mouse brain, relative to wild type mouse brain, indicating that CPE plays a major role in their biosynthesis. Other peptides were only partially reduced in the *Cpe^{fat/fat}* mice, indicating that another enzyme (presumably carboxypeptidase D) contributes to their biosynthesis. Approximately 10% of the secretory pathway peptides were present in the *Cpe^{fat/fat}* mouse brain at levels similar to those in wild type mouse brain. Many peptides were greatly elevated in the *Cpe^{fat/fat}* mice; these peptide processing intermediates with C-terminal Lys and/or Arg were generally not detectable in wild type mice. Taken together, these results indicate that CPE contributes, either directly or indirectly, to the production of the majority of neuropeptides.

Keywords

Peptide processing; Neuropeptide biosynthesis; Carboxypeptidase E; Carboxypeptidase D; Prohormone convertase; ProSAAS

INTRODUCTION

Neuropeptides are secreted from cells in the central nervous system and bind to extracellular receptors, functioning in a wide variety of physiological processes including feeding, body weight regulation, pain, memory, circadian rhythms, depression, anxiety, reproduction, and others (Eipper et al. 1986; Strand 2003). In addition to the known neuropeptides, many other peptides are also secreted from neurons and it is likely that some of these are also functional. In the present study, we collectively refer to established neuropeptides, proposed neuropeptides, and other similar peptides as “secretory pathway peptides” to simplify the

terminology and to distinguish them from peptides arising from the degradation of cytosolic proteins.

Secretory pathway peptides are produced from the selective cleavage of proteins at specific sites (Mains et al. 1990; Seidah and Chretien 1997; Zhou et al. 1999). Often, the cleavage sites contain basic amino acids, usually in pairs, with Lys-Arg, and Arg-Arg the most common (Lindberg and Hutton 1991). For some peptides, the cleavage sites contain a single basic residue although there is often (but not always) another basic residue in the -4 or -6 position (Devi 1991). Endopeptidases that cleave peptide precursors at basic sites have been identified and named proprotein/prohormone convertases (PCs). PC1 (also known as PC3) and PC2 are the major PCs present within mature secretory vesicles (Seidah and Chretien 2004a; Seidah and Chretien 2004b). Other related endopeptidases, such as furin, are located in the trans Golgi network and immature secretory vesicles (Nakayama 1997). The PCs cleave peptide precursors on the C-terminal side of the basic residues. Because the mature forms of most peptides lack these basic residues, a carboxypeptidase is usually required to produce the bioactive form of the peptide.

Carboxypeptidase E (CPE) was the first secretory pathway carboxypeptidase identified (Fricker and Snyder 1982). Like PC1 and PC2, CPE is enriched in mature secretory vesicles (Figure 1). Although CPE is also present in the trans Golgi network and immature vesicles (Varlamov and Fricker 1996), the internal pH of these compartments is not in the optimal range for CPE, which is maximally active at pH 5.0–5.5 and has <1% activity at pH $\geq$ 6.8 (Greene et al. 1992). Thus, the acidification of the vesicles as they proceed from immature to mature vesicles serves to activate CPE. In addition to CPE, carboxypeptidase D (CPD) is also present in the secretory pathway where it is enriched in the trans Golgi network (Eng et al. 1999; Varlamov and Fricker 1998). CPD is also detected in immature secretory vesicles, but is undetectable in mature vesicles (Varlamov et al. 1999b; Varlamov et al. 1999a). The pH optimum of CPD is quite broad and the enzyme is active over the 5–7 range due to the presence of 2 active subunits, each with a different pH optimum (Eng et al. 1998; Novikova et al. 1999). Additional carboxypeptidases are known to transit the secretory pathway, but are not generally thought to function within the cells (Novikova et al. 2000; Wei et al. 2002). Some secretory pathway peptides undergo additional post-translational modifications, many of which are essential to their bioactivity or stability such as C-terminal amidation, sulfation of Tyr, phosphorylation of Ser or Thr, acetylation on the N-terminal amine, pyroGlu (produced from N-terminal Gln), and N- and O-linked glycosylation (Beisswanger et al. 1998; Bennett 1991; Eipper and Mains 1988; Pohl et al. 1991).

In addition to the pathway for the production of secretory pathway peptides described above, other peptidases have been proposed to function in the secretory pathway. Cathepsin L has been detected in secretory vesicles, and studies of cleavage of peptide precursors with this enzyme have found that cleavage occurs at pairs of basic amino acids, as with the PCs, except that cathepsin L cleaves to the N-terminal side of the basic residues (Hook et al. 2008; Hwang et al. 2007a). Thus, cleavage by this enzyme would generate the intact C-terminus of the mature peptide, but the intermediate would contain N-terminal basic residues; these could be removed by aminopeptidase B, which has also been reported to be present in secretory vesicles (Hwang et al. 2007b). In addition, endothelin converting enzyme 2 has been detected in secretory vesicles and proposed to function in the processing of neuropeptides; this enzyme has an acidic pH optimum, consistent with the pH of the mature vesicles, and cleaves a number of neuropeptides and neuropeptide processing intermediates at a variety of sites (Ahn 1998; Emoto and Yanagisawa 1995; Mzhavia et al. 2003).

Mice lacking several of the peptide processing enzymes described above have been studied; the analysis of peptides in these mice reveals the precise role of each processing enzyme

(Scamuffa et al. 2006). In many cases, there is redundancy in the system. For example, mice lacking PC2 have normal levels of many peptides, including some that are clearly PC2 substrates *in vitro* (Furuta et al. 1997; Furuta et al. 1998; Furuta et al. 2001; Pan et al. 2006). In the present study, we used a mass spectrometry-based peptidomics technique to examine peptides in mice lacking CPE activity due to a point mutation in the *cpe* gene. These mice, known as *Cpe^{fat/fat}* mice, arose spontaneously in the early 1970's and the gene defect was identified in 1995 (Naggert et al. 1995). The defect is due to a point mutation that converts Ser²⁰² into a Pro, and this change causes CPE to be inactive (Varlamov et al. 1996). *Cpe^{fat/fat}* mice are obese, and display age-dependent infertility, hyperglycemia, and other behaviors (Fricker and Leiter 1999; Srinivasan et al. 2004). Previous studies investigated peptide levels in these mice using radioimmunoassays, and found decreased levels of the mature forms of several neuropeptides (Cain et al. 1997; Fricker et al. 1996; Naggert et al. 1995; Rovere et al. 1996; Udupi et al. 1997). However, most radioimmunoassays are not specific for a single form of a peptide and cross react with N- or C-terminally extended peptides. Mass spectrometry-based techniques allow for the identification of the precise molecular form of a peptide (Baggerman et al. 2004; Falth et al. 2007; Hummon et al. 2006; Svensson et al. 2007b). Previous studies examined peptides in the *Cpe^{fat/fat}* mice using an affinity column to isolate peptides containing C-terminal basic residues followed by mass spectrometry-based sequencing (Che et al. 2001; Che and Fricker 2002). While these studies revealed a large number of peptide-processing intermediates, they did not provide a reflection of the relative levels of the mature forms of peptides in *Cpe^{fat/fat}* mice, relative to wild type (WT) mice. Recently, we developed a peptidomics technique that permits the analysis of peptides in WT mice and the relative quantification of peptide levels using stable isotopic tags that label N-terminal amines (Che et al. 2005b; Che et al. 2005a; Che et al. 2007; Fricker et al. 2006). This technique can detect both known and novel peptides, unlike radioimmunoassay that are limited to known peptides, and was previously used to examine peptides in WT and *Cpe^{fat/fat}* mouse pituitary and prefrontal cortex (Che et al. 2005a; Lim et al. 2006).

The major purposes of the present study were to examine peptides in various brain regions of WT mice to establish a database of peptides that can be detected with the peptidomics technique, and then to compare the peptide levels in CPE-deficient mice versus WT mice to determine the precise role of CPE in peptide processing. Our findings suggest that CPE is the major carboxypeptidase processing enzyme for the vast majority of secretory pathway peptides, but is not involved in the production of all peptides.

EXPERIMENTAL PROCEDURES

Materials

Hydrochloric acid (6N, sequanal grade, constant boiling), trifluoroacetic acid, and formic acid were purchased from Pierce (Rockford, IL, USA). Acetonitrile of HPLC grade was obtained from Fisher Scientific (Fair Lawn, NJ). Dimethylsulfoxide, sodium hydroxide, sodium phosphate, glycine, and hydroxylamine hydrochloride were obtained from Sigma-Aldrich, Inc. (St Louis, MO, USA). Water was purified with a Milli-Q system (Millipore, Bedford, MS, USA). The stable isotopic labeling reagents, H₉ and d₉-forms of 3-(2,5-dioxopyrrolidin-1-yl)oxycarbonylpropyl trimethylammonium chloride, also known as H₉ and d₉-trimethylammonium butyrate (abbreviated as H₉-TMAB and D₉-TMAB), were synthesized and used for the labeling of endogenous brain peptides as described previously (Che and Fricker 2005).

Mice

C57BKS/J *Cpe^{fat/fat}* and wild type mice were bred in the barrier facility of the Albert Einstein College of Medicine. Breeding pairs of mice heterozygous for the *fat* mutation were used to

produce the homozygous *Cpe^{fat/fat}* mice and their wild type littermates; genotyping was done as previously described (Maddatu and Naggert 1997). Adult mice (age range 8–18 weeks) of both genders were used, but the two groups that were compared using the isotopic labels were always age- and gender-matched with each other. Altogether, 54 mice were used and divided into 18 groups of 3 mice/group. Five of these groups were composed of *Cpe^{fat/fat}* mice, and after extraction of the peptides (described below) the samples were labeled with either H₉-TMAB or D₉-TMAB and then combined with peptides extracted from age- and gender-matched wild type mice that were labeled with the opposite TMAB reagent (i.e. in each set, one group was labeled with H₉-TMAB and the other group with D₉-TMAB). The combined H₉- and D₉-TMAB labeled samples were subsequently analyzed by HPLC and MS, as described below. The other 8 groups consisted entirely of wild type mice, and after extraction of the peptides, 4 of the groups were labeled with H₉-TMAB and the other 4 with D₉-TMAB. Each of the H₉-TMAB groups was combined with an age- and gender-matched D₉-TMAB group and subsequently analyzed by LC/MS as described below. For some of the brain regions, the labeling and subsequent analysis was performed on extracts from all of the above groups of mice, but for other brain regions only some of the samples were extracted and analyzed (see Supplement for a diagram showing the typical labeling scheme and the number of replicates of each scheme analyzed for the various brain regions).

The mice were sacrificed by decapitation and the head was immediately irradiated in a conventional microwave oven for 8 seconds; this has previously been shown to raise the brain temperature to 80°C (Che et al. 2005b). After cooling, the brain was removed and cut with a razor blade into coronal sections for subsequent dissection. First, the prefrontal cortex was removed by cutting at Bregma 1.94 (Paxinos and Franklin 2001). Then additional coronal cuts were made on either side of the hypothalamus at Bregma 0.00 and –3.00 (Paxinos and Franklin 2001). The striatum was dissected from the 1.94 to 0.00 section by removing the cortex. This “striatum” sample includes the caudate putamen, nucleus accumbens, septum, and ventral pallidum. The coronal section of Bregma 0.00 to –3.00 was dissected into the hippocampus, thalamus, amygdala, and hypothalamus. Tissue from three animals was pooled, frozen in dry ice, and stored at –70°C until analysis.

Peptide extraction and isotopic labeling

The dissected tissue pools were sonicated two times for 20 sec at 1 pulse/sec in 600 µl ice-cold water using an ultrasonic processor (W-380, Ultrasonic Inc., Farmingdale, NY, USA). The homogenates were incubated in a 70°C water bath for 20 min and then cooled on ice and acidified with 0.1 M HCl to a final concentration of 10 mM HCl. The homogenates were centrifuged at 13 000 g for 40 min at 4°C and the supernatant was then transferred to a low-retention tube, frozen, and concentrated to 700 µl in a vacuum centrifuge. The pH of the peptide extracts were adjusted to 9.5 by the addition of 0.4 M phosphate buffer (pH 9.5).

The labeling reagents, H₉-TMAB (FW 278) or D₉-TMAB (FW 287), were dissolved in DMSO at 350 µg/µl and used to label either *Cpe^{fat/fat}* or wild-type mouse brain tissue extracts, as described previously (Che and Fricker 2005). Briefly, 6.4 µl of labeling reagent solutions were added separately to each tissue extract and incubated for 10 min at room temperature. Then an appropriate volume of 1.0 M NaOH was added to adjust the pH back to 9.5 and the sample extract solution was incubated another 10 min at room temperature. These two steps were repeated seven times to ensure all the peptides were completely labeled and then the mixture was incubated at room temperature for 40 min, followed by the addition of 20 µl of 2.5 M glycine to quench the remaining TMAB reagents. After labeling, H₉- and D₉-TMAB labeled sample pairs were pooled and filtered through a Microcon[®] YM-10 unit (Millipore) to remove proteins >10 kDa. To remove any labels from Tyr residues in the peptides, the filtrate was adjusted to pH 9.0 and 2.0 M hydroxylamine (in DMSO) was added in three aliquots of 3.3

μl each, with an interval of 10 min between each addition. After desalting with a PepClean™ C-18 spin column (Pierce), peptides were eluted out of the column with 80 μl of 70% acetonitrile and 0.1% trifluoroacetic acid in water, frozen, and concentrated to 20 μl in a vacuum centrifuge. Aliquots of the peptide samples were stored at −70°C until further MS analysis.

Mass spectrometry

To detect and quantify the labeled peptides from *Cpe^{fat/fat}* and wild-type mouse tissues, nano-LC/MS/MS analysis was performed on an API Q-Star Pulsar-i™ quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with a nanoelectrospray ionization source. Peptide samples were first dissolved in 2% acetonitrile and 0.1% formic acid, concentrated on a PepMap™ C18 trapping column (5 μm, 100Å, 300 μm i.d. × 5 mm, LC Packing, Marlton, NJ, USA) and desalted for 20 min with 2% acetonitrile in 0.1% formic acid. Then the peptides were separated on a Vydac MS C18 capillary column (3 μm, 100Å, 75 μm i.d. × 150 mm, Hesperia, CA, USA) with a gradient elution from 2% to 52% solvent B in 45 min, then to 90% solvent B in 20 min at a flow rate of 0.2 μL/min. Solvent A was 2% acetonitrile in 0.1% formic acid. Solvent B was 80% acetonitrile in 0.1% formic acid. A metal-coated fused SilicaTip (with 10 ± 1 μm tip; PicoTip EMITTER, New Objective, Woburn, MA, USA) was used as a nanospray needle. The needle voltage was set at 2 kV. MS/MS was done in the information-dependent acquisition mode. MS survey time and MS/MS accumulation time were 1 and 2 s, respectively. The most intense ion in each MS survey scan was fragmented to generate MS/MS spectra. The dynamic exclusion time for fragmented ions was 240 s. The collision energy was in the 20–45 eV range and dynamically changed based on the m/z value and charge state of the ion.

Data analysis

To identify peptides, multiple strategies were employed. The Mascot program (<http://www.matrix-science.com>) was used to search the MS/MS data for matches to the mouse MSDB database. In Mascot searching, fixed modifications were selected as either GIST-Quat or GIST-Quat:2(H)9 at the N-terminus and on all Lys residues. To reduce the number of false positives, all Mascot hits were manually interpreted and discarded if they did not match the following criteria: (i) the peptide mass had to be within 40 ppm (preferably 20 ppm) of the theoretical mass; (ii) the observed number of TMAB tags on the peptide matched the predicted number of free amines available (i.e., lysine residue and N-terminus); (iii) the observed charge state(s) of the peptide was consistent with the expected number of positive charges; (iv) 80% or more of the major fragments observed in MS/MS matched (within 40 ppm) to predicted fragmentation ions; and (v) at least five fragmentation matched (within 40 ppm) to either b- and/or y-series ions.

Mascot was not able to identify some MS/MS spectra that were of medium-high quality. To identify these, three different strategies were used to get tentative hits (described below), and then these hits were manually evaluated by the above criteria. If the vast majority of MS/MS fragments could be assigned to the peptide, and all other criteria described above also matched, the peptide was considered identified. To obtain the tentative hits for this manual interpretation, one approach involved searching the Swepep database (<http://www.swepep.org/>) for known neuropeptides with the observed monoisotopic mass (±40 ppm). A second approach used the MassXpert program to search a database of all known mouse neuropeptide precursors and related secretory pathway proteins for a peptide with the observed mass, the observed number of free amines (i.e. number of isotopic tags incorporated), and the observed number of basic residues (i.e. the charge state). The third approach involved a bioinformatics search of all known and hypothetical proteins downloaded from GenBank. A hidden Markov model (HMM) based signal sequence predictor was trained on the list of sequences from SwissProt version 40 (Nielsen et al. 1997), essentially the same list that was used for SignalP training

(<http://www.cbs.dtu.dk/services/SignalP-3.0/background/dataset.php>). The HMM was used to process all the protein sequences and label signals using a threshold that results in a detection operating point close to that of HMM SignalP (Nielsen and Krogh 1998). After filtering out proteins without a signal sequence, peptide sequences were identified with the observed monoisotopic mass (± 40 ppm) that occurred between basic residues (Lys and/or Arg), between the end of the signal sequence and a basic residue, or between a basic residue and the end of the protein. In addition, all peptides containing a Gly prior to a Lys or Arg were calculated to terminate with an amide on the residue prior to the Gly. Peptides of the appropriate mass were further screened for the presence of the observed number of free amines (i.e. number of isotopic tags incorporated), and the observed number of basic residues (i.e. the charge state).

Some peptides that were found to reproducibly decrease in the *Cpe^{fat/fat}* mice (based on the MS spectra) either had no MS/MS data or the MS/MS spectra were too weak to provide a conclusive assignment. If the above search strategies found a peptide with a mass within 40 ppm of the theoretical mass that also had the correct number of free amines and positive charges, further analysis was performed. The Allen Brain Atlas (<http://brain-map.org>) was searched to see if the precursor was known to be expressed in the appropriate mouse brain regions. If so, then the precursor sequence was examined for the presence of a prohormone convertase cleavage site on at least one of the sides of the observed peptide (<http://neuroproteomics.scs.uiuc.edu/neuropred.html>). If these additional criteria also matched, then the predicted peptide was listed as potentially identified in Table 1 (i.e. with parentheses surrounding the sequence).

Relative levels of peptides in *Cpe^{fat/fat}* versus wild-type mouse brain regions were determined by calculating the ratio of peak intensity of the D₉-TMAB-labeled (heavy form) versus H₉-TMAB-labeled (light form) peptide pairs, as described previously (Che et al. 2005a; Che and Fricker 2002; Che and Fricker 2005; Lim et al. 2006). Statistical significance was calculated using Student's two-tailed T-test to compare the ratio of each peptide determined from *Cpe^{fat/fat}* versus wild type mouse LC/MS runs to the ratio of the peptide in the wild type versus wild type LC/MS runs.

RESULTS AND DISCUSSION

There were two related goals of this study: one to determine the precise forms of peptides present in various brain regions of WT mice, the second to examine the levels of these peptides in *Cpe^{fat/fat}* mice in order to learn whether CPE activity is required for their biosynthesis. For both of these analyses, we focused on peptides that arise from neuropeptide precursors and other secretory pathway proteins. In addition to the large number of secretory pathway peptides identified in this study (described below) we also detected a comparable number of peptide fragments of cytosolic proteins. These cytosolic protein fragments were not included in the present analysis; all were present in *Cpe^{fat/fat}* mice in levels similar to those in WT mice (data not shown), as previously reported (Che et al. 2007; Lim et al. 2006).

In the present study, over 100 secretory pathway peptides were identified by MS/MS sequence analysis and the other criteria (i.e. correct mass, number of free amines, and charge, and protein known to be present in secretory pathway in brain). The MS/MS sequence analysis of native peptides labeled with the TMAB isotopic tags is not as straightforward as sequence analysis of unlabeled tryptic peptides. The latter are usually 2+ or 3+ ions, whereas native peptides range from 1+ to 7+ (Table 1) and the higher charge states are more difficult to interpret (and usually not possible to interpret with computer programs like Mascot). Also, the TMAB tags used in the present study for quantitative peptidomics often, but not always, show decomposition upon collision-induced dissociation, losing the trimethylamine group. Because the Mascot program only considers the decomposed product, fragments of peptides that retain

the trimethylamine group were unmatched by Mascot and therefore given low scores due to the number of unmatched ions. Therefore, it was important to manually check the MS/MS sequence of every peptide. In addition to requiring that >80% of the ions in the MS/MS spectra matched the predicted fragment ions, our criteria also included that the number of tags incorporated into the peptide matched the predicted number based on the free amines (Lys residues and N-terminus, if free and not pyroGlu or acetylated). Furthermore, the observed charge state of the peptide had to generally agree with the number of positive charges expected on the peptide (isotopic tags plus Arg and His residues). Finally, the assignment matched a product of a secretory pathway protein known to be present in that brain region, based on the literature and/or the distribution of the precursor mRNA in the Allen Brain Atlas (<http://brain-map.org>).

Not all of the peptides detected in the present study provided suitable MS/MS analysis for conclusive identification. Some of these peptides were tentatively identified based on several criteria. First, the observed mass had to match the theoretical mass of a known secretory pathway peptide (within 40 ppm). In addition, the observed charge state and number of free amines (i.e. the number of isotopic tags incorporated) had to match the values predicted for that peptide, as mentioned above. In contrast to the criteria when MS/MS sequence was available, when a sufficient MS/MS sequence was not available we required the additional criteria that a known or predicted endopeptidase cleavage site was present on at least one side of the peptide.

Altogether, 158 peptides from 32 precursors were detected in the present study. In general, the precursors that are known to be abundant in brain showed a large number of peptide fragments (Table 1). For some precursors, all or nearly all of the peptides derived from the precursor were detected. Proneuropeptide Y is cleaved at a single pair of basic residues to yield neuropeptide Y and a C-terminal fragment; both were detected (Table 1). For all other precursors, the coverage was less than 100%. In most cases, the “missing” peptides were larger or smaller than the 500–5000 Da range that is optimal for the mass spectrometer used in the present study (Figure 2). In other cases, peptides were not detected because they did not contain a free amine, which was required for the isotopic labeling and subsequent analysis. For example, the N-terminal fragment of procholecystokin was not detected as an isotopic peak pair (Table 1, Figure 2); this peptide does not have Lys residues and there is a Gln residue on the N-terminus following removal of the signal peptide (N-terminal Gln is usually converted into a pyroGlu residue). In all cases where the peptide had a C-terminal amide group, the precursor contained a Gly on the C-terminus (Table 1); this is consistent with the known enzymatic reaction that produces C-terminal amides (Eipper and Mains 1988; Prigge et al. 2000).

For most peptides that were detected in multiple brain regions, the difference between levels in *Cpe^{fat/fat}* and WT mouse brain observed in one brain region were comparable to the differences in other brain regions. Therefore, Table 1 shows the average level for all brain regions; the full data are included in the supplemental Table. Many peptides were below the detection limit in one or more tissues, and so to determine the average level among tissues, the detection limit was used and the overall average is indicated in Table 1 as “less than” the resulting average.

The peptides can be roughly divided into four groups based on the differences between *Cpe^{fat/fat}* and WT brain levels. One group of peptides is decreased more than 70% in the mutant mice (*Cpe^{fat/fat}* to WT ratios less than 0.30). Another group of peptides is also decreased in the *Cpe^{fat/fat}* mice, but to a lesser extent (20–70% decrease, ratios between 0.80 and 0.30). A third group of peptides is not affected by the *fat* mutation (ratios between 0.80 and 1.10). A fourth group of peptides is elevated in the *Cpe^{fat/fat}* mice (ratios ≥ 1.75). In contrast, all but a handful of peptides in the comparison of WT and WT groups of mice fell within 25% of the expected

1.00 ratio (i.e. 0.75 to 1.25) and the few exceptions were all proopiomelanocortin-derived peptides; this precursor is part of the stress-response pathway and the variation among levels of some of these peptides in WT animals may therefore reflect stress-induced changes.

The majority (~75%) of the secretory pathway peptides detected in WT mice are greatly affected by the *fat* mutation. The simplest interpretation of the data is that for these peptides, CPE is directly involved in their biosynthesis, and other enzymes (CPD), or other pathways that do not require a carboxypeptidase (cathepsin L) do not contribute much to the production of these peptides. Most of the peptides greatly affected by the *fat* mutation have C-terminal flanking sequences of one or two basic residues, or in the case of amidated peptides, a Gly residue followed by basic residues (Table 1). There are, however, several exceptions where the C-terminal flanking residue is not a basic residue or a Gly followed by basic residues; examples include a series of procholecystokinin peptides (46–58 to 46–63) and proSAAS peptides (PEN 1–18 to 1–20). These are discussed further below.

Approximately 10% of the peptides detected in WT mice show an intermediate degree of decrease in the *Cpe^{fat/fat}* mice. In those cases where the precursor has basic residues that need to be removed after endoprotease cleavage by a PC-like enzyme, the partial reduction of the peptide levels in the *Cpe^{fat/fat}* mice implies that CPD (or another enzyme) contributes substantially to the processing of the peptide. The simplest mechanism for this to happen is for the endopeptidase reaction to begin in the trans Golgi network or immature secretory vesicles where CPD is located (Varlamov et al. 1999b; Varlamov et al. 1999a; Varlamov and Fricker 1998). Two peptides in this category, little SAAS and PEN, are produced from the proSAAS precursor which is known from previous studies to be cleaved into intermediates early in the secretory pathway (Fricker et al. 2000; Mzhavia et al. 2001; Mzhavia et al. 2002). Another peptide in this category is a fragment of 7B2, which is also known to be cleaved early in the secretory pathway (Zhu et al. 1996; Zhu and Lindberg 1995). Other peptides in this category include several without downstream basic residues; examples include several peptides that terminate in Pro-Arg, with no additional basic residues (chromogranin B 357–374; procholecystokinin 43–55), and the C-terminal peptides of provasopressin, prohormone convertase 2, and prodynorphin (Table 1). The changes in the levels of these peptides in the *Cpe^{fat/fat}* mouse brain regions presumably reflect a decrease in endopeptidase activity required for the production of these peptides. This effect is likely to be specific to the neurons and/or secretory vesicles in which these peptides are produced because only selected peptides are affected. Previous studies have found an accumulation of prohormones such as proinsulin and promelanin concentrating hormone in the *Cpe^{fat/fat}* mouse, indicating that some endopeptidase reactions proceed more slowly in absence of CPE (Naggert et al. 1995; Rovere et al. 1996). A likely mechanism is product inhibition of the PCs, either by normal neuropeptide-processing intermediates or by specific inhibitors of PC1 and PC2 such as proSAAS and 7B2, respectively (Fricker et al. 2000; Martens et al. 1994). For example, the inhibition of PC1 by proSAAS and proSAAS processing intermediates requires the presence of a Lys-Arg sequence on the C-terminus of the peptide named PEN, and a carboxypeptidase is therefore required to inactivate the inhibitor (Sayah et al. 2001). Alternatively, the products of typical PC substrates are also inhibitory towards these enzymes and the presence of a carboxypeptidase in the PC reaction leads to increased endopeptidase activity (Day et al. 1998).

Approximately 10% of the peptides detected in the present study were present in the *Cpe^{fat/fat}* mouse brain regions in levels comparable to the WT mouse brain (Table 1). Some of the peptides in this group represent the C-terminal region of their peptide precursor and are therefore not expected to require any carboxypeptidase for their biosynthesis. Other peptides in this group include cerebellin 1 and related peptides (Table 1). Cerebellin 1 is produced from its precursor by cleavage following an N-terminal Arg and a C-terminal His residue. However, multiple forms were detected lacking the N- and/or C-terminal residues, reflecting either

alternative cleavage by endopeptidases or by amino- and carboxypeptidases. Related peptides derived from cerebellin 2 and 4 precursor proteins were also found in the present study. The function of these peptides is not known, although the presence of signal peptides in their precursors indicates that these peptides will be secreted from cells.

One of the peptides present in comparable levels in *Cpe^{fat/fat}* and WT mice was big SAAS, a product of proSAAS processing that was expected to require CPE and/or CPD for its production. As noted above, pulse-chase studies of proSAAS have shown that this precursor is rapidly processed early in the secretory pathway into intermediate-sized peptides (Fricker et al. 2000). Although the previous studies did not identify the exact cleavage sites used in the early processing steps, the size and immunoreactivity of the intermediates suggested cleavage of big SAAS was one of the early steps (Fricker et al. 2000). Thus, the observed result is consistent with, and extends the previous observations. The cleavage site in proSAAS that generates big SAAS (RLRR-A, with cleavage between the R and A) matches the consensus site for furin and related trans Golgi network endoproteases (Nakayama 1997).

A large number of peptides were present in *Cpe^{fat/fat}* mice and not detected in WT mouse brain; a representative peptide is indicated in Figure 3. All of the peptides that increased dramatically in the *Cpe^{fat/fat}* mice and which were not found in the WT mice corresponded to peptide processing intermediates containing C-terminal Lys and/or Arg residues (data not show), consistent with previous studies that found greatly elevated levels of peptide-processing intermediates in *Cpe^{fat/fat}* mice (Che et al. 2001; Che et al. 2005a; Lim et al. 2006). Only one Arg-extended peptide processing intermediate was detected in WT mouse brain extracts: the Arg-extended form of the proenkephalin-derived peptide metorphamide, which is elevated approximately 600% in the *Cpe^{fat/fat}* mice (Table 1). Previous studies found that CPE removes C-terminal Lys or Arg residues from nearly all peptides examined, although peptides with Pro or Gly in the penultimate position are cleaved relatively slowly by this enzyme (Chen et al. 2001; Novikova et al. 1999). The detection of Gly-Arg-extended metorphamide in the WT mouse brain is consistent with these previous studies.

Three peptides were slightly elevated in the *Cpe^{fat/fat}* mice, relative to WT mice: all represent the C-termini of their precursors either directly (big LEN) or after the removal of a small number of residues (big LEN lacking a C-terminal Pro, and the C-terminal peptide of protachykinin A lacking four basic residues). Protachykinin A contains three Arg residues and one Lys residue on the C-terminus, and these would be removed rapidly by CPD in the *trans* Golgi network. The increase in big LEN and the protachykinin C-terminal peptide observed in *Cpe^{fat/fat}* mice presumably reflects an increase in the production of their precursors. Interestingly, the apparent increase in production of proSAAS is not the same in all brain regions; levels of big LEN in the amygdala, hippocampus, and prefrontal cortex are only 30–50% elevated in the *Cpe^{fat/fat}* mice whereas levels in the hypothalamus, striatum, and thalamus are approximately 100% higher (supplemental table). The upregulation of protachykinin is also region specific, with an 18% increase in prefrontal cortex, a 79% increase in striatum, and a 164% increase in hypothalamus (supplemental Table 1).

In addition to those peptides identified by MS/MS or tentatively identified by the match to a predicted secretory pathway protein, a number of other peptides were detected in the MS mode but did not have suitable MS/MS spectra for identification and did not match the above criteria for tentative matches to predicted peptides. Twenty one of these peptides were decreased in *Cpe^{fat/fat}* mouse brain regions, relative to WT mouse brain regions, suggesting that they arise from secretory pathway proteins. These 21 peptides, which are included in the supplemental table, may represent novel fragments of previously known secretory pathway proteins or novel post-translational modifications (only amidation was included in the searches for all peptides). Alternatively, they may represent products of proteins not included in our searches. Some of

these “unknowns” appear to be broadly distributed; two of the peptides (1151.67 and 1178.15) were detected in all six of the brain regions analyzed, and another two (1822.01 and 1980.01) were detected in four of the six brain regions (see supplemental table).

Most, but not all of the peptides identified in the present study have been previously found in peptidomics studies of rodent brain (Che et al. 2005b; Che et al. 2007; Dowell et al. 2006; Falth et al. 2007; Lim et al. 2006; Skold et al. 2002; Svensson et al. 2003; Svensson et al. 2007b; Svensson et al. 2007a). Peptides identified in the present study by MS/MS sequencing that have not been previously reported, to the best of our knowledge, include chromogranin B 313–330, 357–374 with and without a phosphoSer, and 64–86; procholecystokinin 56–63, 46–60, 46–61, and 72–94; prothyrotropin releasing hormone 161–169, 63–74, 95–106, and 57–74; and secretogranin III 23–36. All of these peptides have consensus sites for PC cleavages on at least one side and show large decreases in the *Cpe^{fat/fat}* mice (Table 1). N- and C-terminally truncated forms of cerebellin 1 (cerebellin 2–15) and also related peptides from the cerebellin 2 and 4 precursors also appear to be novel peptides (Table 1). Another novel peptide found in the present study is the N-terminal peptide of the cystatin C precursor. Unlike the precursors discussed above, the cystatin C precursor is not generally thought to be routed into the regulated secretory pathway or processed, and is a serum marker for various disease states. Cystatin C was recently reported to be present in neuroendocrine secretory vesicles (Wegrzyn et al. 2007), and the present finding supports and extends this observation to include the processing of the protein at an Arg-Arg near the N-terminus. Two additional pairs of basic residues (Arg-Lys) are present in the protein, but fragments resulting from cleavage at these sites were not detected in the present study. It is possible that the cleaved N-terminal fragment of the cystatin C precursor functions as a neuropeptide, although this sequence is not highly conserved among mammals which suggests it is not functional.

As mentioned above, the vast majority of the peptides that are substantially lower in the *Cpe^{fat/fat}* mice have a Lys or Arg immediately downstream of the peptide, or are amidated and therefore contain a Gly followed by a Lys or Arg residue (Table 1). There are, however, several exceptions. The procholecystokinin fragments 46–60 through 46–63 are all much lower in the *Cpe^{fat/fat}* mice, but only the longest of these peptides has a downstream basic residue (Table 1). This implies that the shorter peptides are derived from the 46–63 peptide, and not directly from a precursor such as the 46–64 peptide that contains a C-terminal Arg and is the immediate product of PC cleavage of the precursor (and is present in the *Cpe^{fat/fat}* mice at elevated levels; data not shown). The simplest interpretation is that the Arg-extended peptide (46–64) is processed by CPE into the 46–63 peptide, and this then undergoes additional cleavages by another carboxypeptidase that is able to remove C-terminal Ala and Leu. Another peptide precursor shows similar processing at a related sequence; the proSAAS-derived PEN 1–20 is trimmed by a carboxypeptidase that removes C-terminal Leu residues to produce PEN 1–19 and PEN 1–18 (Table 1 and Figure 2). Because the shorter fragments of PEN are also much lower in the *Cpe^{fat/fat}* mice than in WT controls, it is clear that CPE is involved in their biosynthetic pathway, presumably by removing the Arg from PEN 1–21 (i.e. the predicted product of endopeptidase cleavage of the precursor). The shorter peptides therefore are more likely to arise from carboxypeptidase trimming than by direct endopeptidase action. It is interesting that both PEN 1–20 and procholecystokinin 46–62 have a Leu-Gly-Ala-Leu-Leu sequence at the C-terminus. Previous studies using an antiserum raised against PEN found several forms of this peptide in brain extracts; one of these forms co-eluted with full length PEN but the other forms eluted earlier from a reverse phase column and were generally comparable in abundance to PEN (Mzhavia et al. 2001; Mzhavia et al. 2002). Taken together, it is likely that the C-terminally truncated forms of PEN represent major forms of this peptide in brain, and because of the similar C-terminal sequence with the procholecystokinin peptides it is conceivable that these forms have biological activity. The enzyme(s) responsible for the formation of the truncated forms of PEN and the procholecystokinin peptides are not known,

but several carboxypeptidases with predicted selectivity for aliphatic/aromatic residues have been detected in brain (Fontenele-Neto et al. 2005; Normant et al. 1996; Turner et al. 2002; Wei et al. 2002; Wei et al. 2003).

A central question in the field is “why does the *fat* mutation cause the mice to be overweight?” This phenotype is also seen when the CPE gene is disrupted, indicating that it is not due to the point mutation but rather to the absence of CPE activity (Cawley et al. 2004). Many peptides are involved in body weight regulation, but while some peptides lower body weight others function in the opposite direction and therefore it was not clear why the absence of CPE would have a net positive effect on body weight (Che et al. 2005c; Fricker 2007; Hillebrand et al. 2002; Williams et al. 2000; Woods et al. 2000). Based on the results of the present study together with previous studies using radioimmunoassays, it is possible to speculate on the reasons for the increase in body weight. Several peptides are known to decrease body weight and many of these are greatly reduced in the *Cpe^{fat/fat}* mouse brain. For example, levels of α -melanocyte stimulating hormone, neurotensin, and peptides derived from Cocaine- and Amphetamine-Regulated Transcript (CART), prothyrotropin, and procholecystokinin are much lower in *Cpe^{fat/fat}* mouse brain compared to wild type mouse brain (Table 1). In contrast to the peptides that decrease body weight, some of the hypothalamic peptides that increase body weight are present at normal levels in *Cpe^{fat/fat}* mouse brain. For example, enkephalin heptapeptide and β -endorphin 1–31 are present in the *Cpe^{fat/fat}* mouse hypothalamus at levels close to those in WT mice (Table 1), and melanin concentrating hormone was previously reported to be elevated in these mice (Rovere et al. 1996). All three of these peptides are present on the C-terminus of their precursors and do not require a carboxypeptidase for their production. Several proSAAS-derived peptides are also detected at either WT levels, or at elevated levels in the *Cpe^{fat/fat}* mice (Table 1); proSAAS has been implicated in body weight regulation because transgenic mice overexpressing this protein are overweight (Wei et al. 2004). Although not all peptides that increase body weight are normal or elevated in the *Cpe^{fat/fat}* mouse brain, the overall balance in the *Cpe^{fat/fat}* mouse is tipped towards increasing body weight due to the severe effect of the mutation on the body weight lowering peptides.

In conclusion, the major finding of the present study is that CPE activity is required for the biosynthesis of the vast majority of secretory pathway peptides. CPE was previously proposed to be a neuropeptide processing enzyme based on its localization to the peptide-containing secretory vesicles and its ability to remove C-terminal Lys and Arg residues from most peptides tested (Fricker 1988). However, final proof of an enzyme’s physiological role requires showing altered levels of products in a biological system from which the enzyme activity has been removed. While previous studies have examined peptides in selected brain regions of the *Cpe^{fat/fat}* mouse using radioimmunoassay or peptidomic techniques, the present report is the most comprehensive study and firmly establishes CPE’s role as a peptide processing enzyme in multiple brain regions. The present study also argues against a major contribution from alternative pathways that do not require a carboxypeptidase step, such as the putative cathepsin L/amino peptidase B pathway (Hwang et al. 2007a; Hwang et al. 2007a).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CART	cocaine- and amphetamine-regulated transcript
CPE	carboxypeptidase E
CPD	carboxypeptidase D
HMM	hidden Markov model
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PC	prohormone convertase
TMAB	trimethylammonium butyrate
WT	wild type

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Figure 1.

Diagram of typical processing of peptide precursors by selective peptidases, and model of the intracellular location of these enzymes. Left: Typical peptide precursors contain multiple sites for proteolytic processing that are usually pairs of basic amino acids, with Lys-Arg (KR) and Arg-Arg (RR) the most common. These sites (as well as sites with single basic residues) are initially cleaved by an endopeptidase such as prohormone convertase 1 or 2, which cleaves to the C-terminal side of the basic residue. A carboxypeptidase is then required to remove the C-terminal basic residues from the processing intermediates. On occasion, additional post-translational processing (amidation, sulfation, and others) is required for biological activity (not shown). Right: Carboxypeptidase D (CPD) is enriched in the trans Golgi network and immature secretory vesicles, along with endopeptidases such as furin (not shown). However, CPD is retrieved from the immature vesicles to the trans Golgi network and is not detected in mature secretory vesicles. In contrast, carboxypeptidase E (CPE) is present throughout the secretory pathway but is inactive at the neutral pH of the Golgi. CPE is activated by the drop in pH that occurs during the late secretory pathway, and is maximally active in mature vesicles where the pH is typically 5–5.5.

Figure 2.

Selected propeptides and processed peptides identified in the present study in WT mice; some of these peptides were also detected in *Cpe^{fat/fat}* mice, but others were only present at detectable levels in the WT mice (see Table 1). When basic amino acids are present in the cleavage sites, they are indicated above the prohormone. Solid lines indicated peptides identified in the present study. If the peptide has not previously been named, the amino acid location within the precursor is indicated (i.e. as for procholecystokinin); if the peptide has been named, the name is indicated and then shorter fragments of this peptide (if detected) are indicated by the numbering within this peptide. Abbreviations: A8, dynorphin A8; Ac, acetyl; BAM18, bovine adrenal medulla peptide of 18 residues; C-term, C-terminal; DesAc, des-acetyl; End,

endorphin; HP, enkephalin heptapeptide; J-peptide; joining peptide; LE, Leu-enkephalin; MA, metorphamide; ME, Met-enkephalin; MSH, melanocyte stimulating hormone; NE, neoendorphin; and OP, enkephalin octapeptide. Note that ProSAAS and the various names of the proSAAS-derived peptides are not abbreviations.

Figure 3.

Representative MS data showing peptides that are either increased or decreased in *Cpe^{fat/fat}* mice, relative to WT mice. Left panels: Extracts from WT mice were labeled with H₉-TMAB and those from *Cpe^{fat/fat}* mice were labeled with D₉-TMAB. Right panels: The labeling scheme was reversed; extracts from WT mice were labeled with D₉-TMAB while those from *Cpe^{fat/fat}* mice were labeled with H₉-TMAB, as described in Experimental Procedures and also shown in the diagram included as supplemental data. The top panels represent the proenkephalin-derived “octapeptide” with a monoisotopic mass (after subtraction of the TMAB tag and one proton) of 929.45 Da, as determined from MS/MS sequence analysis (data in supplement). This peptide incorporated 1 TMAB group and is doubly charged, with the resulting m/z values of approximately 529.19 and 533.72 for the light and heavy isotopic forms, respectively. Note that levels of this peptide are much lower in the *Cpe^{fat/fat}* mice, as compared to the WT mice. The bottom panels represent the peptide-processing intermediate of the octapeptide (YGGFMRS�KR), as determined by MS/MS analysis, which incorporated 2 TMAB tags and 2 protons to produce m/z ions of 367.91 and 372.43 for the light and heavy isotopic forms, respectively. Note that this peptide cannot be detected in the WT samples.

Table 1
Neuropeptides and other fragments of secretory pathway proteins identified in wild type and/or *Cpe^{fat/fat}* mouse brain.

Precursor and Peptide Name	Peptide Sequence	C-term Flanking	Theor. Mass	Regions	WT:WT Avg±SEM(n)	<i>Cpe^{fat/fat}</i> :WT Avg±SEM(n)
7B2						
167–176	(LLYEKMKGGQ)	RRKRRS	1165.60	A,Hi,Hy,PC,S,T	1.02±0.07(8)	0.58±0.05(4)**
Cerebellin 1 precursor						
Cerebellin 2–15	GSAKVAFSAIRSTN	HEPSEM	1407.75	A,Hi,Hy,PC,S,T	1.06±0.04(8)	0.82±0.03(11)**
Cerebellin 1–15	SGSAKVAFSAIRSTN	HEPSEM	1494.78	A,Hi,Hy,PC,S,T	0.92±0.05(11)	0.94±0.08(14)
Cerebellin 2–16	GSAKVAFSAIRSTNH	EPSEMS	1544.81	A,Hi,Hy,PC,S,T	1.01±0.08(8)	0.88±0.05(10)
Cerebellin	SGSAKVAFSAIRSTNH	EPSEMS	1631.84	A,Hi,Hy,PC,S,T	1.05±.05(10)	1.04±0.08(15)
Cerebellin 2 precursor						
89–902	GSAKVAFSATTRSTN	HEPSEM	1395.71	A,S	0.93±0.09(2)	0.97±0.05(3)
88–902	(SGSAKVAFSATTRSTN)	HEPSEM	1482.74	A,S,T	1.03±0.08(3)	0.81±0.08(6)
89–903	(GSAKVAFSATTRSTNH)	EPSEMS	1532.77	Hi,S	0.92±0.02(2)	0.93±0.07(3)
88–903	(SGSAKVAFSATTRSTNH)	EPSEMS	1619.80	A,Hy,S,T	1.09±0.04(3)	0.89±0.06(6)
Cerebellin 4 precursor						
66–77	SKVAFSAVRSTN	HEPSEM	1265.67	A,Hi,Hy,S,T	1.07±0.05(5)	0.97±0.05(13)
64–77	ANSKVAFSAVRSTN	HEPSEM	1450.75	Hy,St	0.93±0.06(2)	1.15±0.06(4)
63–77	AANSKVAFSAVRSTN	HEPSEM	1521.79	Hy,St,Th	1.06±0.06(3)	1.06±0.03(6)
Chromogranin A						
392–402	AYGFRDPGPQL	RRGWRP	1219.60	A,Hy,PC,S	1.05±0.10(7)	<0.17(10)**
358–371	WSRMDQLAKELTAE	KRLGE	1676.82	A,Hi,Hy,PC,S,T	1.17±0.09(10)	<0.15(13)**
374–390	LEGEDDPPDRSMKLSFRT	RAYGFR	1994.94	A,Hi,Hy,PC,S	1.06±0.06(8)	0.21±0.03(11)**
Chromogranin B						
592–597	(APQLDL)	KRQYDG	655.35	PC	0.91(1)	0.20(1)
438–446	LLDEGHYPV	RESPID	1041.52	A,Hi,Hy,PC,S	1.04±0.06(8)	<0.11(12)**
588–597	SFARAPQLDL	KRQYDG	1116.59	A,Hi,Hy,PC,S	1.08±0.10(8)	<0.15(12)**
457–466	YPOSKWQEQE	KNYLYN	1321.59	PC	0.98±0.08(3)	0.23±0.12(2)**
186–199 + pSer	HIEDpSGEKPNTFESN	KRSEAS	1653.67	PC	0.96±0.18(3)	0.18(1)
600–613	QYDGVAEILDQLLHY	RKKADE	1662.79	A,Hy,PC	0.94±0.08(6)	<0.10(5)**

Precursor and Peptide Name	Peptide Sequence	C-term Flanking	Theor. Mass	Regions	WT:WT Avg±SEM(n)	Cpe ^{full} :WT Avg±SEM(n)
438-453	LLDEGHYPVRESPIDT	AKRYPQ	1839.90	A,Hi,Hy,PC	0.94±0.07(6)	<0.10(9) ^{**}
438-454	LLDEGHYPVRESPIDTA	KRYPQS	1910.93	A,Hi,Hy,PC,S	0.94±0.03(10)	<0.15(15) ^{**}
313-330	PSPKESKEADVATVRLGE	KRSHHL	1911.99	A,Hi,Hy,PC,S,T	1.07±0.05(8)	<0.14(13) ^{**}
357-373	(GLQYRGRGSEEDRAPRP)	RSEESQ	1942.97	Hi	1.09(1)	<0.10(2)
357-373 + pSer	(GLQYRGRGpSEEDRAPRP)	RSEESQ	2022.97	A,Hy,S	1.08±0.05(3)	<0.10(6) ^{**}
357-374	GLQYRGRGSEEDRAPRR	SEESQE	2099.07	A,Hi,Hy,S,T	1.05±0.05(5)	0.62±0.04(13) ^{**}
357-374 + pSer	GLQYRGRGpSEEDRAPRR	SEESQE	2179.07	A,Hi,Hy,PC,S,T	0.96±0.04(8)	0.66±0.03(14) ^{**}
516-535	LGALFNYPFDPLQWKNSDFE	KRGNDP	2400.15	A,Hy,PC,S	1.02±0.07(7)	0.25±0.04(8) ^{**}
64-66	SGKEVKGEKGENQSKFEVRL	RDPADA	2604.35	A,Hi,Hy,S,T	1.18±0.11(5)	<0.10(11) ^{**}
287-310	(RPRHHGRSGNSKSSYEGHPLSEE)	RRPSPK	2740.29	A	0.93(1)	<0.10(2)
Cocaine- and amphetamine-regulated transcript (CART)						
33-40	APGAMLQIEALQEVLLKLL	KSKRIP	1951.12	A,Hy,S	0.97±0.09(4)	<0.10(3) ^{**}
33-32	APGAMLQIEALQEVLLKLLKS	KRIPIY	2166.25	A,Hy,S	1.00±0.05(4)	<0.16(6) ^{**}
Cystatin C precursor						
N-terminal	ATPKQGPRMLGAPEEADANEQGV	RRALDF	2366.11	A Hy,PC,S	0.95±0.05(7)	<0.10(8) ^{**}
Procholecystinin						
56-63	ARLGALLA	RYIQVQ	783.50	A,S	1.10±0.01(2)	<0.10(5) ^{**}
36-64	(AQEAPRRQL)	RAVLR	1067.58	S	1.21(1)	<0.10(2)
46-58 amide	(AVLR TDGEP RARL- amide)	GALLAR	1451.82	A,Hi,S	0.92±0.05(3)	<0.10(6) ^{**}
43-55	(QLRAVLR TDGEP R)	ARLGAL	1509.84	A,S	0.77±0.08(2)	0.51±0.16(4)
46-60	AVLR TDGEP RARLGA	LLARYI	1580.88	A,Hi,Hy,S	1.22±0.17(4)	0.31±0.06(7) ^{**}
46-61	AVLR TDGEP RARLGA	LARYIQ	1693.97	A,Hi,PC,S	1.09±0.08(6)	0.35±0.10(6) ^{**}
46-62	AVLR TDGEP RARLGA	ARYIQQ	1807.04	A,Hi,Hy,PC,S	1.04±0.04(9)	<0.12(10) ^{**}
46-63	AVLR TDGEP RARLGA	RYIQVQ	1878.08	A,Hi,Hy,PC,S	0.96±0.03(7)	0.17±0.02(11) ^{**}
77-94	(MSVLKNIQSLDPSHRISD)	RDYMGW	2039.05	A	0.91(1)	0.29±0.07(2)
72-93	(APSGRMSVLKNIQSLDPSHRIS)	DRDYM	2392.26	A,Hi,S	0.96±0.10(3)	<0.10(6) ^{**}
72-94	APSGRMSVLKNIQSLDPSHRISD	RDYMGW	2507.29	A,Hi,PC,S	0.99±0.08(6)	<0.15(13) ^{**}
71-94	KAPSGRMSVLKNIQSLDPSHRISD	RDYMGW	2635.39	A,Hi,PC,S	1.08±0.05(6)	<0.10(9) ^{**}
46-69	(AVLR TDGEP RARLGAARYIQVQ)	RKAPSG	2665.51	A,Hi,PC,S	1.05±0.05(6)	<0.15(10) ^{**}

Precursor and Peptide Name	Peptide Sequence	C-term Flanking	Theor. Mass	Regions	WT:WT Avg±SEM(n)	Cpe ^{full} :WT Avg±SEM(n)
65–94	(YIQQVRKAPSGRMSVLKQLSDPSHRISD)	RDYMGW	3422.82	A,S	0.96±0.04(2)	<0.10(4) ^{**}
Prodynorphin						
Dynorphin A8	YGGFLRRI	RPKLKW	980.56	A,Hi,Hy,PC,S,T	1.11±0.05(9)	<0.11(14) ^{**}
Dynorphin A10–17	PKLKWDNQ	KRYGGF	1027.55	A,Hi,Hy,S,T	0.99±0.02(5)	<0.10(10) ^{**}
β-neoendorphin	(YGGFLRKYP)	KRSSEM	1099.58	Hy	0.87±0.14(2)	0.25±0.02(2)
α-neoendorphin	YGGFLRKYPK	RSSEMA	1227.68	A,Hi,Hy,PC,S,T	1.04±0.04(9)	0.25±0.02(17) ^{**}
Dynorphin B13	(YGGFLRRQFKVVT)	RSQENP	1569.88	A,Hi,Hy,PC,S,T	1.02±0.05(9)	<0.17(14) ^{**}
C-terminal	SQENPTYSEDLDV	end	1609.67	Hy	0.85±0.10(3)	0.65(1)
183–99	DEGGQDQVGHEDLY	KRYGGF	1847.71	Hy	1.11±0.27(3)	<0.10(2) [*]
Dynorphin A17	(YGGFLRRIRPKLKWDNQ)	KRYGGF	2146.19	Hy	0.83(1)	<0.10(2)
177–99	SSEMARDEGGQDQVGHEDLY	KRYGGF	2508.99	Hy	0.84±0.12(3)	<0.15(3) ^{**}
Proenkephalin						
Leu-Enkephalin	YGGFL	KRFAES	555.27	A,S	1.10±0.06(2)	<0.10(4) ^{**}
Met-Enkephalin	YGGFM	KR, KK, RR	573.23	A,Hy,PC,S	1.10±0.05(7)	0.17±0.05(10) ^{**}
Heptapeptide	YGGFMRF	end	876.40	A,Hi,Hy,PC,S,T	1.03±0.04(10)	0.88±0.07(14)
Heptapep. (ox)	YGGF-Mox-RF	end	892.40	A,S	1.07±0.27(2)	0.91±0.06(4)
Octapeptide	YGGFMRS�	KRSPQL	929.45	A,Hi,Hy,PC,S,T	1.02±0.05(10)	<0.14(16) ^{**}
Metorphamide	YGGFMRRV-amide	GRPEWW	983.50	Hi,Hy,S,T	1.05±0.03(4)	<0.17(8) ^{**}
221–228	PEWWMDYQ	KRYGGF	1153.45	Hy	0.93±0.22(3)	0.34(1)
Metorphamide precursor	YGGFMRRVGR	PEWWMD	1197.62	Hi,S	1.16±0.13(2)	6.90±1.25(7) [*]
197–207	SPQLEDEAKEL	QKRYGG	1257.61	Hy	0.91±0.13(2)	<0.10(1)
197–208	SPQLEDEAKELQ	KRYGGF	1385.67	Hy,PC,S	0.94±0.07(6)	<0.13(8) ^{**}
218–228	VGRPEWWMDYQ	KRYGGF	1465.65	A,Hy,PC,S,T	0.92±0.07(10)	<0.11(14) ^{**}
114–133	MDLYPMEPEEEANGGEILA	KRYGGF	2235.96	Hy	0.84±0.08(3)	<0.15(3) ^{**}
BAM18	(YGGFMRRVGRPEWWMDYQ)	KRYGGF	2333.06	A,Hy	1.25±0.05(2)	<0.10(4) ^{**}
238–261	FAESLPsDEEGENYSKEVPEIE	KRYGGF	2497.10	Hy,S	0.93±0.14(3)	0.23±0.13(3) [*]
238–261 + pSer	FAESLPsDEEGENYSKEVPEIE	KRYGGF	2577.06	Hy,S	0.97±0.24(2)	<0.14(4) ^{**}
Progalanin						
65–72	(ELQLEVEE)	RRPGSV	987.48	Hy	0.83±0.08(3)	<0.10(3) ^{**}

Precursor and Peptide Name	Peptide Sequence	C-term Flanking	Theor. Mass	Regions	WT:WT Avg±SEM(n)	Cpe ^{del/del} ; WT Avg±SEM(n)
33–61	(GWTLNSAGVLLGPHAIDNHRFSFDK)HGLT-amide	GKRELQ	3162.57	Hy,S	1.07±0.10(2)	<0.10(4) ^{**}
Progastrin-releasing peptide						
43–52 amide	(GSHWAVGHLM-amide)	GKKSTD	1092.51	A,Hi,Hy,T	1.12±0.09(4)	<0.13(8) ^{**}
Prohormone convertase 1						
619–628	GVEKMNVVVE	KRPTQK	1102.57	Hy,PC	0.98±0.08(4)	0.14±0.04(2) ^{**}
90–104	(LSDDDRVTVAEQQYE)	KERSKR	1853.81	Hy	0.81±0.06(3)	<0.10(2) ^{**}
90–108	LSDDDRVTVAEQQYEKERS	KRSVQK	2354.08	Hy	1.34(1)	<0.20(2)
Prohormone convertase 2						
C-terminal	SLQSLRK	end	1057.62	A,Hi,Hy,PC,S,T	0.98±0.06(8)	0.51±0.03(11) ^{**}
94–104	IKMALQQEGFD	RKKRGY	1278.64	A,Hy,PC	1.00±0.11(7)	0.19±0.04(8) ^{**}
C-terminal	pyroE-ELEELDEAEERSLQSLRK	end	2610.31	Hy	0.91±0.01(2)	nd
Promelanin-concentrating hormone						
Neuropeptide EI	EIGDEENSAKFPI-amide	GRRDFD	1446.68	Hi,Hy,PC,S	1.02±0.05(9)	<0.15(9) ^{**}
Pronorepinephrine						
C-terminal	SSPETLISDLLMKESTENAPTRLEDPMSW	end	3432.65	A,Hi,Hy,PC,S	1.09±0.08(9)	0.81±0.05(15) ^{**}
Neuropeptide Y (YP)	KPDNPGEDAPAEEDMARYYALRHYINLITRQY-amide	GKRSSP	4269.07	Hy,PC	1.09±0.14(4)	<0.10(3) ^{**}
Pronorepinephrine						
Neuropeptide N	KIPYIL	KRQLYE	745.47	A,Hi,Hy,S,T	0.93±0.05(6)	<0.12(12) ^{**}
Neuropeptide N	pyroE-LYENKPRRPYIL	KRGSYI	1671.91	A,Hy,S	1.05±0.05(6)	<0.11(9) ^{**}
Pronociceptin						
101–107	(VRSLVQV)	RDAEPG	799.49	Hy	1.11(1)	<0.10(2)
Nociceptin	FGGFTGARKSARKLANQ	KRFSEF	1807.98	A,Hi,Hy,PC,S,T	1.00±0.05(9)	<0.10(13) ^{**}
109–138	(DAEPGADAEFGADAEFGADAEVEQKQLQ)	KRFGGF	3051.31	Hy	0.92±0.17(3)	0.36(1)
Proopiomelanocortin						
J-peptide 5–18	(AVWGDGSPSPRE-amide)	GKRSYS	1481.69	Hy	0.99±0.20(3)	<0.10(2) [*]
α-MSH 9–13	(WGKPV-amide)	GKKRRP	584.33	Hy	0.66(1)	<0.10(2)
γ-1-MSH 1–7	(KYVMGHE)	RWDRFG	880.43	Hy	0.59(1)	<0.10(2)
γ-1-MSH	KYVMGHEFRWD	RFGPRN	1337.64	Hy	0.66±0.17(3)	<0.10(2)
Des-acetyl-MSH	SYSMEHFRWGKPV-amide	GKKRRP	1621.77	Hy	1.08±0.17(3)	<0.10(3) ^{**}

Precursor and Peptide Name	Peptide Sequence	C-term Flanking	Theor. Mass	Regions	WT:WT Avg±SEM(n)	Cpe ^{del/del} :WT Avg±SEM(n)
Dehydro α-MSH	Ac-dehydroS-YSMEHFRWGKPV-amide	GKKRRP	1645.79	Hy	0.76(1)	<0.10(2)
α-MSH	Ac-SYSMEHFRWGKPV-amide	GKKRRP	1663.79	Hy	0.88±0.32(4)	<0.10(4) **
α-MSH(oxide)	Ac-SYS-Mox-EHFRWGKPV-amide	GKKRRP	1679.80	Hy	0.96±0.07(2)	<0.10(2)
J-peptide 2-18	EEEEVWGDGSPSPRE-amide	GKRSYS	1868.80	Hy	1.02±0.08(2)	nd
J-peptide	AEEEA V WGDGSPSPRE-amide	GKRSYS	1939.86	Hy	0.63±0.23(3)	<0.10(3) *
CLIP	RPVKVYPNVAENESAEAFPLEF	KRELEG	2505.26	Hy	0.64±0.21(3)	0.12±0.03(4) **
CLIP + pSer	RPVKVYPNVAENEPsAEAFPLEF	KRELEG	2585.23	Hy	0.67±0.16(3)	0.13±0.08(2) *
β endorphin 1-31	YGGFMTSEKSTPLVTLFKNAHKHKKGQ	end	3435.83	Hy	0.80(1)	0.92±0.12(2)
Prooxytocin						
Oxytocin	CYIQNCPLG-amide	GKRAVL	1006.44	Hy	1.07±0.30(2)	0.13±0.04(4) **
Propeptidyl α-amidating monooxygenase (PAM)						
Pro peptide	FRSPLSVF	KRFKET	951.53	A,Hi,Hy,PC,S,T	0.97±0.04(12)	0.23±0.03(18) **
Propituitary adenylate cyclase-activating polypeptide (PACAP)						
111-28	GAGENLGSAVDDPAPLT	KRHSDG	1639.77	Hy	0.99±0.08(3)	0.18±0.03(3)
ProSAAS						
Little EN	LENPSQAPA	RRLLPP	1022.50	Hy	nd	<0.10(2)
Little SAAS 5-16	ASAPLVESTPL	RLRRAV	1184.63	Hy	0.90±0.10(3)	<0.10(2) **
GAV 1-15	AVPRGEAAGAVQELA	RALAH	1437.76	Hy	0.85(1)	<0.10(1)
Little SAAS 5-18	ASAPLVESTPLRL	RRAVPR	1453.81	Hy	0.94±0.10(3)	0.57±0.20(3)
Little SAAS 1-16	SLSAASAPLVESTPL	RLRRAV	1542.81	A,Hy,S,T	0.91±0.08(7)	<0.10(11) **
Big LE 1-15	(LENPSQAPARRLLP)	P-end	1657.93	Hy	0.92±0.01(2)	1.81±0.71(3)
Big EN	LENPSQAPARRLLPP	end	1754.98	A,Hi,Hy,PC,S,T	0.96±0.04(11)	1.75±0.11(16) **
Little SAAS	SLSAASAPLVESTPLRL	RRAVPR	1812.01	A,Hi,Hy,PC,S,T	1.00±0.05(11)	0.41±0.03(18) **
PEN 1-18	(SVDQDLGPEVPPENVLGA)	LLRVKR	1834.90	Hy	0.86±0.14(3)	0.11±0.08(3) **
PEN 1-19	(SVDQDLGPEVPPENVLGAL)	LRVKRL	1947.98	Hy	0.88±0.13(3)	0.15±0.14(3) **
PEN 1-20	SVDQDLGPEVPPENVLGALL	RVKRLE	2061.06	Hy	0.82±0.10(3)	0.08±0.04(3) **
PEN	SVDQDLGPEVPPENVLGALLRV	KRLNRP	2316.23	A,Hy,PC,S	0.99±0.04(8)	0.64±0.05(13) **
Big SAAS 1-24	ARPVKEPRSLAASAPLVESTPL	RLRRAV	2476.37	Hy	0.85±0.19(3)	<0.10(2) *
GAV 5-28	GEAAGA VQELARALAHLEAEQRE	RARAEA	2531.31	A,Hy,PC,S,T	1.00±0.05(8)	<0.10(9) **

Precursor and Peptide Name	Peptide Sequence	C-term Flanking	Theor. Mass	Regions	WT:WT Avg±SEM(n)	Cpe ^{full} ;WT Avg±SEM(n)
Big SAAS	ARPVKEPRSLSAASAPLVETSTPLRL	RRAVPR	2745.55	A,H ₁ ,H ₂ ,S	1.03±0.05(6)	1.10±0.06(10)
GAV	AVPRGEAAGAVQELARALAHLLAEARQE	RARAEA	2954.58	A,H ₁ ,H ₂ ,PC,S,T	0.95±0.04(10)	<0.12(16)**
62–91	(AVPRGEAAGAVQELARALAHLLAEARQERA)	RAEAQE	3181.71	A,H ₂	1.11±0.02(2)	0.24±0.04(2)**
Protachykinin A						
Neurokinin A	HKTDSFVGLM-amide	GKRALN	1132.55	A,H ₁ ,H ₂ ,PC,S,T	1.05±0.07(8)	0.24±0.04(13)**
Substance P	RPKPQQFFGLM-amide	GKRDAD	1346.74	A,H ₁ ,H ₂ ,S,T	0.98±0.04(8)	0.19±0.02(16)**
C-terminal	ALNSVAYERSAMQNYE	RRRK-end	1844.84	H ₂ ,PC,S	1.08±0.09(7)	2.06±0.27(10)**
72–94	(DADSSVEKQVALLKALYGHGQIS)	HKRHKHT	2428.26	S	1.11(1)	<0.10(2)
72–95	DADSSVEKQVALLKALYGHGQISH	KRHKTD	2565.32	H ₂ ,S,T	1.25±0.04(3)	<0.15(7)**
Protachykinin A, isoform CRA_a						
Neuropeptide γ	(DAGHGQISHKRHKTDTSFVGLM-amide)	GKRALN	2319.17	A,H ₂ ,S,T	0.99±0.07(6)	0.39±0.11(6)**
Protachykinin B						
Neurokinin B	DMHDFVGLM-amide	GKRNSQ	1209.52	A,H ₂ ,PC,S,T	1.06±0.10(9)	0.20±0.04(14)**
Prothyrotropin releasing hormone						
53–99	(FLRKDLQ)	RVRGDL	918.53	H ₂	0.85(1)	<0.10(2)
53–61	(FLRKDLQRV)	RGDLGA	1173.70	H ₂ ,S	0.81±0.03(2)	0.32±0.06(7)**
160–269	SFPWMESDVT	KRQHPG	1197.50	H ₂	0.80±0.12(4)	0.19±0.06(5)**
63–244	GDLGAALDSWIT	KRQHPG	1217.59	H ₂	1.04±0.07(3)	0.21±0.11(2)**
95–206	GDLGEVGAWRPH	KRQHPG	1292.63	A,H ₂	0.96±0.15(2)	0.20±0.05(4)**
57–244	DLQVRGDLGAALDSWIT	KRQHPG	1985.03	H ₂	0.78±0.12(2)	nd
133–251	(PSWLPPDFLDSWFSDAPQV)	KRQHPG	2253.04	H ₂	0.87±0.23(3)	<0.20(2)
53–74	(FLRKDLQVRGDLGAALDSWIT)	KRQHPG	2529.39	H ₂	0.92(1)	<0.10(1)
178–200	FIDPELQRSWEE/TEGEGGLMPE	KRQHPG	2677.19	H ₂	0.95±0.20(3)	0.29±0.16(3)*
83–106	EEKEDVEAEERGDLGEVGAWRPH	KRQHPG	2765.25	H ₂ ,S	1.02±0.06(5)	<0.10(5)**
25–50	LLEAAQEEGAVTPDLPGLKQVQRPE	RRFLRK	2787.48	A,H ₂ ,S	0.96±0.05(6)	<0.10(7)**
Provasoactive intestinal peptide						
146–152	(YLNILN-amide)	GKRSSE	834.46	A	1.11(1)	0.35±0.18(2)
111–122	ISSISEDVPVI	KRHSDA	1242.63	H ₂ ,PC	0.98±0.06(5)	<0.15(4)**
Provasopressin						

Precursor and Peptide Name	Peptide Sequence	C-term Flanking	Theor. Mass	Regions	WT:WT Avg±SEM(n)	<i>Cpe^{fat/fat}</i> ; WT Avg±SEM(n)
151–165	VQLAGTRESVDSAKP	R VY-end	1556.82	Hy	0.90(1)	<0.10(2)
154-end	(AGTRESVDSAKPRVY)	end	1634.84	Hy	1.04(1)	0.50±0.12(2)
151-end	VQLAGTRESVDSAKPRVY	end	1975.05	Hy	0.93±0.22(3)	0.90±0.18(5)
Secretogranin II						
516–524	(PELLNTNQL)	K RVPSP	1040.55	Hy	0.92±0.20(2)	<0.30(3)
287–297	SGQLGLPDEEN	R RESKD	1157.52	Hy	1.20(1)	0.21(1)
300–316	ESKDQLSEDASKVITYL	R RLVNA	1924.96	A,Hi,Hy,PC,S,T	1.03±0.04(10)	<0.10(13)**
Secretogranin	TNEIVEEQYTPQSLATLESVFQELGKLTGPSNQ	K RERVD	3649.80	Hy	0.85±0.21(2)	0.31(1)
Secretogranin III						
23–6	FPKPEGSDKSLHN	R ELSAE	1582.77	A,Hi,Hy,PC,S,T	1.02±0.04(8)	0.20±0.03(11)**
38–55	(ELSAERPLNEQIAEAEAD)	K IKKAF	1983.94	Hy	0.87±0.16(3)	<0.10(2)*
VGF						
211–218	(ASWGEFQA)	R VPERA	894.39	PC	0.94±0.14(3)	0.23(1)
220–236	VPERAPLPVPVPSQFQA	R MSESA	1828.98	PC	1.03±0.26(3)	0.13±0.02(3)**
489–507	NAPPEVPVPPRAAPAPTHV	R SPQPP	1914.01	A,Hi,Hy,PC,S,T	1.04±0.10(10)	<0.13(18)**
487–507	KKNAPPEVPVPPRAAPAPTHV	R SPQPP	2170.20	A,Hi,Hy,PC,S,T	1.06±0.06(8)	<0.10(16)**
348–372	(DLGGRELQETQGERENEREAEQE)	R RGGE	3030.34	Hy,PC	0.93±0.07(6)	0.14±0.02(4)**
180–209	(QQETAAAEETRTHTLTRVNLESPGPERVW)	R ASWGE	3406.69	S	1.07(1)	<0.10(2)
588–617	(AQEEADAERRLQEQELENYIEHVLLHRP)	end	3672.78	A,Hy,PC,S,T	1.07±0.07(6)	0.26±0.05(8)**

Abbreviations: Column 1: CLIP; corticotropin-like intermediate peptide; Dynorphin; J-peptide, Joining-peptide; MSH, melanocyte stimulating hormone; ox, oxidized; pSer, phosphoserine; Column 2: Met, Met-oxide; pS, phosphoserine. Sequences in parentheses were tentatively identified by a number of criteria, but not by MS/MS data; all others were identified by these criteria and by MS/MS data. Column 3: C-term flanking, the amino acid sequence to the C-terminal side of the observed peptide, with basic residues indicated in bold. Column 4: Theor. mass, theoretical mass of the unprotonated peptide (without isotopic tags). Column 5: A, Amygdala; Hi, Hippocampus; Hy, Hypothalamus; PC, Prefrontal Cortex; S, Striatum; T, Thalamus. Column 6: WT:WT Avg±SEM(n), the average ratio of different groups of WT versus WT mice ± standard error of the mean (number of replicates from different groups of mice and LC/MS runs). Column 7: *Cpe^{fat/fat}*; WT Avg±SEM(n), the average ratio of groups of *Cpe^{fat/fat}* versus WT mice ± standard error of the mean (number of replicates).

* p<0.05;

** p<0.01 versus the WT:WT ratio, using Student's 2-tailed T-test. See Supplementary Table for additional information on each peptide, including observed mass, number of isotopic tags, charge state, relative elution time, and individual data for each brain region.