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Hemopressins and other hemoglobin-derived peptides in mouse brain: Comparison between brain, blood, and heart peptidome and regulation in *Cpe^{fat/fat}* mice

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Abstract

Many hemoglobin-derived peptides are present in mouse brain, and several of these have bioactive properties including the hemopressins, a related series of peptides that bind to cannabinoid CB1 receptors. Although hemoglobin is a major component of red blood cells, it is also present in neurons and glia. To examine whether the hemoglobin-derived peptides in brain are similar to those present in blood and heart, we used a peptidomics approach involving mass spectrometry. Many hemoglobin-derived peptides are found only in brain and not in blood, whereas all hemoglobin-derived peptides found in heart were also seen in blood. Thus, it is likely that the majority of the hemoglobin-derived peptides detected in brain are produced from brain hemoglobin and not erythrocytes. We also examined if the hemopressins and other major hemoglobin-derived peptides were regulated in the *Cpe^{fat/fat}* mouse; previously these mice were reported to have elevated levels of several hemoglobin-derived peptides. Many, but not all of the hemoglobin-derived peptides were elevated in several brain regions of the *Cpe^{fat/fat}* mouse. Taken together, these findings suggest that the post-translational processing of alpha and beta hemoglobin into the hemopressins, as well as other peptides, is upregulated in some but not all *Cpe^{fat/fat}* mouse brain regions.

Keywords

peptide processing; neuropeptide; cannabinoid; hemoglobin; carboxypeptidase E

INTRODUCTION

Neuropeptides play a large role in a variety of physiological processes including sleep, feeding, pain, and many more (Strand 2003). Most neuropeptides are produced within the secretory pathway by the selective cleavage of precursors at specific sites (Eipper et al. 1986). After synthesis, the bioactive molecules are stored in vesicles and secreted upon stimulation. In addition to peptides produced within the secretory pathway, a number of peptides have been described that are derived from cytosolic proteins. In some cases, these cytosolic protein-derived peptides have been found to have neuropeptide-like signaling properties. Some

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examples of this class of peptides include diazepam binding inhibitor (DBI), hippocampal cholinergic neurostimulating peptide (HCNP), hemorphins, neokytorphin, and the hemopressins. DBI is a fragment of acyl-Coenzyme A-binding protein that displaces diazepam binding to GABA_A receptors (Costa & Guidotti 1991). HCNP is a fragment of phosphatidylethanolamine-binding protein that enhances the differentiation of hippocampal neurons (Ojika et al. 2000). Alpha and beta chains of hemoglobin are precursors of numerous bioactive peptides, some of which are the hemorphins, neokytorphin, and the hemopressins. The hemorphins, derived from the beta chain, bind to opiate receptors and neokytorphin, derived from the alpha chain, binds angiotensin receptors (Fukui et al. 1983, Nyberg et al. 1997, Zhao et al. 1997). The hemopressins are a family of peptides derived from either alpha hemoglobin (RVD-hemopressin- α and VD-hemopressin- α) or beta hemoglobin (VD-hemopressin- β) which bind to the CB1 cannabinoid receptor (Gomes et al. 2009). This family also includes the 9 amino acid hemopressin peptide which is antinociceptive, reduces blood pressure, and acts as an inverse agonist at the CB1 receptors (Dale et al. 2005b, Heimann et al. 2007, Rioli et al. 2003, Dale et al. 2005a).

Carboxypeptidase E (CPE) is an enzyme involved in the biosynthesis of numerous neuropeptides and other secretory pathway-derived peptides (Fricker 1988). *Cpe^{fat/fat}* mice lack functional CPE due to a point mutation in the gene that causes the substitution of a Pro for a Ser and results in mis-folded enzyme (Naggert et al. 1995). In *Cpe^{fat/fat}* mice, the mature forms of many neuropeptides are substantially reduced and the processing intermediates containing C-terminal basic residues are greatly elevated (Zhang et al. 2008, Fricker et al. 1996, Rovere et al. 1996, Che & Fricker 2005, Che et al. 2005a, Lim et al. 2006). Additionally, previous reports identified several hemoglobin-derived peptides in *Cpe^{fat/fat}* mice that contained C-terminal basic residues, some of which were present at higher levels in the *Cpe^{fat/fat}* mice than in the wild-type littermates (Bures et al. 2001, Lim et al. 2006, Che et al. 2005a).

Hemoglobin is responsible for oxygen delivery to the respiring tissues of the body and is a well-known constituent of red blood cells. In addition, hemoglobin has recently been found to be expressed in many diverse cell types. In 1999, Liu and colleagues found beta hemoglobin mRNA and protein in activated macrophages (Liu et al. 1999). In the past few years, hemoglobin mRNA and/or protein has been detected in lens cells, alveolar epithelial type II and Clara cells, kidney mesangial cells, and epithelial and stromal cells of the endometrium (Wride et al. 2003, Bhaskaran et al. 2005, Newton et al. 2006, Nishi et al. 2008, Haase 2008, Dassen et al. 2008, Ullal et al. 2008). Most recently, alpha and beta hemoglobin mRNA and protein expression were detected in nigral and mesencephalic dopaminergic, striatal GABAergic, and cortical pyramidal neurons and glial cells (Richter et al. 2009, Medvedeva et al. 2009, Pinto et al. 2009, Biagioli et al. 2009). Hemoglobin mRNA expression was also detected in cultured neurons and in differentiated but not in undifferentiated human neuroblastoma cells (Richter et al. 2009, Medvedeva et al. 2009, Pinto et al. 2009, Biagioli et al. 2009). Moreover, hemoglobin expression in mouse brain was suggested to be upregulated in response to ischemia, and the processing of hemoglobin into peptides in mouse brain was rapidly elevated by global ischemia (Gomes et al. 2009, He et al. 2009). Together, these data suggest that hemoglobin-derived peptides could be endogenous signaling molecules within the central nervous system.

To gain a better understanding of the regulation of hemoglobin-derived peptides within the central nervous system, we used mass spectrometry and quantitative peptidomics techniques to compare peptides from wild-type (WT) and *Cpe^{fat/fat}* mice brains. Because it is reasonable to expect that peptides detected upon peptidomics analysis of mouse brain could be derived from blood cells, we also compared the peptidome of brain and blood and analyzed if the major hemoglobin-derived peptides detected in brain were common to blood; these analyses revealed

substantial differences between hemoglobin-derived peptides, as well as many other peptides, between blood and brain. We also investigated the hemoglobin-derived peptidome of the heart, a highly vascularized tissue that presumably has hemoglobin only from blood. We found that unlike the brain, hemoglobin derived peptides in heart were not different from those seen in blood. Accordingly, we observed that many hemoglobin peptides were increased in the *Cpe^{fat/fat}* mice brain whereas most peptides derived from other cytosolic proteins were not substantially affected in these mice. Western blot analysis and quantitative real-time PCR indicate that the increase in hemoglobin-derived peptides was not simply due to elevated hemoglobin protein and mRNA production. These data indicate that the effect of the *fat* mutation was not a non-specific increase in protein turnover, and suggest a more general mechanism for the post-translational processing of hemoglobin not directly involving CPE. Taken together, these findings suggest that hemoglobin-derived peptides are regulated and specifically produced in mouse brain.

MATERIALS AND METHODS

Materials

Hydrochloric acid (6 N, sequanal grade, constant boiling) and trifluoroacetic acid were purchased from Pierce (Rockford, IL, USA). Acetonitrile of HPLC grade was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Dimethylsulfoxide (DMSO), 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, MA, USA). The 4-trimethylammoniumbutyryl (TMAB) stable isotopic labeling reagents, D₀ and D₉ forms of 3-(2,5-dioxopyrrolidin-1-yloxycarbonyl)propyl trimethylammonium butyrate (abbreviated as D₀-TMAB and D₉-TMAB), were synthesized as described previously (Che & Fricker 2005, Zhang *et al.* 2002).

Quantitative peptidomics

For peptidomics of the blood, C57BKS/J WT animals were decapitated and 300µl of peripheral blood was collected into low-retention tubes. The samples were immediately placed in a boiling water bath for 1 minute and frozen in dry ice. Samples were then prepared for mass spec analysis as previously described for brain tissues (Che *et al.* 2005b, Che *et al.* 2007, Zhang *et al.* 2008, Che & Fricker 2002). Briefly, for peptide extraction, each sample of blood was sonicated 40 times at 1 pulse/second 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, cooled on ice and then acidified with 0.1 M HCl to a final concentration of 10 mM HCl. The homogenates were centrifuged at 13,000g for 30 min at 4°C and the supernatant was transferred to a low-retention tube and 1/3 final volume of 0.2 M phosphate buffer (pH 9.5) was added.

Because the data on the blood peptidome was to be compared to previous studies on the brain peptidome that involved isotopically-labeled samples to provide quantitative information, the same isotopic labels were used for the analysis of the blood peptidome. For isotopic labeling, 15 mg of D₀-TMAB or D₉- was dissolved in 37.5 µl DMSO. Labeling was performed as described previously (Che & Fricker 2005). Briefly, 5.7 µl of TMAB solutions were added separately to each blood extract and incubated for 10 min at 23–25°C. Then 1.0 M NaOH was added to adjust the pH to 9.5 and the sample extract solution was incubated another 10 min at 23–25°C. These two steps were repeated six more times to ensure that all peptides were completely labeled. The mixture was then incubated at 23–25°C for two hours and then 60 µl of 2.5 M glycine was added to quench the remaining TMAB reagents. After labeling, D₀ and D₉ TMAB labeled samples were pooled and filtered through an Amicon Ultracel 10 kDa centrifugal filter device (Millipore) to remove proteins larger than 10 kDa in size. To remove

any labels from Tyr residues in the peptides, the filtrate was adjusted to pH 9.0 with 1.0 M NaOH, six microliters of 2.0 M hydroxylamine in DMSO was added, and the sample was incubated for 10 min. This was repeated two more times, for a total added volume of 18 μ l hydroxylamine. The peptides were desalted with a PepClean™ C-18 spin column (Pierce) and eluted out of the column with 160 μ l of 70% acetonitrile and 0.5% trifluoroacetic acid in water. The samples were frozen, evaporated in a vacuum centrifuge, and stored at -70°C until analysis on a quadrupole time-of-flight mass spectrometer (Ultima Q-TOF, Waters/Micromass, Manchester, UK). The peptide mixture was desalted online for 15 min using a Symmetry C18 trapping column (5 μ m particles, 180 μ m i.d. \times 20 mm, Waters, USA) and the trapped peptides were then separated by elution with a water/acetonitrile 0.1% formic acid gradient through a BEH 130 -C18 column (1.7 μ m particles, 100 μ m i.d. \times 100 mm, Waters, USA), at a flow rate of 600 nl/min, as previously described (Berti et al. 2009). Data were acquired in data-dependent mode and selected peptides dissociated by collisions with argon.

For peptidomics of heart, BALB/C animals were decapitated and heart was extracted, placed in a low retention tube, and microwave irradiated to raise the temperature of the tissue to 80°C . Heart was then immediately frozen and samples were processed for mass spec analysis as previously described for brain (Che et al. 2005b, Che et al. 2007, Zhang et al. 2008, Che & Fricker 2002)

Experiments using *Cpe^{fat/fat}* and WT mice were previously published (Zhang et al. 2008, Lim et al. 2006). However, in these previous publications these data were analyzed specifically for neuropeptides and a small number of other protein fragments. In the present study, we reanalyzed the data for hemoglobin-derived peptides as well as a large number of abundant peptides that arise from cytosolic proteins. The quantitative analysis and identification of peptides was performed as previously described (Zhang et al. 2008, Lim et al. 2006).

Western blot

C57BKS/J *Cpe^{fat/fat}* and WT mice were transcardially perfused with 40 ml ice cold sterile saline to reduce peripheral blood. Brains were then removed and olfactory bulb and striatum were excised. Peripheral blood was used as a positive control. Protein was extracted after sonication in lysis buffer (pH = 8.0) containing 50 mM Tris, 120 mM NaCl, 0.5% NP-40, 100 mM Sodium Fluoride, 200 μ M sodium vanadate with protease inhibitor (complete mini, EDTA-free, Roche Diagnostics). The protein concentration was determined using the Bradford method (BioRad). A 10–20% Tris-HCl gel was loaded with 70 μ g protein (olfactory bulb), 100 μ g protein (striatum), and 0.25 μ g protein (blood) per lane and electrotransferred to a nitrocellulose membrane. The membrane was then incubated overnight at 4°C with 1:1000 dilution of either alpha or beta hemoglobin antisera (mouse monoclonal Anti-HBA1 and HBB, Sigma). Following repeated washes, the membrane was then incubated with 1:3000 dilution of IRDye800 conjugated affinity purified anti-mouse IgG antibody (Rockland Immunochemicals, Inc.) for 1 hour. Following several washes, the membrane was scanned and bands were quantified on Li-Cor using Odyssey V3.0 software.

Quantitative real-time PCR

C57BKS/J *Cpe^{fat/fat}* and WT Mice were transcardially perfused with ice cold sterile saline. Brains were removed and olfactory bulb and striatum were extracted. Total RNA was isolated from each brain region using the RNeasy Lipid Tissue Mini Kit (Qiagen). cDNA was generated from 1 μ g total RNA and random hexamers using the superscript III first strand kit (Invitrogen). A 1:10 dilution of the cDNA template was used for the real-time PCR. Primers for hemoglobin α and β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed and purchased from Invitrogen. Primer sequences are alpha hemoglobin forward 5'TGT GGA TCC CGT CAA CTT CAA; alpha hemoglobin reverse 5'TTT GTC CAG AGA GGC ATG CAC;

beta hemoglobin forward 5'ACC ACT AAG CCC CTT TTC TGC T; beta hemoglobin reverse 5'GCT AGA TGC CCA AAG GTC TTC A; GAPDH forward 5'GCA AAG TGG AGA TTG TTG CCA T; GAPDH reverse 5'CCT TGA CTG TGC CGT TGA ATT T. The amplicon size for hemoglobin alpha is 106 bp, for hemoglobin beta is 106 bp, and for GAPDH is 107 bp. For these PCRs, SYBR green (Power SYBR Green PCR Master Mix, Applied Biosystems) was the fluorescent tag. All PCRs were performed on a 7900HT Real Time Thermal cycler (Applied Biosystems). The thermal cycling conditions comprised of an initial uracil-DNA glycosylase decontamination step at 50°C for 2 min, a denaturing step at 95°C for 10 min, and 40 cycles of 10 sec at 95°C, 20 sec at 60°C, and 30 sec at 72°C, followed by a dissociation curve stage at 95°C, 60°C, and 95°C, each for 15 sec. All samples were run in triplicate. Quantitative values were obtained from the threshold cycle number (C_t) at which the increase in the signal associated with exponential growth of PCR products was first detected with SDS 2.1 software (Applied Biosystems). The fold-change in expression was calculated using the $\Delta\Delta C_t$ method, with GAPDH as an internal control.

RESULTS

To assess whether hemoglobin-derived peptides in blood could contribute to the peptides detected in the brain, we performed peptidomic analysis of blood and brain using the same methods. Overall, of the 38 alpha hemoglobin-derived peptides detected in brain, only 19 were also detected in the analysis of blood (Table 1, Figure 1A, and Table S1). Similarly, of the 10 beta hemoglobin-derived peptides detected in brain, only 4 were also detected in blood (Table 1, Figure 1A and Table S1). Only one of the three hemopressin peptides (RVD-hemopressin- α) found in brain tissue (Gomes et al., 2009) was detected in blood (Table 1 and Table S1). Conversely, 12 peptides derived from alpha and beta hemoglobin were found only in blood and not detected in brain (Table 1, Figure 1A). Additionally, 39 non-hemoglobin-derived peptides were detected in the blood analyses; these peptides were derived from 18 blood proteins including albumin, apolipoprotein, fibrinogen, kininogen, kallikreins, and several others (Table 1 and Table S1). Of these 39 peptides, 37 were detected in blood but not in brain, while only 2 were detected in both blood and brain. Thus, the comparative profile of peptides in brain versus blood is much different and it is likely that the hemoglobin-derived peptides detected in the brain tissue samples arise from hemoglobin-expressing brain cells and not from blood contamination.

As a control for the brain peptidomic analysis, we also analyzed the hemoglobin-derived peptidome of the heart. The heart is a tissue that is highly vascularized and there is no evidence to suggest that the heart expresses hemoglobin mRNA or protein. Therefore, we compared the peptidome of the heart and blood to see if there is a distinction between the peptide pools, as observed between brain and blood. Altogether, 14 hemoglobin-derived peptides were detected in the heart extracts, and all 14 of these were also seen in blood; no peptides were specific to only heart (Figure 1B). We also compared the hemoglobin-derived heart peptidome to that of brain, and found that of the 48 hemoglobin peptides found in brain, only 11 were also seen in heart (Figure 1C). These data further suggest that many of the hemoglobin-derived peptides in brain are produced from brain hemoglobin.

Next, we investigated the peptide levels in distinct brain regions of wild-type and *Cpe^{fat/fat}* mice, using a quantitative peptidomics approach. This method uses stable isotopic tags to label peptides present in different samples. Then, the samples are pooled, purified, and analyzed by liquid chromatography and mass spectrometry. Previous analysis of these data detected >150 secretory pathway peptides and a large number of peptides derived from cytosolic proteins, although this latter group were not included in the previous analysis (Che & Fricker 2002, Lim et al. 2006, Zhang et al. 2008). The present study re-analyzed the previous mass spectrometry data to focus on hemoglobin-derived peptides and other commonly detected peptides derived

from cytosolic proteins. Representative data are shown in Figure 2. A number of peptides derived from alpha and beta hemoglobin were detected, including RVD-hemopressin- α and VD-hemopressin- α (Table 2). Both RVD-hemopressin- α and VD-hemopressin- α were elevated in *Cpe^{fat/fat}* mice as compared to WT controls in olfactory bulb, with levels 3-4-fold higher (Table 2). RVD-hemopressin- α was also found to be elevated in striatum (>6 fold) but not in the prefrontal cortex, which showed a *Cpe^{fat/fat}* mice to WT mice ratio of 1.2 (Table 2). Other alpha hemoglobin derived peptides showed an increase in *Cpe^{fat/fat}* mice as compared to WT mice, and these increases were also limited to the olfactory bulb and striatum, with no increases observed in the prefrontal cortex. A beta hemoglobin-derived peptide, VV-hemorphin-7, was found to be elevated >10 fold in the hypothalamus (Table 2).

To test if the increase in hemoglobin-derived peptides was a result of elevated levels of hemoglobin mRNA levels in brain, we examined levels of alpha and beta hemoglobin mRNA using quantitative real-time PCR and compared WT and *Cpe^{fat/fat}* mice that were perfused with saline to reduce the contamination from blood. For this, oligonucleotides were created that gave products of ~100 nucleotides and had comparable melting temperatures to each other and to the control oligonucleotides (GAPDH). Analysis of the PCR product showed bands of the correct size (data not shown). Both male and female mice were included in this analysis because the peptidomics analysis also used both genders in roughly equal numbers. No major differences were observed in comparing the male and female data, so these groups were pooled to increase the overall n value for the experiment. No significant difference in the levels of alpha and beta hemoglobin mRNA were found in any of the *Cpe^{fat/fat}* mouse brain regions, relative to wild-type mice (Figure 3). In addition, hemoglobin protein levels in the various brain regions of saline-perfused WT and *Cpe^{fat/fat}* mice were determined using Western blot analysis. Representative data are shown in Figure 4A. Quantitation of the results from striatum and olfactory bulb for alpha and beta hemoglobin showed generally comparable levels between wild-type and *Cpe^{fat/fat}* mice, with no statistical significance between groups (Figure 4B).

Because the levels of hemoglobin mRNA and protein are not altered in *Cpe^{fat/fat}* mouse brain, relative to WT mouse brain, it is likely that the increase in some of the hemopressins and other hemoglobin-derived peptides in the mutant mice (Table 2) is due to elevated processing of hemoglobin. To test if other peptides derived from cytosolic proteins are elevated in the *Cpe^{fat/fat}* mice, we analyzed the data comparing *Cpe^{fat/fat}* and WT mice for a number of different cytosolic peptides that were previously identified in brain peptidomics analyses (Table S3). For most of the cytosolic peptides *Cpe^{fat/fat}*/WT ratios were approximately 1.0, indicating that the vast majority of peptides from cytosolic proteins are not altered in the *Cpe^{fat/fat}* mice (Table S3). A small number of peptides show ratios around 2.0 or higher, but in most cases the error range for these is large, indicating that the peptide is variable among mice and not necessarily elevated in the *Cpe^{fat/fat}* mice. For example, one peptide that appears to be much higher in the *Cpe^{fat/fat}* mice than in WT mice is KTEWLDGKHVVF from peptidylprolyl isomerase A (Table S3). This peptide appeared to be elevated approximately 6-fold in *Cpe^{fat/fat}* mouse striatum, but had a large error range (± 3.7). Furthermore, this peptide was not comparably altered in several other brain regions in which it was detected, and many other fragments of the same protein were only slightly elevated in the *Cpe^{fat/fat}* mice. Interestingly, one other cytosolic protein that showed consistent increases in processing in the *Cpe^{fat/fat}* was the precursor of DBI, acyl-CoA-binding protein (Costa & Guidotti 1991); all three different fragments of this protein detected in our analysis were found to be present in the *Cpe^{fat/fat}* mice at levels ~2 fold higher than the levels in WT mice (Table S3).

DISCUSSION

The discovery that alpha- and beta-hemoglobin derived peptides are present in mouse brain and bind to CB1 cannabinoid receptors raised the possibility that these molecules represent

novel endogenous signaling molecules. A central question is whether these peptides are actually produced in brain, or are merely a result of the blood that is inevitably present in brain. Although perfusion can be employed to reduce or eliminate blood cells from tissues, to avoid postmortem production of hemoglobin-derived peptides due to ischemia for peptidomics analyses it is necessary to rapidly heat the brain using microwave irradiation (Che et al. 2005b) or other methods (Skold et al. 2007). Thus, the brain, blood, and heart peptidome were compared in the present study in order to evaluate whether the hemoglobin-derived peptides were common to all samples or if brain peptides were distinct from those present in blood and heart. The finding that hemoglobin-derived peptides detected in brain are largely distinct from those in blood and heart implies that the processing pathways are tissue-selective and that the brain peptides are not simply the result of blood contamination. This was further supported by a comparison of the heart hemoglobin-derived peptidome with those of blood; the large overlap between heart and blood suggests that blood is the source of the hemoglobin peptides found in heart, while the small overlap between brain and either blood or heart suggests distinct origin for the brain peptides. Furthermore, of the 39 non-hemoglobin-derived peptides detected in blood, only 2 were also found in the analyses of brain peptides. Collectively, these results strongly suggest that the brain hemoglobin peptides are locally produced and that the contribution of the blood peptidome to the brain peptidome is minimal.

Another important question from the present studies concerns the mechanism of production of the hemopressins and the physiological regulation of these molecules. The previous finding that Lys- and Arg-extended forms of hemoglobin were elevated in the *Cpe^{fat/fat}* mice (Bures et al. 2001, Lim et al. 2006, Che et al. 2005a) raised the possibility that the processing involved CPE; most of the peptides found to be elevated in these mutant mice are substrates of this enzyme (Che et al. 2005a, Che & Fricker 2005, Fricker et al. 1996, Lim et al. 2006, Rovere et al. 1996, Zhang et al. 2008). Although CPE is in the secretory pathway and hemopressins are expected to be produced in the cytosol, it is conceivable that transporters like TAP1 and TAP2 could pump these peptides into the endoplasmic reticulum and then they would encounter CPE along the secretory pathway (Koopmann et al. 1997). However, our finding that the increase in many hemoglobin peptides in the *Cpe^{fat/fat}* mice is not limited to those peptides with C-terminal Lys- or Arg-residues (Table 2) indicates that this cytosolic peptide increment is indirect and unrelated to the catalytic action of CPE. The finding that the hemopressins and several other hemoglobin-derived peptides increase in some, but not all brain regions of *Cpe^{fat/fat}* mice without accompanying changes in hemoglobin protein or mRNA levels, suggests regulation at the post-translational processing level.

Overall, the level of upregulation of hemoglobin-derived peptides in *Cpe^{fat/fat}* is generally much lower than the degree of upregulation of neuropeptide processing intermediates. For example, peptides derived from pro-opiomelanocortin that have C-terminal lysine or arginine are >20 fold more abundant in *Cpe^{fat/fat}* mice brains as compared to WT mice (Che et al. 2005a). Furthermore, a fragment of procholecystokinin containing a C-terminal -Gly-Arg-Arg extension was found at levels estimated to be 13–51-fold higher in *Cpe^{fat/fat}* mice as compared to WT controls (Cain et al. 1997, Wang et al. 1998). Although some neuropeptides are not greatly affected by the absence of CPE activity in the *Cpe^{fat/fat}* mice, this is presumably due to the presence of a related enzyme, carboxypeptidase D, in the *trans* Golgi apparatus and immature secretory vesicles (Varlamov & Fricker 1998). In contrast to the large increases in levels of most Lys/Arg-extended neuropeptide intermediates and large decreases in levels of the mature neuropeptides previously found in *Cpe^{fat/fat}* mice, the hemopressins and other related hemoglobin-derived peptides detected in this study either showed relatively modest 2–10 fold increases or no change, depending on brain region. The magnitude of the changes in hemoglobin peptides detected in the present study is in general agreement with a previous peptidomics studies that reported changes in hemoglobin-derived peptides with C-terminal Lys and/or Arg in *Cpe^{fat/fat}* mice (Bures et al. 2001). The Bures et al study used affinity columns

to isolate the Lys-and/or Arg-extended peptides, a technique developed to identify neuropeptide processing intermediates in *Cpe^{fat/fat}* mice (Che *et al.* 2001, Fricker *et al.* 2000). Because the hemopressins, and most other hemoglobin-derived peptides, do not contain C-terminal Lys/Arg residues, they would not have been detected in the previous studies that used this technique.

Because CPE resides in the secretory pathway and removes basic residues from the C-terminus of peptides (Fricker & Snyder 1982, Fricker & Snyder 1983), it makes sense that many neuropeptide processing intermediates that contain C-terminal Lys/Arg residues are increased in the *Cpe^{fat/fat}* mice which lack functional CPE (Fricker *et al.* 1996). However, it is not clear by what mechanism a CPE defect in *Cpe^{fat/fat}* mice leads to altered levels of the hemopressins and other hemoglobin-derived peptides. This upregulation is not likely due to increased levels of hemoglobin production as levels of its mRNA and protein were not significantly different between *Cpe^{fat/fat}* mice and WT mice (Figures 2 and 3). Therefore, it is likely that the post-translational processing of alpha and beta hemoglobin is upregulated in some brain regions of *Cpe^{fat/fat}* mice. The upregulation of the hemoglobin derived peptides is not due to a general large-scale breakdown of all cytosolic proteins because most peptides derived from cytosolic proteins are present in *Cpe^{fat/fat}* and WT mice at generally comparable levels (Figure 5). This suggests that there is specificity for hemoglobin breakdown in the *Cpe^{fat/fat}* mice and that by some unknown mechanism the post translational processing of both alpha and beta hemoglobin is affected, leading to increased hemoglobin derived peptides.

In addition to alpha and beta hemoglobin, the only other protein detected in the present study that appeared to consistently show elevated processing into peptides in the *Cpe^{fat/fat}* mice was DBI, a putative peptide antagonist of the binding of benzodiazepine to the GABA_A receptor (Costa & Guidotti 1991). It is interesting that peptides derived from all three of these proteins have been found to be bioactive, and more specifically, to interact with neurotransmitter receptors (Gomes *et al.* 2009, Nyberg *et al.* 1997, Ivanov *et al.* 1997, Costa & Guidotti 1991, Heimann *et al.* 2007). The hemopressins, hemorphins, and DBI are distinct from classical neuropeptides that are produced in the secretory pathway and released from cells upon stimulation. It is possible that the cytosol-derived bioactive peptides are analogous to signaling molecules such as nitric oxide or anandamide which are not stored in vesicles. Instead, these “non-classical neurotransmitters” are made on demand by enzymes that are stimulated by various pathways, and then constitutively secreted. It is therefore plausible that hemopressins, hemorphins, and other peptides derived from cytosolic proteins represent “non-classical neuropeptides” which are synthesized upon stimulation and then constitutively secreted. Regulation, which is central to a proposed role as a signaling molecule, would occur at the level of synthesis rather than secretion. The major finding of the present study suggesting that the hemopressins and other hemoglobin derived peptides are upregulated in the *Cpe^{fat/fat}* mice supports this proposed role as non-classical neuropeptides.

Increased levels of hemoglobin-derived peptides have also been reported in another mutant animal model, *Purkinje cell degeneration (pcd)* mice (Berezniuk *et al.* 2010). *Pcd* mice have a spontaneous mutation in the cytosolic carboxypeptidase CCP1/Nna1 (Fernandez-Gonzalez *et al.* 2002, Mullen *et al.* 1976). In a recent peptidomics study comparing peptides in *pcd* and WT mouse brain extracts, all hemoglobin-derived peptides were found to be elevated greater than 2-fold in the *pcd* mutant brains; these peptides included four alpha hemoglobin-derived and two beta hemoglobin-derived peptides (Berezniuk *et al.* 2010). Although the hemopressins were not reported in the Berezniuk *et al.* study, RVD-hemopressin- α was seen in *pcd* brains in an additional study and was found to be elevated >5 fold as compared to control brains (I. Berezniuk and L. Fricker, unpublished). Furthermore, this increase in RVD-hemopressin- α and other hemoglobin-derived peptides was not seen in the hearts of *pcd* animals as compared to WT controls (J. Sironi and L. Fricker, unpublished). These studies further support the idea

that hemopressins detected in brain extracts are produced from brain hemoglobin and are not contaminants from erythrocytes present within the brain.

There are many remaining questions that need to be answered to complete our understanding on this emerging field. Key questions involve the enzymatic pathway required for the production of the cytosolic peptides, the mechanism(s) by which this pathway is regulated, whether the peptides are secreted, and if so, the mechanism of this secretion. It is likely that the ubiquitin-proteasome pathway is involved in the production of hemopressin and other cytosolic peptides, although this needs to be directly investigated. It is also likely that the peptides are secreted from the cytosol; many intracellular peptides, such as annexins, thymosin, and interleukin- β are known to be secreted from the cytosol (Goya & Bolognani 1999, Gerke & Moss 2002, Simi *et al.* 2007). Further studies are required to understand the pathophysiological function of the hemopressins and the other cytosolic-derived peptides and to determine the mechanism by which hemopressins are increased in *Cpe^{fat/fat}* mouse brain. In the present study, RVD-hemopressin- α and VD-hemopressin- α were detected in the olfactory bulb; in rodent brain, very high levels of CB1 receptor expression are detected in the anterior olfactory nucleus (Tsou *et al.* 1998, Lein *et al.* 2007). Hemopressin peptides were also found in the striatum and prefrontal cortex in the present study, which also have high levels of CB1 receptor expression. An interesting area for further research will be to investigate if hemoglobin is differentially processed in various cells/brain regions, as suggested by the present data.

Supplementary Material

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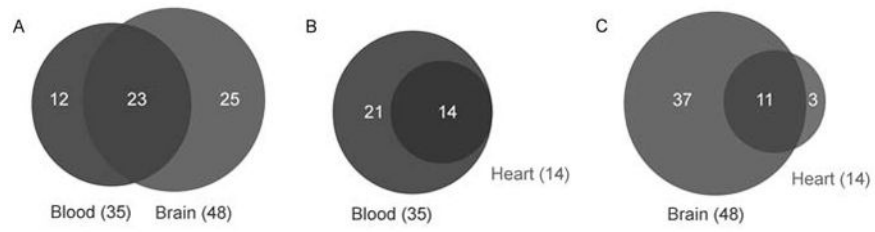


Figure 1.

Comparison of hemoglobin derived peptidome between tissues. A. Hemoglobin-derived peptides found in blood versus those found in brain, overlap of circles distinguishes those peptides that were seen in both blood and brain. Comparison of blood and heart is in panel B and brain versus heart is in panel C.

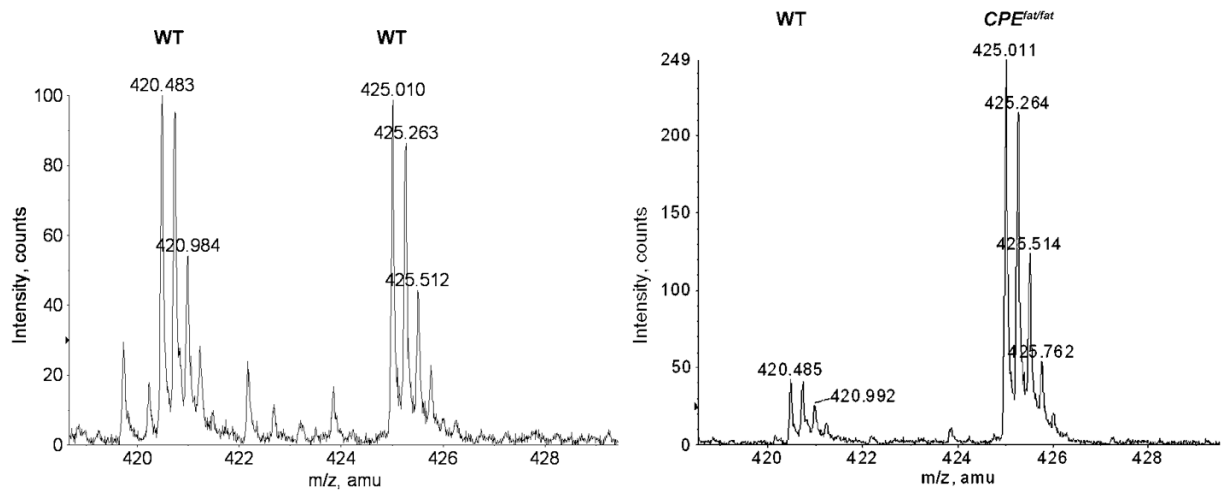


Figure 2.

Representative spectra showing levels of peptides in wild-type and *Cpe^{fat/fat}* mice. Left panel: ions at m/z 420.483 and 425.010 (monoisotopic peaks) correspond to mono-D₀- and D₉-TMAB labeled RVD-hemopressin- α in olfactory bulb in an experiment evaluating WT vs WT mice. Ratio of D₀ to D₉ is 1.0, illustrating no change in peptide level in WT mice. Right panel: ions at 420.485 and 425.011 (monoisotopic peaks) correspond to mono-D₀- and D₉-TMAB labeled RVD-hemopressin- α in olfactory bulb in an experiment evaluating *Cpe^{fat/fat}* mice vs WT mice. Quantification of this spectra illustrates RVD-hemopressin- α is 5.5 fold higher in the *Cpe^{fat/fat}* mouse sample as compared to the WT mouse sample.

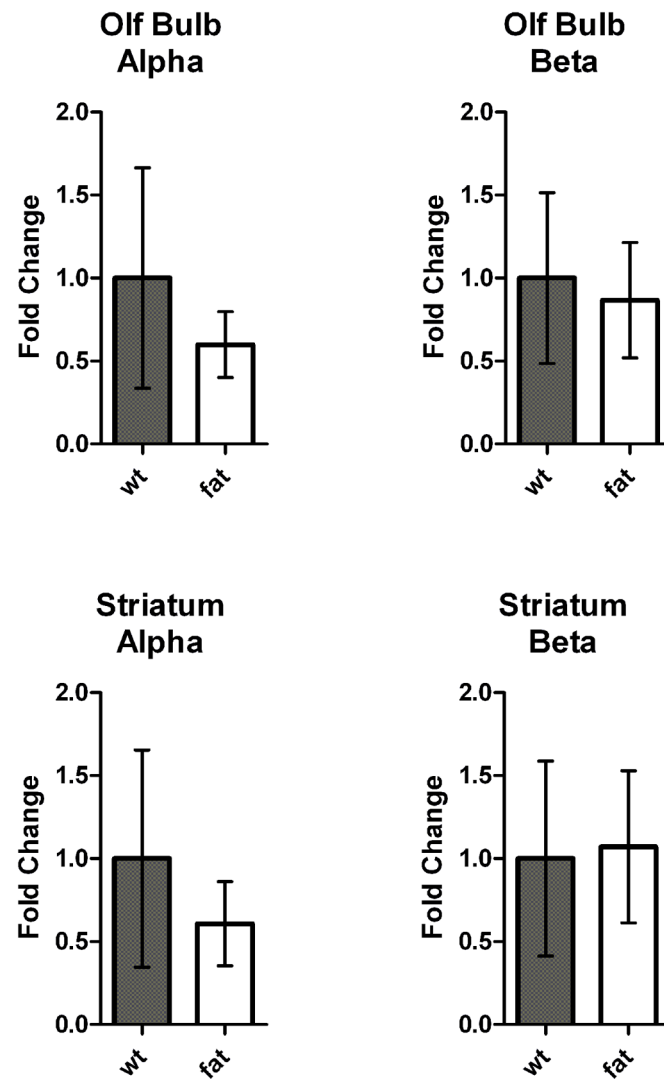


Figure 3. Quantitative real-time PCR of hemoglobin mRNA levels in wild-type and *Cpe^{fat/fat}* mice. Results are shown as the fold-change in expression. Fold change was calculated using the $\Delta\Delta C_t$ method, with GAPDH as an internal control. Both male and female mice used. $n=5$ *Cpe^{fat/fat}* mice and 6 WT mice. Error bars represent standard error of the mean.

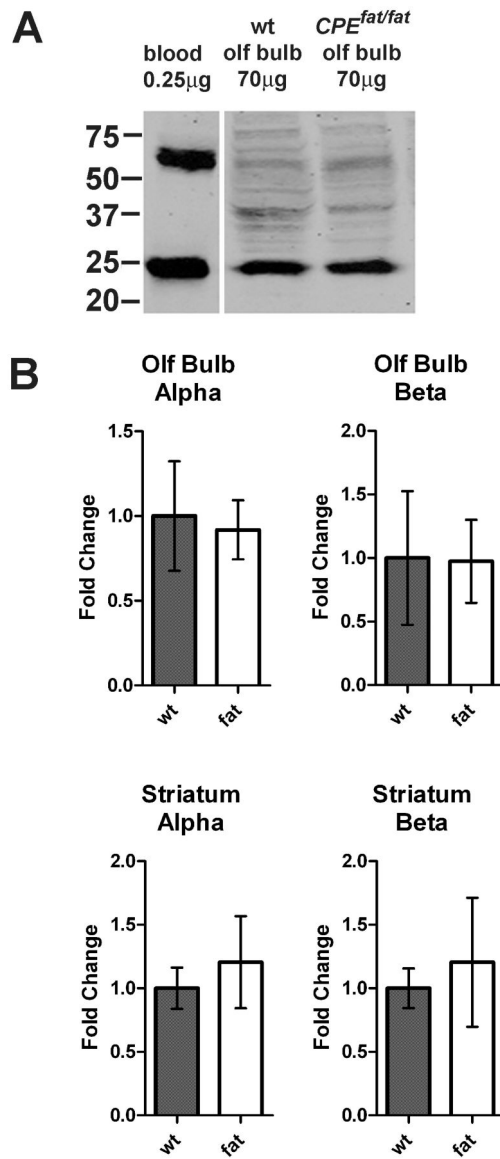


Figure 4. Western blot analysis of hemoglobin protein levels in wild-type and *Cpe^{fat/fat}* mice. A: Representative data for alpha hemoglobin in olfactory bulb and blood. Hemoglobin monomer is seen at approximately 24 kD. B: Quantitation of results from multiple analyses. n= 5 *Cpe^{fat/fat}* mice and 6 WT mice. Error bars represent standard error of the mean.

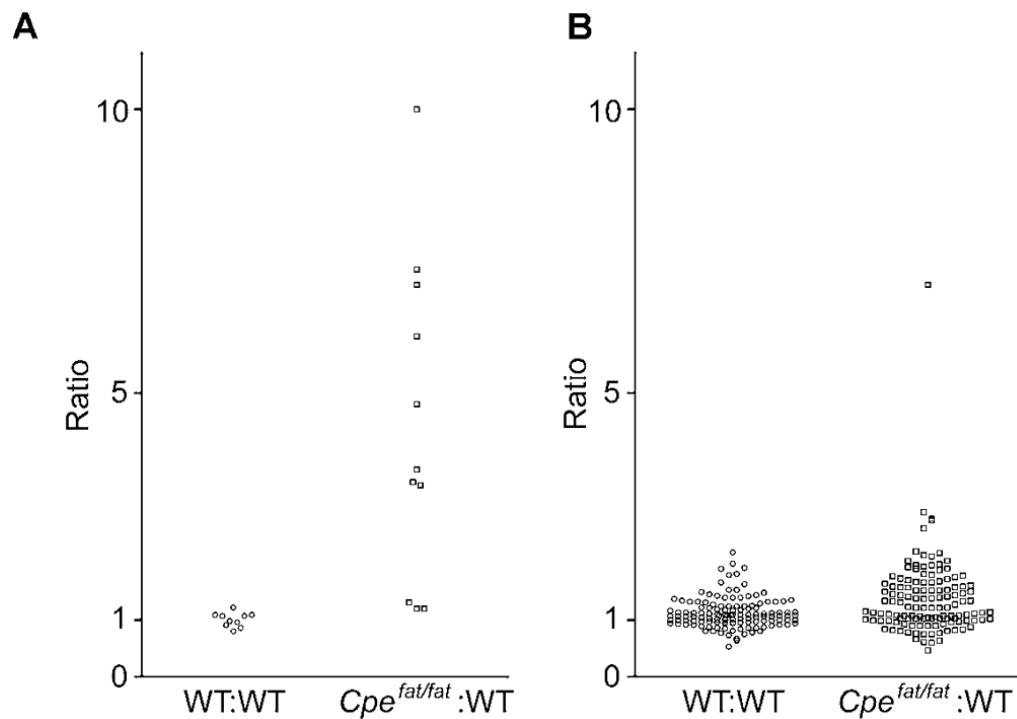


Figure 5. Ratio of hemoglobin-derived and other cytosolic protein-derived peptides in WT:WT and *Cpe^{fat/fat}:WT* mice. A. Relative amounts of hemoglobin-derived peptides in groups of WT mice as compared to other WT mice (left) and *Cpe^{fat/fat}* mice as compared to WT mice (right). Data points with a ratio of “10” refer to a ≥ 10 fold increase in *Cpe^{fat/fat}* mice as compared to WT mice. For identity of peptides, see Table 2. B. Ratios of peptides derived from cytosolic proteins other than hemoglobin. For identity of peptides, see Table S3.

Table 1

Summary of peptidomic analysis of blood and brain.

Protein	Number of different peptides found in:		
	Blood Only ^a	Brain Only ^b	Blood and Brain ^c
Albumin	3	1	0
Alpha-2-HS-glycoprotein	1	0	0
Apolipoprotein A-I	2	0	0
Apolipoprotein A-II	3	0	0
Apolipoprotein C-I	2	0	0
Apolipoprotein C-III	1	0	0
Apolipoprotein E	1	0	0
Apolipoprotein J	1	0	0
Complement component 3	1	0	0
Enhancer of polycomb homolog 1	1	0	0
Fibrinogen alpha	8	0	1
Fibrinogen beta	2	1	0
Kallikrein 1-related peptidase b1	1	0	0
Kallikrein 1-related peptidase b22	2	0	0
Kallikrein 1-related peptidase b27	2	0	0
Kallikrein 1-related peptidase b3	1	0	0
Kallikrein 1-related peptidase b9	1	0	0
Kininogen-1	2	0	1
Pregnancy zone protein	2	0	0
Hemoglobin alpha	5	19	19
Hemoglobin beta	7	6	4

^aData from Table S1^bHemoglobin data from (Gomes et al. 2009). Albumin peptide sequence is GEYGFQNAILVR and Fibrinogen beta peptide is ADDDYDEPTDSDLAR^cData from Table S1 and Gomes et al. 2009

Table 2Relative levels of hemoglobin-derived peptides in *Cpe^{fat/fat}* mice.

Protein	Sequence	Brain Region	<i>Cpe^{fat/fat}</i> :WT \pm SEM (n)
Alpha Hemoglobin			
	LSDLHAHKL	Olf Bulb	3.65 \pm 1.06 (4)
	VDPVNFKLLSH	Olf Bulb	4.81 \pm 3.31 (2)
	AGHLDDLPGALSAL	Olf Bulb	6.91 \pm 5.39 (2)
	RVDPVNFKLLSH	Olf Bulb	3.43 \pm 0.68 (8)
	RVDPVNFKLLSH	PFCx	1.20 \pm 0.19 (3)
	RVDPVNFKLLSH	Striatum	>6 (4)
	ASHHPADFTPAVHA	Olf Bulb	3.37 \pm 1.72 (2)
	ANAAGHLDDLPGALSAL	Olf Bulb	7.18 \pm 5.42 (2)
	KFLASVSTVLTSKYR	PFCx	1.20 \pm 0.21 (2)
	(FTPAVHASLDKFLASVSTVLTSKYR)	PFCx	1.31 \pm 0.31 (2)
Beta Hemoglobin			
	VVYPWTQRY	Hypo	>10 (2)

The relative levels of peptides were calculated by the ratio of peak intensity of the D₀-TMAB-and the D₉-TMAB-labeled peptide pairs.

Cpe^{fat/fat}:WT indicates the ratio of peptide in *Cpe^{fat/fat}* mice relative to WT mice with standard error of the mean (SEM) for n replicates.

Abbreviations: Olf Bulb, olfactory bulb; PFCx, prefrontal cortex; Hypo, hypothalamus; ND, not detected.