New Roles of Carboxypeptidase E in Endocrine and Neural Function and Cancer

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Carboxypeptidase E (CPE) or carboxypeptidase H was first discovered in 1982 as an enkephalin-convertase that cleaved a C-terminal basic residue from enkephalin precursors to generate enkephalin. Since then, CPE has been shown to be a multifunctional protein that subserves many essential nonenzymatic roles in the endocrine and nervous systems. Here, we review the phylogeny, structure, and function of CPE in hormone and neuropeptide sorting and vesicle transport for secretion, alternative splicing of the CPE transcript, and single nucleotide polymorphisms in humans. With this and the analysis of mutant and knockout mice, the data collectively support important roles for CPE in the modulation of metabolic and glucose homeostasis, bone remodeling, obesity, fertility, neuroprotection, stress, sexual behavior, mood and emotional responses, learning, and memory. Recently, a splice variant form of CPE has been found to be an inducer of tumor growth and metastasis and a prognostic biomarker for metastasis in endocrine and nonendocrine tumors. **(*Endocrine Reviews* 33: 216–253, 2012)**

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Abbreviations: AGRP, Agouti-related peptide; BDNF, brain-derived neurotrophic factor; BMD, bone mineral density; CART, cocaine- and amphetamine-regulated transcript; Caspr2, contactin-associated protein 2; CCK, cholecystokinin; CPE, carboxypeptidase E; CPE-ΔN, spliced variant of CPE without its N-terminus; CSP, constitutive secretory pathway; E, embryonic day; EAE, experimental autoimmune encephalomyelitis; ER, endoplasmic reticulum; EST, expressed sequence tag; FH, familial hyperproinsulinemia; GEMSA, guanidinoethylmercaptosuccinic acid; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; KO, knockout; LDCV, large dense-core vesicle; LTP, long term potentiation; MC4R, melanocortin 4 receptor; MEN2, multiple endocrine neoplasia type 2; NOS1AP, nitric oxide synthase 1 adaptor protein; NPY, neuropeptide Y; PC, prohormone convertase; PGL, paraganglioma; PHEO, pheochromocytoma; POMC, proopiomelanocortin; RER, rough ER; RPE, retinal pigmented epithelium; RSP, regulated secretory pathway; SDHD, succinate dehydrogenase subunit D; siRNA, small interfering RNA; SLMV, synaptic-like microvesicle; SNP, single nucleotide polymorphism; SV, synaptic vesicle; T2DM, type 2 diabetes mellitus; TGN, *trans* Golgi network; TIRF, total internal reflection fluorescence; T/N, tumor *vs.* normal surrounding tissue; UTR, untranslated region; WT, wild-type.

I. Introduction to Functions of CPE

Although discovered in 1982, carboxypeptidase E (CPE) has remained a molecule of keen interest to many investigators. In addition to its carboxypeptidase activity, numerous studies over the last 1.5 decades have indicated that CPE is a multifunctional protein that plays many nonenzymatic roles in the endocrine and nervous systems. This review presents a comprehensive look at CPE from structure to function and disease, with a focus on new roles that this unique protein plays in many physiological systems.

A. Discovery of CPE as a prohormone-processing enzyme

Processing of propeptides often begins with the endoproteolytic cleavage at paired or sometimes at single basic amino acid residues. Since the discovery of the prohormone convertases (PC enzymes), which cleave prohormones between or on the carboxyl side of pairs of basic residues (1), it became clear that another enzyme was needed to remove the basic residues from the intermediates to produce the mature bioactive hormone. CPE, which was first identified as enkephalin convertase (2, 3), was subsequently found to be the enzyme responsible for cleaving the C-terminally extended basic residues from peptide intermediates in endocrine cells and neuropeptides in peptidergic neurons. CPE differed from other carboxypeptidases in that its optimal pH was in the acidic range, consistent with its localization to acidic compartments of the *trans* Golgi network (TGN) and to dense core secretory granules of endocrine cells and peptidergic vesicles of neurons where processing occurs. CPE is localized primarily to endocrine tissues and to specific areas of the central nervous system. The importance of CPE as a processing enzyme was further realized when a mutation in the *Cpe* gene was found in the $Cpe^{fat/fat}$ mouse that presented with severe obesity, diabetes, and infertility. Studies on the structure, biosynthesis, forms, tissue distribution, and enzymatic properties of CPE are discussed in *Sections II.A to II.D*.

B. Role of CPE in prohormone sorting, vesicle transport, and secretion

Prohormones/propeptides are synthesized in the rough endoplasmic reticulum (RER) and then inserted into the RER cisternae. From there the precursors are transported in vesicles to the Golgi apparatus where they are sorted at the TGN into budding granules along with their processing enzymes such as the PCs and CPE, as well as other cargo proteins such as the granins [for review, see Bartolomucci *et al.* (4)]. The precursors are then processed within the secretory granules en route to the storage and release sites at the periphery of the cell. Intracellular transport of proteins to various organelles has been found to be mediated by sorting signal motifs and respective receptors. For example, the KDEL signal motif and KDEL receptors mediate the retention of endoplasmic reticulum (ER) resident proteins in the ER (5). The search for sorting motifs for targeting prohormones/propeptides at the TGN into the granules of the regulated pathway has been challenging. The identification of sorting signal motifs on these precursors and evidence supporting a role for a membrane form of CPE as a sorting receptor are discussed in *Section III.A*.

Prohormone-containing granules that have budded off from the TGN are transported to the plasma membrane via microtubule- and actin-based transport systems. In *Section III.B*, the role of a transmembrane form of CPE in facilitating the post-Golgi transport of these granules to the cell periphery is discussed. The cytoplasmic tail of the transmembrane form of CPE appears to interact with microtubule motors, actin, and other cytoskeletal proteins to enable granules to be transported via the regulated secretory pathway (RSP) to the plasma membrane for exocytosis.

Recently, the transmembrane form of CPE was also found in synaptic vesicles (SV) in a subset of hypothalamic neurons. The localization of these vesicles to the active zone of the synapse for neurotransmitter release was shown to be dependent on the interaction of the CPE cytoplasmic tail with cytoskeletal proteins. The significance of CPE in facilitating SV localization to the synapse is discussed in *Section III.C*.

C. Biomedical implications of CPE in physiological function and disease

Much has been learned about the physiological functions and disease states caused by the lack of CPE from $Cpe^{fat/fat}$ (6) and knockout (KO) mice (7). In *Sections IV and V*, insights gained from using these mouse models are discussed. These include revealing the role of CPE in obesity, bone remodeling, diabetes, reproduction, neuroprotection, mood, and emotional responses.

More recently, a splice variant form of CPE was discovered that had activity in promoting the growth and migration of cancer cells (8). Unlike wild-type (WT) full-length CPE, this splice isoform (*i.e.*, CPE-ΔN) lacks the signal peptide at the N terminus that normally directs it into the secretory pathway. It exists in the cytoplasm and in metastatic tumor cells. CPE-ΔN moves into the nucleus and functions to activate metastatic and antiapoptotic genes, thus inducing or promoting tumor metastasis. Use of CPE-ΔN as a biomarker for diagnosing and predicting future metastasis in several types of endocrine and epithelial cancers is discussed in *Section VI*.

Several single nucleotide polymorphisms (SNP) occurring in the *CPE* gene have been found in humans. Some result in the loss of enzymatic activity as identified in type II diabetic patients, whereas others lead to unstable molecules that are misfolded and degraded in the ER (see *Section VII*).

We conclude the review with a summary of the new nonenzymatic roles for CPE, its splice isoform CPE-ΔN, and discuss the mechanisms by which CPE controls functions in health and disease, and the potential use of CPE as a therapeutic drug target (*Section IX*).

II. The *CPE* Gene, Biosynthesis, Protein Structure, and Distribution

A. Phylogenic analysis of CPE

CPE falls into the peptidase M14-like superfamily of enzymes (9). The M14 family of metallocarboxypeptidases is a group of zinc-binding carboxypeptidases that hydrolyze single, C-terminal amino acids from polypeptide chains and have a recognition site for the free C-terminal carboxyl group, a key determinant of specificity. Based on substrate specificity, CPE is classified into carboxypeptidase B-like (CPB-like) enzymes because it only cleaves the basic residues lysine or arginine. Various metallocarboxypeptidase proteins containing a Zn-carboxypeptidase domain have been highly conserved from bacteria to mammals (10, 11). To date, 23 genes encoding Zn-carboxypeptidase domain proteins have been identified in the human genome (10). Comparison of the sequence of CPE with carboxypeptidase A (A1, A2, A3, A4, A5 and A6) and carboxypeptidase B (B1 and B2) show a 21% identity at the protein level with 55% and 37% sequence coverage, respectively. Carboxypeptidase O is 22% identical at the protein level with just 32% sequence coverage. Additionally, carboxypeptidase M is 46% identical at the protein level with 82% sequence coverage, and carboxypeptidase N1 is 51% identical at the protein level with 86% sequence coverage. Interestingly, carboxypeptidae D has multiple Zn-carboxypeptidase domains and is 50% identical at the protein level with 86% sequence coverage. There is very little conservation outside the Zn-carboxypeptidase domain between CPE and CPA/B proteins, suggesting that these proteins diverged very early during evolution.

The orthologous protein sequences of the *Cpe* gene have been identified in many species, covering a phylogenetic distance from invertebrate *Protostomia* (*Ecdysozoa*, nematodes) to vertebrate *Deuterostomia*. The phylogenetic relationships, as estimated from amino acid sequence similarities, are shown in a cladogram tree (Fig. 1). According to this tree, the CPE sequences of *Caenorhabditis elegans* (nematode) were found at separate branches from those of the other species. CPE has been biochemically characterized only in a few species so far. It was first cloned from bovine adrenal chromaffin granules (12) and has been well studied in mouse (*Mus musculus*) (6) and in rat (*Rattus norvegicus*) (13–15). Human CPE was first characterized by Manser *et al.* (16). CPE was also studied in chicken (*Gallus gallus*), thymus (17), and in nematode (*C. elegans*) (18). Subsequently, by blast search and data mining, CPE was also found to exist in genome sequences of the chimpanzee (*Pan troglodytes*) (19), macaque (*Macaca fascicularis*) (20), boar (*Sus scrofa*) (21), clawed frog (*Xenopus tropicalis*) (22), sea-slug (*Aplysia californica*) (23), and zebrafish (*Danio rerio*) (24) (Table 1). The remarkable degree of similarity at the protein level of CPE across the different phyla suggests its functional importance early in evolution.

Alternative splicing of CPE transcripts

In mammals, the *CPE* gene contains nine exons (Fig. 2) (13–16). In humans, two alternatively spliced transcripts of *CPE* mRNA have been found, one of which encodes a truncated protein lacking a partial N-terminal region (CPE-ΔN) due to alternative splicing of the first exon (Fig. 2) (8). CPE-ΔN is alternatively spliced with noncanonical alternative 3′ and 5′ splice sites, which remove 98 nucleotides of the first exon. This type of noncanonical splice site is more inclined to occur at a 5′ untranslated GC-rich region (25–28) and is common in certain cancers (29, 30). Interestingly, the spliced region also contains G-quadruplex-like elements (31); these elements are thought to modulate gene expression at the translational level by forming stable RNA hairpin secondary structures in the 5′ untranslated region (UTR) of mRNA inhibiting the process of translation (32). Splicing of such elements found in the CPE-ΔN transcript could result in a translational "advantage" during tumor metastasis (see *Section VI*). The second splice variant *CPE* mRNA transcript is derived from alternative splicing at the 3′ donor site of exon 6 and

Figure 1.

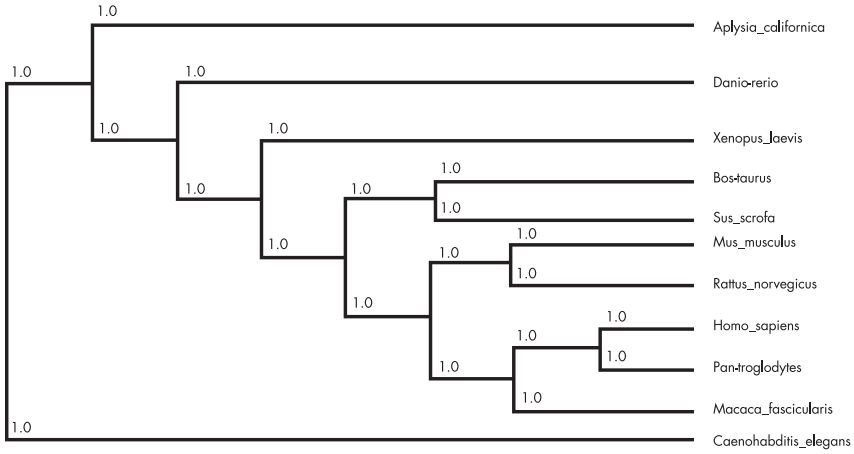

Figure 1. Phylogenetic analysis of CPE protein. The phylogenetic tree was built using 476 representative amino acids using Phylogeny.fr platform (www.phylogeny.fr/version2_cgi/phylogeny.cgi) and determined by the program Gblocks (328), which eliminates poorly aligned positions and divergent regions (removes alignment noise) after sequence alignment using multiple sequence comparison by log-expectation (MUSCLE) (329). Bootstrap values above 50% (0.5) are shown. Conserved position for at least half the number of sequences is represented by +1.

the 5′ donor site of exon 7 (Fig. 2, variant 1). This splicing event results in an 18-amino acid deletion within the area of the active site of the CPE protein, rendering this protein enzymatically inactive. This variant has a signal peptide and is therefore translocated into the RER cisternae and the secretory pathway. It is likely secreted into the extracellular space and may function as a ligand or signaling molecule. The evidence for these splice variants was derived from expressed sequence tag (EST) database searches. Interestingly, all these variants were found in human brain tissue, suggesting that the brain contains cells that are active in alternative splicing of *CPE* (33, 34).

B. Structure, biosynthesis, and intracellular trafficking of CPE

A schematic of the primary structure of the CPE protein is shown in Fig. 3A. It has a signal peptide directing the protein into the RER cisternae, a catalytic domain, and a highly acidic C-terminal domain. The three-dimensional structure of CPE (Fig. 3B) has been modeled based on the crystal structure of carboxypeptidase D, an enzyme homologous to CPE (35). The model (Fig. 3B) indicates several functional domains of CPE: the enzymatic active site showing the zinc (cofactor) binding site; a prohormone sorting signal binding site (see *Section III.A*), an amphipathic α-helical transmembrane domain, and the cytoplasmic tail that interacts with microtubule proteins for vesicle transport (see *Section III.B*).

CPE is a 476-amino acid protein synthesized as a precursor with a 25-amino acid signal peptide that directs proCPE into the cisternae of the RER and is then removed. The proCPE is transported from the ER through the Golgi complex to the granules of the RSP where the 17-amino acid "pro" region is removed after a penta-arginine sequence ($RRRRR_{42}$) (Fig. 3A), to generate the mature protein ($CPE_{43–476}$) (36). Processing of the pro region is required neither for enzymatic activity because proCPE is enzymatically active as a carboxypeptidase (37) nor for intracellular trafficking of CPE (38). The CPE protein is glycosylated at two N-linked glycosylation consensus sites, Asn_{139} and Asn_{390}. Under mildly acidic conditions and increasing calcium concentrations similar to that of the TGN, CPE has been shown to aggregate *in vitro* (39) with granule cargo proteins (40), suggesting that this occurs *in vivo* as a mechanism of condensation and sorting to the RSP. Binding of potential prohormone cargo to CPE via a prohormone sorting signal, such as that found in proopiomelanocortin (POMC) (41), could also occur in this compartment (42) through interaction of its prohormone binding site, composed of the amino acids Arg_{255} and Lys_{260} on CPE (43). In addition, the carboxyl terminus of CPE forms an amphipathic α-helix under acidic conditions and is involved in binding tightly to cellular membranes (44, 45). This binding step is important for its trafficking from the TGN to the granules of the RSP (46, 47). Binding of the carboxyl terminus of CPE with cholesterol-sphingolipid-rich domains (lipid-rafts) in the TGN membrane has been demonstrated (48), allowing it to act as a sorting receptor for prohormones (see *Section III.A*). A subpopulation of CPE molecules appears to have a transmembrane topology (49). CPE together with bound prohormones are packaged into immature granules budding from the TGN. Indeed, analysis of secretory granule membranes from bovine pituitary indicates that it is highly enriched with cholesterol, consistent with the notion that granules are budded from lipid raft domains of the TGN (50). Within these granules, some of the CPE molecules are further processed (51) at Arg_{455}-Lys_{456} (Fig. 3A) to yield a soluble form (molecular mass, ~50 kDa) (51), which is enzymatically more active than the membrane-associated form (molecular mass, ~53 kDa) (52). Soluble CPE then functions to cleave the C-terminal extended basic residues from the peptide hormone intermediates liberated by PC1 and PC2 in the granules. Sub-

TABLE 1. Different species with a conserved CPE gene

Species	Nucleotide sequence accession no.	Nucleotide identities (%)	Protein		Chromosome
			Identities (%)	Similarities (%)	
Homo sapien (man)	NM_001873	100	100	100	4q32.3
Pan troglodytes (common chimpanzee)	NM_001098559	99	99	99	4
Macaca fascicularis (crab-eating macaque)	AB169871	98	99	99	4
Bos taurus (cow)	NM_173903.3	88	93	96	17
Sus scrofa (boar)	NM_001097439.1	87	92	95	8
Mus musculus (mouse)	NM_013494.3	88	97	98	8, 32.6 cM
Rattus norvegicus (rat)	NM_013128.1	88	96	98	16p13
Gallus gallus (chicken)	CR388992.1	85	94	97	4
Danio rerio (zebrafish)	NM_214810.1	53	83	94	1
Xenopus laevis (African clawed frog)	NM_001127813.1	78	83	92	
Aplysia californica (sea slug)	NM_001204485	11	47	65	
Caenorhabditis elegans (nematode)	NM_069534.5	9	44	60	4

All the species except *Macaca fascicularis* and *Gallus gallus* have annotated gene entry in the NCBI database. The percentage identity and similarities were compared with human mRNA and protein. Chromosome loci for the Cpe gene have not been assigned for *Xenopus laevis* and *Aplysia californica*. cM, Centimorgan.

sequent to granule exocytosis, membrane CPE can recycle from the plasma membrane through the early endosomes and back to the TGN where it gets reused (53, 54).

Much debate has surrounded whether the membrane form of CPE does and can assume a transmembrane topology in the granules membrane. For instance, the primary structure of CPE at the C terminus that associates with the membrane does not contain a typical transmembrane domain as predicted from modeling programs (45) and as shown in biochemical experiments (45, 46, 51) that involved carbonate extraction studies (45) and secretion experiments (46). Additionally, the C-terminal membrane binding domain is highly acidic (Fig. 3A) and theoretically does not favor localization in a membrane lipid bilayer. However, studies on the insertion of a CPE C-terminal peptide (last 22 amino acids) into model membranes have indicated that it can shallowly embed in a lipid bilayer by itself at an acidic pH (49). Also, evidence from cell biological experiments strongly support the existence of a cytoplasmic tail in CPE that interacts with various cytoplasmic molecules such as dynactin to mediate granule transport in neuroendocrine cells (see *Section III.B*). In addition, after granule exocytosis, the C-terminus cytoplasmic tail of CPE has been shown to interact with Arf6, a small

Figure 2.

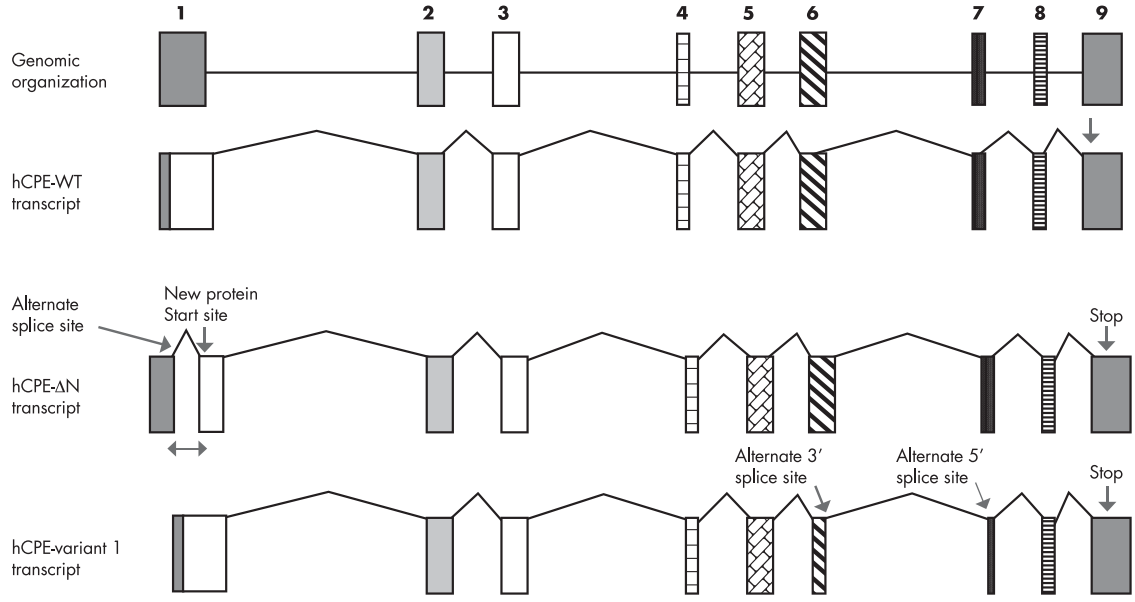

Figure 2. Schematic representation of the CPE gene and alternatively spliced variants. *Rectangular boxes* denote the exons (1–9). *Dark solid boxes* are the UTR, and *light and textured boxes* are the coding exons. Alternative splice sites to yield hCPE-ΔN and variant 1 transcripts are indicated with *arrows* on the exons. Stop codons are also indicated with *arrows* and introns as *solid lines*.

Figure 3.

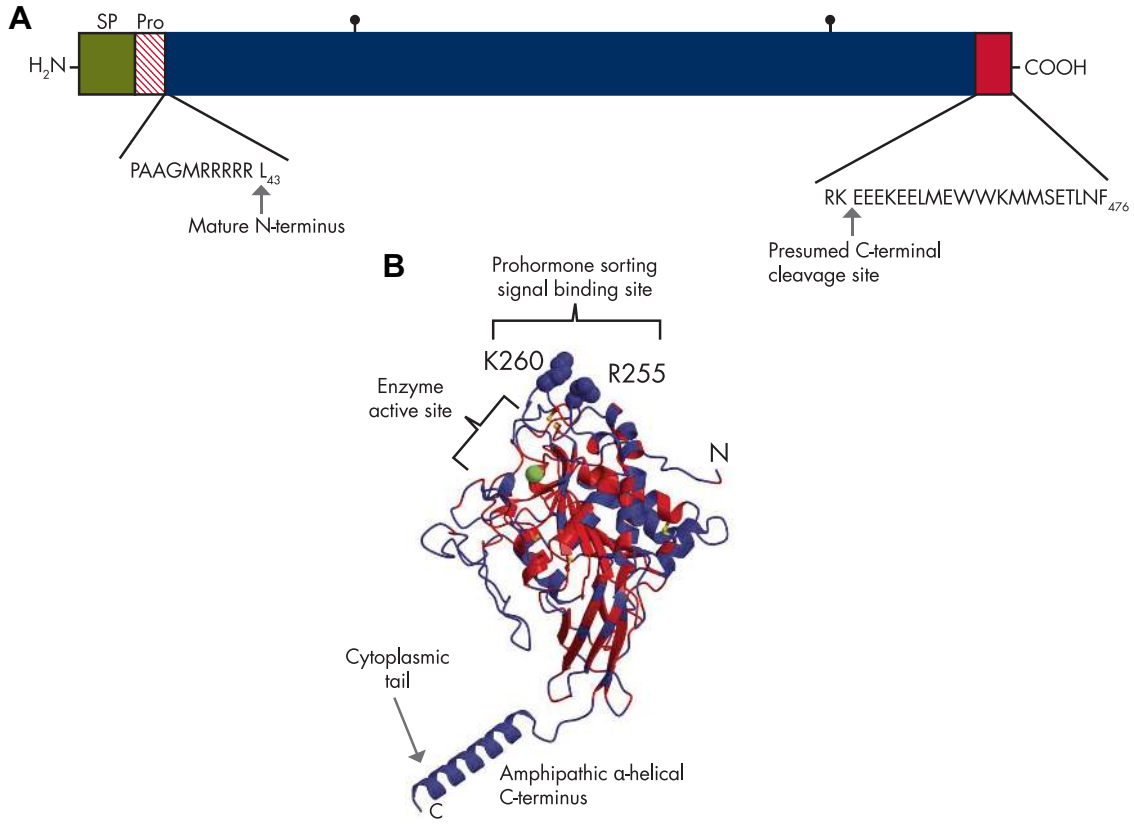

Figure 3. Schematic diagram and molecular model of the CPE protein. A, The preproCPE protein is 476 amino acids in length and contains a signal peptide (SP) that directs it into the ER. After cleavage of the SP, the proCPE (57 kDa) is trafficked through the Golgi and sorted into the granules of the RSP via interaction of its C-terminal amphipathic α-helical domain with cholesterol-sphingolipid-rich microdomains in the TGN. The Pro region (Pro) is processed within a post-Golgi compartment to generate the mature full-length membrane-bound CPE (55 kDa). Within the granules, the C terminus of this membrane-associated CPE can be cleaved presumably by a PC at a paired-basic residue cleavage site to generate a soluble form of mature CPE (53 kDa). B, The molecular model of CPE was based on the crystal structure of CPD. The *red areas* indicate the common overlapping homologous sequences shared between the two proteins, demonstrating a high degree of structural similarity. Unique to CPE are the two basic residues, Arg255 and Lys260, that were demonstrated to interact with the acidic residue-based prohormone sorting-signal found in POMC, proinsulin, and proBDNF. CPE also contains a unique C-terminal sequence that forms an amphipathic α-helix under acidic conditions. This C-terminal region is involved in tight membrane association and for a subset of CPE molecules can traverse the lipid bilayer, resulting in a small cytoplasmic tail that interacts with cytoplasmic proteins such as Arf6 and dynactin. The *green ball* represents the zinc atom in the active site. *Lollipop symbols* indicate the asparagine-linked glycosylation sites.

cytoplasmic GTPase, to mediate recycling of CPE from the plasma membrane back to the TGN for reuse (53). Furthermore, in another study, it was demonstrated that Arf6-dependent recycling of CPE mediated the endocytosis of the eosinophil cationic protein, a CPE-interacting protein (54). All these studies indicate the existence of a cytoplasmic tail and a transmembrane orientation of some CPE.

There are two possibilities by which CPE could achieve a transmembrane orientation. One is the insertion of the C terminus of CPE, under acidic conditions, through the TGN membrane with the help of a chaperone protein that could shield the acidic charges of the CPE C terminus in the lipid bilayer, allowing it to penetrate the membrane. As an example of this kind of mechanism, the diphtheria toxin α-subunit only partially penetrates artificial lipid bilayers by itself but is able to move across the membrane upon introduction of globule-like proteins as a chaperone (55). In addition, whereas it is known that C-terminal tail-anchored proteins (*e.g.*, cytochrome b5) are transmembrane proteins, the mechanism by which large domains of polypeptide are translocated across the phospholipid bilayer in an unassisted manner is still not fully understood because it is independent of the Sec61 translocon (56, 57). Of particular interest is the finding that CPE specifically interacts with Wolframin (58), an ER resident protein of unknown function, but may be involved in protein folding and intracellular transport. Mutations in the *WFS1* gene cause Wolfram syndrome—a syndrome characterized by diabetes insipidus, childhood-onset diabetes mellitus, optic atrophy, and deafness. Additional supporting evidence for a functional partnership

between CPE and Wolframin derives from the observation that both CPE and Wolframin are co-up-regulated in the amygdala of male rats subjected to cat odor (fear response) (59). These observations raise the possibility that Wolframin may assist CPE in its folding and/or trafficking as a chaperone through the ER to allow CPE to function efficiently downstream. Another report has noted the interdependence of CPE and phogrin, a receptor tyrosine phosphatase-like protein found in mature secretory granules of AtT20 cells (60). Under the mildly acidic environment of the TGN (61), CPE can interact with the N-terminal luminal domain of phogrin. Additionally, small interfering RNA (siRNA) silencing of CPE or phogrin reduces sorting of phogrin and CPE, respectively, into granules. Hence, the interaction of CPE with phogrin in the TGN may, in part, facilitate and stabilize a nonclassical transmembrane domain of CPE in that compartment.

The second possibility is that CPE does not fully enter the ER cisternae during synthesis because the C terminus is retained by an interacting protein on the outside of the organelle, leaving a cytoplasmic tail exposed (62). Understanding how CPE assumes a transmembrane orientation awaits further studies.

C. Distribution of CPE in embryonic and adult tissues

After the initial identification and characterization of CPE (2, 3), antisera specific for CPE became available. Initial studies were performed by immunohistochemistry (IHC) (63, 64), autoradiography, and binding studies using tritiated guanidinoethylmercaptosuccinic acid (GEMSA), a potent inhibitor of CPE (65–69). The IHC studies in rats showed a general localization of CPE in neuropeptide-rich areas of the brain and endocrine tissues such as the median eminence, supraoptic nucleus, paraventricular nucleus, and suprachiasmatic nucleus of the hypothalamus; the neural, intermediate, and selected cells in the anterior lobe of the pituitary; and the bovine adrenal medulla. Staining was also evident in the pyramidal neurons of the hippocampus, dentate gyrus, and amygdala. Using radiolabeled GEMSA, staining was reported in the rat epithelial cells of the stomach, colon, oviduct, and the acinar cells of the submandibular gland as well as the pancreatic islets of Langerhans (70) and the adrenal medulla (67). Staining in the rat heart atrial tissue (71, 72) also suggests a role for CPE in the physiology of atrial natriuretic factor in this organ. Other IHC studies have reported the expression of CPE in somatostatin-producing cells in rat brain (15); the gastrin cells and progenitor gastrin-somatostatin cells of the antropyloric mucosa of the gut in rats (73); and in different areas of the lung as part of an opioid network involved in respiratory function in humans (74, 75).

Subsequent to cloning of bovine and rat *Cpe* cDNA (12, 76), oligonucleotide probes were used for Northern blot and *in situ* hybridization. The expression patterns of CPE have been studied extensively in the rat brain, during embryonic development (77), and in the adult (78–80). In the adult brain, *Cpe* mRNA is highly expressed in pyramidal neurons of the hippocampus, amygdala, supraoptic nucleus, paraventricular nucleus, and ependymal cells of the lateral ventricle. Other areas of the brain include the piriform and entorhinal cortex, cerebellar cortex, thalamus, medial geniculate, and lateral septal nuclei. High expression is also found in the anterior and intermediate lobes of the pituitary, the adrenal medulla (77, 79), and pancreatic islets. With the characterization of fat cells as an endocrine tissue (81), CPE has also been found in sc and visceral fat (82), although its role in this tissue is unclear at this time.

During development, *Cpe* mRNA is first seen at embryonic day (E) 10, specifically in the diencephalon and spinal cord in rats (77). Because PC1 and PC2 are not expressed at this time nor is there a defined endocrine system, the role that CPE plays in these tissues at this developmental stage is unknown. It is possible that other PC-like enzymes not identified here are present; or furin, a ubiquitously expressed PC found in the TGN involved in processing constitutively secreted proproteins, could function upstream of CPE to provide proprotein intermediates as substrates for CPE. However, the furin expression pattern at this stage does not overlap with that of CPE. By E12, CPE expression is seen more extensively throughout the embryo, specifically in the nervous system in areas such as the neuroepithelium, peripheral ganglia, mesenchymal cells around the midgut mesentery, and the epithelia of the branchial arch. At E13, whereas the expression of PC1 and PC2 transcripts is restricted to the developing nervous system, high levels of CPE expression are seen throughout the embryo that overlap with PC1 and PC2 expression in addition to that for furin. There have been many other characterizations of CPE in specific cells/tissues under different experimental paradigms that can be found in Table 2.

D. Biochemical and enzymatic properties of CPE

CPE is a Zn^{++} metallocarboxypeptidase that cleaves the carboxy-terminal arginine (Arg) or lysine (Lys) residues from protein substrates; however, it can also cleave histidine poorly under acidic conditions. CPE prefers Arg over Lys residues with K_M values at approximately 50–100 μM and approximately 200 μM, respectively, for Met-enkephalin extended peptides (2). CPE has an optimum activity at pH 5–6 with the V_{max} declining sharply at pH below 5.0 due to a single ionizing group in the active site (83). Multiple ionizing groups in the active site at pH above 7.0 also have deleterious effects on activity such that

TABLE 2. Literature summary of the identification or expression of CPE in various tissue and cell systems

Tissue/cell	Experimental context	Experimental procedure	Ref.
Mouse cDNA library	Screen for caspase substrates	Cleavage of expressed proteins	337
Corpus luteum	Changes during luteal phase	Gene array, qRT-PCR	338
Dorsal root ganglia	Effect of monensin	Activity	339
Eye ciliary body	Ciliary epithelium as a neuroepithelium	Expression and subtractive libraries	203
H4-II-E-C3 hepatoma	Angiotensin II processing	RT-PCR	340
Rat CNS	Compare CPE and CPD	ISH, IHC	341
Breast cancer cells	Vasopressin processing	qRT-PCR, Western blot	342
Pituitary neurointermediate lobe	Regulation of expression	Northern blot	343
Testicular and epididymal transcriptomes	Analysis of epididymal segments	cDNA microarray	344
Bovine DNA	SNP analysis for meat quality	RFPL sequencing	345
Gastric enterochromaffin-like cells	Components of vesicle release	IHC	251
Antropyloric mucosa	Gastrin localization	IHC	73
Nervous system/mouse model of MS	Development of disease state	Oligonucleotide microarrays	325
RPE/choroid	Age-related changes in genes	cDNA microarray, RT-PCR	204
Intestine 407, human fetal epithelial cells	Overexpression of NeuroD	cRNA microarray	346
C. elegans neuromuscular junction	Impaired acetylcholine release	Paralysis assay	18
Pancreatic β-cells	Palmitate-induced apoptosis	Proteomics, Western blot	238
Brain	Global ischemia	Western blot	231
Lungs	CPE fat/fat mouse	Response to ozone	347
Airway responsiveness in mice	Effect of ozone inhalation	Compare CPE fat/fat mice to WT	348
Olfactory bulb, amygdala	Rat exposure to cat odor	Differential gene expression	59
Synaptic vesicles and PC12 cells	Characterization	EM, TIRF, subcellular organelles	142
Lung epithelium	Effect of smoke inhalation	cDNA microarray	349
Seminal plasma	Identification of proteins	LC-MS/MS	350
Immunocytes	Inflammation/pain	IHC	351
Rats	Alcohol effects on behavior	CPE activity	352
Multiple cancer tissues	Review	Database mining	247
Cultured lens tissue	Calcium influx by ionophore	2D-PAGE/MS	353
Small-cell carcinoma	Peptide processing	qRT-PCR, Western blot	253
N/A	PI3K-mTOR pathway analysis	Yeast-2-hybrid, interactome mapping	354
Prefrontal cortex of piglets	Social isolation stress	Microarray, qRT-PCR	235
Cat visual cortex	Young *vs.* adult cats	Subtractive hybridization	355
Avian pancreas	Embryonic development	IHC	356
Placenta, umbilical cord	Compare CPD and CPE	IHC	357
Ovary	FSH-responsive cells	cDNA microarray	358
Neural complex	Analysis in chordates	EST sequencing, ISH	359
Placenta	Stages of gestation	ISH	360
Retinal tissue/cells	Processing of NPY	Activity, ICC, Western blot	361
RGC-5 retinal cells	Ischemia	Western blot, activity	362
Cultured astrocytes and neurons	Secretion and tissue analysis	CPE activity and Northern blots	363
Basophilic mast cells/Jurkat cells	Secretory vesicles	ICC	364
Chicken thymus	Colocalization with CgA	RT-PCR, Western blot, IHC	17
Brain	Ischemia	ISH, ICC, Western blot	232
Retinal photoreceptors	CPE KO and fat/fat mouse	IHC	141

Table includes published literature where CPE expression was identified and/or characterized in a wide variety of tissues and cell systems. The experimental context and technique of the characterization of CPE is annotated. Some citations made in the text may be duplicated here. CNS, Central nervous system; ISH, *in situ* hybridization; qRT-PCR, quantitative real time PCR; ICC, immunocytochemistry; LC/MS, liquid chromatography/mass spectroscopy; EM, electron microscopy; 2D-PAGE, two-dimensional PAGE; N/A, not applicable; MS, mass spectroscopy; PI3K-mTOR, phosphatidylinositol 3-kinase-mammalian target of rapamycin.

CPE becomes inactive at pH 7.4. Aside from pH requirements, CPE utilizes zinc as the coordinating metal in its active site to mediate peptide hydrolysis (84). Cobalt, another divalent transition-state metal, can stimulate enzymatic activity (2). Although calcium can bind CPE (85) and this binding decreases the thermostability of the enzyme (85), it exerts no effects on the aggregation of the soluble CPE (39). CPE is a metallocarboxypeptidase; therefore, chelating agents, such as 1,10-*o*-phenanthroline, are effective inhibitors. Several thiol-directed inhibitors are also highly effective, such as *p*-chloromercuriphenylsulfonate and $HgCl_2$ (86). In addition, mimetic substrates designed as active site-directed inhibitors, *e.g.*, GEMSA, with a Ki of approximately 8 nM (87), are used as potent inhibitors.

The importance of CPE in peptide hormone and neuropeptide processing has become especially evident with the CPE-deficient mice. Specifically, using a peptidomics approach comparing WT and $Cpe^{fat/fat}$ mice, new potential substrates of CPE have been identified (88, 89). Indeed, identification of proSAAS, an endogenous inhibitor of PC1/3, was made by this approach (90), as was the

discovery of the involvement of CPE in the processing of hemopressins (91)—hemoglobin peptides found in the brain that bind cannabinoid CB1 receptors (92). Other studies include WT and *Cpe*$^{fat/fat}$ mice comparisons of peptides in the prefrontal cortex, pituitary, and brain (93–95). Additional effects of food deprivation and exercise on hypothalamic peptides have been investigated (96). Peptidomic analyses have also been applied to hypothalamus and hypothalamic responses to chronic morphine (97) and cocaine treatments (98) to study the possible role of neuropeptides in drug addiction.

III. CPE in Prohormone Sorting and Vesicle Transport

Endocrine and neuroendocrine cells have two different secretory pathways: constitutive and regulated. The constitutive secretory pathway (CSP) supports continuous protein secretion, independent of stimulation (99). In the CSP, small secretory vesicles formed at the TGN are constantly transported to and fused to the plasma membrane without storage. Because CSP proteins are continuously secreted, this pathway is driven primarily by the biosynthesis of secretory proteins at the ER (100). The CSP provides membrane proteins, such as receptors (101), to the plasma membrane, as well as various secretory proteins (102, 103), for maintenance of cell survival, differentiation, and growth. Although the CSP is present in all cells, the RSP is unique to endocrine and exocrine cells and neurons. Hormones and neuropeptides that are critical for mediating various endocrine functions, neurotransmission, and neuronal plasticity (104–107) are secreted via the RSP.

RSP proteins such as prohormones and proneuropeptides are synthesized at the RER, inserted into the RER cisternae, and then transported to the Golgi complex. At the TGN, the prohormones and their processing enzymes are sorted away from constitutively secreted and lysosomal proteins and are packaged into specialized budding vesicles called immature granules destined for regulated secretion. The newly synthesized hormone-containing immature granules are then transported from the post-Golgi network to storage sites in the proximity of secretion sites at the plasma membrane while undergoing maturation that includes acidification of the granule, processing and condensation of cargo proteins, as well as removal of constitutive proteins via the clathrin-dependent constitutive-like secretory pathway (108, 109) to give rise to the mature granule or large dense-core vesicle (LDCV). Upon stimulation of endocrine cells or neurons, the mature granules dock at the plasma membrane and undergo exocytosis to release their contents (Fig. 4). The mechanisms by which CPE mediates prohormone sorting at the TGN to the RSP, peptide processing, and granule transport to the release sites are reviewed below.

A. CPE function as a prohormone sorting receptor for the regulated pathway

The search for the mechanisms involved in the sorting of prohormones at the TGN into vesicles of the RSP has been difficult and challenging. Several primary sequence domains, as well as regions representing loop structures stabilized by disulfide bridges, have been proposed as motifs for sorting various prohormones to the RSP (110–112). These have been reviewed elsewhere (110). However, the mechanism by which these domains mediate sorting is unclear. Nevertheless, it has also been proposed that prohormones are passively sorted into the RSP by aggregation that segregates them from other proteins (113–116). It is clear that whereas aggregation is important as a concentration step, it is insufficient to sort prohormones to the RSP [for review, see Dikeakos and Reudelhuber (117)]. Interaction of a specific domain of the prohormone with the TGN membrane seems to be necessary for sorting to occur. Molecular modeling studies have identified a three-dimensional consensus sorting motif that is comprised of two acidic amino acid residues located a specific distance apart from each other (12–15 Å) and two hydrophobic residues (5–7 Å apart) exposed on the surface of the molecule. This motif has been identified for POMC, proinsulin, and proenkephalin that facilitate their sorting into the RSP of endocrine cells (41, 110, 118, 119). The same conformation-dependent sorting motif has been subsequently identified in the structure of brain-derived neurotrophic factor (BDNF) and shown to be necessary for sorting this molecule to the RSP (120).

Molecular modeling studies of CPE have indicated a binding domain on CPE that contains two basic amino acid residues (Arg_{255} and Lys_{260}; see Fig. 3) with the appropriate molecular distance from each other that allows docking with the two acidic residues in the sorting motif of POMC, proinsulin, proenkephalin, and pro-BDNF (119–121). Binding studies further demonstrated a specific interaction of membrane CPE with N-$POMC_{1–26}$, a peptide containing the POMC sorting motif, with a K_D of 6 μM.

Evidence in support of membrane CPE as a sorting/retention receptor came from using antisense or RNA interference technology to down-regulate CPE expression in model cell lines, as well as by using CPE KO and *Cpe*$^{fat/fat}$ mice that show diminished regulated secretion and missorting of proinsulin (118), POMC (41), and BDNF (120) to the CSP in the absence of CPE. These early studies were

Figure 4.

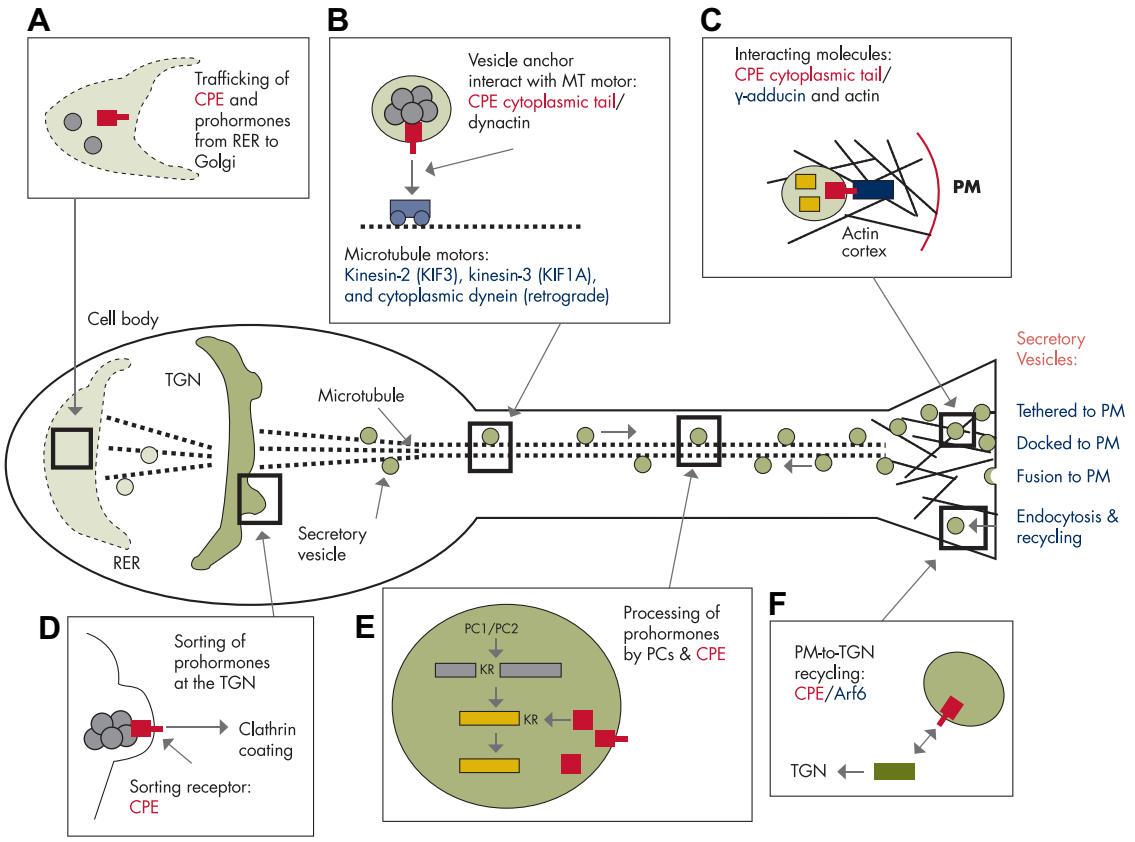

Figure 4. Trafficking of CPE in the RSP of (neuro)endocrine cells. A, Newly synthesized CPE (*red*) and prohormones (*gray balls*) in RER move from the RER to the Golgi complex via a microtubule-based vesicle transport. *Thick dotted lines* represent microtubules. B, Within the TGN (pH = 6.0–6.5), the amphipathic region of C-terminal CPE forms an α-helix structure that embeds into lipid raft domains in the TGN. In a subpopulation of CPE molecules, this C-terminus domain penetrates through the lipid-raft-rich domains of the TGN membrane to form a cytoplasmic tail. Prohormones aggregate and bind to membrane CPE (sorting receptor) at the TGN and are then sorted into the RSP. C, Budded RSP vesicles containing prohormones bound to CPE recruit dynactin, an anchor for microtubules and microtubule motors, via the CPE cytoplasmic tail. Kinesin-2 and kinesin-3 mediate anterograde vesicle transport toward the secretion sites in the neurite terminals, whereas cytoplasmic dynein mediates retrograde transport toward the cell body. MT, Microtubules. D, During Golgi-to-plasma membrane (PM) transport, proprotein convertases 1 and 2 (PC1 and PC2) cleave prohormones between or on the carboxyl side of paired-basic residues, usually lysine (K) and arginine (R). CPE then cleaves off the extended basic residue(s) from the C terminus to generate mature neuropeptides/hormones. E, At the proximity of the plasma membrane in endocrine cells and at the presynaptic terminal for neurons, respectively, the CPE cytoplasmic tail can interact with γ-adducin, an actin cortex-interacting molecule. The interaction localizes/transports vesicles containing CPE and mature neuropeptides/hormones to the preactive zone beneath the plasma membrane, which is required for the activity-dependent secretion of neuropeptides and hormones. F, After exocytosis of hormones and neuropeptides, the transmembrane CPE is endocytosed and recycled back to the TGN via the interaction of its cytoplasmic tail with Arf6. PM, Plasma membrane.

considered controversial because another study using pancreatic β-cells from the *Cpe*$^{fat/fat}$ mouse revealed no significant differences in the regulated secretion of insulin *vs.* the normal mouse, suggesting that there is no defect in sorting insulin to the RSP in the absence of CPE (122). However, it was subsequently found that up to approximately 45% of mutant CPE escaped degradation within 2 h of expression and was found in the secretory vesicles in the pancreatic β-cell line derived from the *Cpe*$^{fat/fat}$ mice (123), compared with the apparent complete degradation in the anterior and intermediate pituitary cells used in the initial studies by others (6, 124). The significant amount of mutant CPE present in the pancreatic β-cells may be sufficient to function as a sorting/retention receptor, thus explaining the differences in results. More recently, Hosaka *et al.* (125), using anterior pituitary cells from the *Cpe*$^{fat/fat}$ mice, found that there was a significant increase in the amounts of the secretory granule protein, secretogranin III, in these cells that could bind and sort POMC at the TGN in these mice. This effect could in part compensate for the lack of CPE, leading to some, although diminished, regulated secretion of POMC/ACTH compared with WT mice. More importantly, they also demonstrated increased constitutive secretion of POMC/ACTH from the

anterior pituitary cells of these mice, supporting a role for CPE in sorting POMC to the RSP (125). Thus, in the analysis of prohormone sorting, it is necessary to consider not only stimulated secretion, but also constitutive secretion, as well as possible effects on synthesis and degradation of the prohormone in the experimental condition, especially after suppression of CPE expression, to fully follow the routing and fate of the prohormone molecules. However, a recent report studying the secretion behavior of newly synthesized ACTH in AtT20 cells concluded that CPE did not play a role in POMC sorting in these cells (126). Unfortunately, the significantly elevated levels of constitutively secreted POMC, observed in this experiment when CPE expression was acutely reduced by siRNA silencing, were not addressed adequately. The amount of newly synthesized POMC was similar between scrambled and CPE siRNA-treated cells; hence, the elevated level of newly synthesized POMC in the medium was not due to elevated expression. This observation suggests that normal trafficking of POMC is grossly perturbed in the absence of CPE. Hence, CPE appears to be involved in POMC trafficking in AtT20 cells despite the production and stimulated secretion of ACTH.

B. CPE mediates post-Golgi hormone vesicle transport

The precursors of hormones and neuropeptides are packaged at the TGN into immature granules, which become mature granules or LDCV as they are transported to the secretion sites for activity-dependent secretion in endocrine cells and neurons (Fig. 4). To reach the secretion sites at the plasma membrane of endocrine cells or at the nerve terminals of neurons, LDCV use microtubule-dependent transport systems mediated by kinesins (127–133). Final movement of LDCV to just beneath the plasma membrane or active zone for release involves an actin-myosin-based mechanism (134–136). Unused LDCV in the transiting pool can be trafficked back to the cell body (137) by the retrograde microtubule motor complex, cytoplasmic dynein (138) for either reuse or degradation by the endosome/lysosome system.

Support for the role of CPE in vesicle transport comes from a number of correlative reports. Enhanced expression of CPE leads to increased trafficking of the dopamine transporter to the presynaptic membrane at the axonal terminal of dopaminergic neurons, thereby enhancing dopamine uptake (139). The neural cell adhesion molecule, contactin-associated protein 2 (Caspr2), directly interacts with CPE in the Golgi complex within the cell body of rat cortical neurons. This interaction increases transport of Caspr2 to apical dendrites (140). In *C. elegans*, the homolog of CPE (*egl-21*) has been shown to be involved in the release of acetylcholine at the neuromuscular junction (18), whereas studies with CPE KO and $Cpe^{fat/fat}$ mice have suggested that transport of SV from the cell body at the inner segment of the retina to the nerve terminals at the outer plexiform layer is defective (141). More direct evidence comes from live cell-imaging studies, which showed that overexpression of the cytoplasmic tail (~10 amino acids; see Fig. 3) of CPE directly reduced real-time movements of POMC/ACTH LDCV containing CPE tagged with green fluorescent protein (GFP; CPE-GFP) (133). The final movement of LDCV through the actin cortex just beneath the plasma membrane may also involve interaction of the CPE tail with actin-associated proteins, such as γ-adducin (142). Total internal reflection fluorescence (TIRF) microscopy studies suggest that localization of synaptic-like microvesicles (SLMV) to the plasma membrane (within <200 nm) in PC12 cells also involves the CPE tail (142).

To understand the mechanism of CPE involvement in granule transport, glutathione-S-transferase pull-down, copelleting, and coimmunoprecipitation experiments with AtT20 cell cytosol were carried out (133). These studies showed that the tail bound to a complex containing the anterograde motors, kinesin-2 and kinesin-3, as well as the retrograde motor, cytoplasmic dynein (138), and the dynactin complex (143); kinesin-1 was not involved (Fig. 5). Kinesin-2 consisting of KIF3A, KIF3B, and KAP moves along microtubules at speeds of 0.3–0.5 μm/sec. Kinesin-3, also known as KIF1A, is the fastest motor (~1 μm/sec) and has also been reported by others to mediate anterograde transport of CPE (*Egl-21*)-containing peptidergic vesicles to the neuromuscular junction in *C. elegans* (18). Thus, kinesin-2, kinesin-3, and cytoplasmic dynein are associated with the CPE cytoplasmic tail via dynactin (144). Indeed, endogenous dynactin significantly colocalized with POMC/ACTH vesicles along the processes of AtT20 cells.

Thus, the current studies indicate that the CPE cytoplasmic tails on LDCV bind dynactin that, in turn, recruits a motor complex of kinesin-2, kinesin-3, and cytoplasmic dynein. The recruitment is required for rapid processive movement of POMC/ACTH granules toward and along the processes of anterior pituitary cells for delivery to the release site for secretion (see model in Figs. 4 and 5), as well as for retrograde transport. Similar studies on BDNF vesicle transport in hippocampal neurons (132) also revealed involvement of the CPE cytoplasmic tail in the recruitment of dynactin for anterograde transport of BDNF for activity-dependent release and retrograde transport of these vesicles to the cell body, presumably for degradation to maintain homeostasis of the number of granules/vesicles at the storage depot in the proximity of the secretion sites (137).

Figure 5.

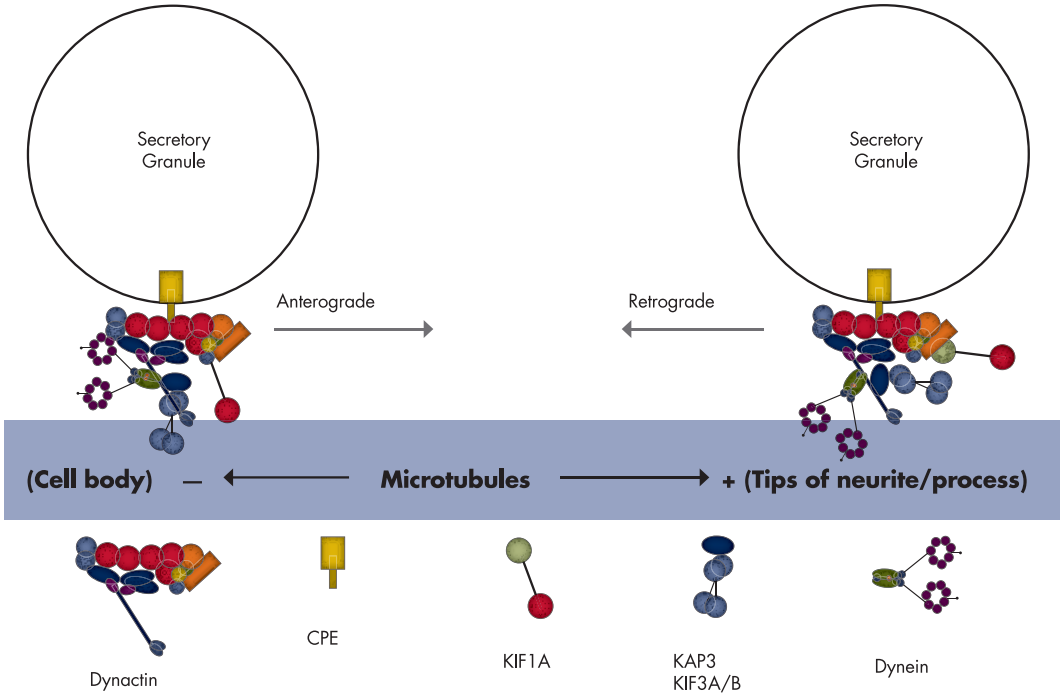

Figure 5. Schematic diagram of the interaction of CPE to microtubule motors. The cytoplasmic tail of transmembrane CPE in secretory peptidergic granules recruits dynactin that associates with and confers processivity to KIF3A (kinesin 2) and KIF1A (kinesin 3). Kinesin 2 that consists of two motor proteins, KIF3A and KIF3B, and a cargo binder, KAP3, is known to bind dynactin directly. Kinesin 3 is a fast-moving (>1 μm/sec) plus-end microtubule-based motor and forms a homodimer. Kinesin 2 and kinesin 3 simultaneously bind dynactin and microtubules to mediate delivery of these vesicles to the release site for activity-dependent secretion of hormones and neuropeptides in (neuro)endocrine cells. Cytoplasmic dynein, a minus end-directed motor complex, also binds dynactin and mediates return of secretory granules from the end of the process back to the cell body under nonstimulated conditions.

C. CPE mediates synaptic vesicle localization to nerve terminal preactive zone

The presynaptic terminals of peptidergic/neuroendocrine neurons in the hypothalamus contain both synaptic and peptidergic vesicles, although SV predominate (142, 145, 146). Likewise, endocrine cells, such as chromaffin cells, have SLMV and LDCV beneath the plasma membrane (147–150). For SV and LDCV, there are different groups of vesicles that have been characterized on the basis of their sensitivity to extracellular stimuli: the reserve pool, the slow-response pool, and the readily releasable pool (151–154). For SV, approximately 80% at the terminal belong to the reserve pool that responds to stimulation very slowly (within minutes). The slow-response pool (approximately 19%) secretes its contents more acutely (within a few seconds). The reserve and slow-response pools are mixed and are held within the presynaptic bouton and at the proximity of the plasma membrane of neuroendocrine and endocrine cells, respectively, by actin-based tethering. The readily releasable pool (approximately 1%) of vesicles is docked to the presynaptic and plasma membrane at the active zone and responds immediately to stimulation. After stimulation, vesicular membrane proteins are endocytosed to form empty SV or SLMV, which are then refilled with neurotransmitters by vesicle-associated transporters, such as vesicular glutamate transporters and vesicular acetylcholine transporters (155, 156). Some vesicles formed by endocytosis are recycled back to late endosomes and lysosomes at the cell body for degradation to prevent overpopulation of vesicles at the nerve terminal and at the plasma membrane of (neuro)endocrine cells (157, 158).

Recently, transmembrane CPE has been found in SV in hypothalamic peptidergic neurons, but not in SV in the rest of the brain (142). Recruitment of transmembrane CPE into SV membranes is most likely achieved by recycling of the presynaptic membranes containing CPE deposited by LDCV after fusion, after stimulation of these neurons. In the hypothalamus of CPE KO mice, electron microscopy revealed a significant (~3-fold) reduction of docked SV within the preactive zone (between 0 and 100 nm from the presynaptic membrane aligned with the postsynaptic density) in presynaptic boutons, compared with that in WT mice. Notably, the pool of SV that was absent

within the 0- to 100-nm zone was found above 300 nm in the CPE KO mice. Consistent with impaired localization of SV at the proximity (<100 nm) of the presynaptic membrane from CPE KO mice, stimulated glutamate release from hypothalamic neurons in these mutants was decreased compared with WT mice (142). These data suggest that the pool of hypothalamic SV containing the cytoplasmic tail of CPE may interact with cytoplasmic proteins to mediate retention of SV within the less than 100-nm preactive zone. Although it is difficult to demonstrate this point in hypothalamic neurons, studies using TIRF microscopy on the neuroendocrine chromaffin cell line, PC12, were carried out to investigate this further (142). PC12 cells express CPE and contain LDCV as well as SV counterparts called "synaptic-like microvesicles" (SLMV). It was found that the average intensity of synaptophysin-red fluorescent protein containing SLMV in the TIRF zone was decreased approximately 2-fold when GFP-CPE cytoplasmic tail (C-terminal 15 amino acids acting as a dominant negative) was overexpressed compared with cells overexpressing GFP alone. Hence, excess CPE cytoplasmic tail peptides interfered with retention of SLMV within the TIRF zone, similar to the observation of the lack of SV within the preactive zone (<100 nm) in the hypothalamus of CPE KO mice, suggesting a common mechanism.

Defective glutamate-mediated neurotransmission has also been reported in the photoreceptors of CPE KO and $Cpe^{fat/fat}$ mice. Electroretinograms showed reduced glutamate-mediated b-wave activity and decreased number of SV per synapse in the photoreceptors, likely due to impairment of SV transport and glutamate secretion in these CPE-deficient mice (141). Collectively, these studies indicate a critical role for the CPE cytoplasmic tail both in SV localization and in tethering to the active zone at the nerve terminal in some neurons to mediate neurotransmitter secretion.

IV. CPE Action in the Endocrine System—Insights from Mouse Models

One of the CPE animal models arose from a spontaneous autosomal recessive mutation identified in a colony of mice at Jackson Laboratories (159). Because the mice were observed to be obese, diabetic, and infertile, they were termed *fat/fat* mice. Gene mapping studies identified the *fat* mutation to be on chromosome 8, near the locus for *Cpe*. Subsequently, the mutation was localized to the *Cpe* gene and was termed $Cpe^{fat/fat}$. The mutation gave rise to a Ser202Pro amino acid change in the mature CPE protein (6) that rendered the protein unstable and subject to degradation (6, 160). When expressed in a baculovirus expression system, the CPE(Ser202Pro) mutant was shown to lack enzymatic activity and to be deficient in trafficking through the RSP, and it failed to be secreted when expressed in AtT20 cells but was degraded in the ER (161). Similar degradation and lack of secretion was reported in immortalized pancreatic β-cells (NIT3) from the $Cpe^{fat/fat}$ mouse (162). Despite these findings, another study demonstrated that a portion (~45%) of the newly synthesized CPE(Ser202Pro) protein escaped degradation in the ER, was colocalized in mature β-granules, and was secreted in a regulated manner from the NIT3 cells of the $Cpe^{fat/fat}$ mouse (123). These latter findings demonstrated that whereas the $Cpe^{fat/fat}$ mice were defective in CPE enzymatic activity, they were not completely devoid of CPE protein in all tissues. To clarify this point, a KO mouse was generated with deletion of exons 4 and 5 in *Cpe* (7). The CPE KO mice showed a complete absence of CPE and shared many of the phenotypic characteristics of the $Cpe^{fat/fat}$ mice. In *Sections IV.A to IV.C*, insights into the role of CPE in diabetes, obesity, bone remodeling, and infertility gained from these two CPE-deficient mouse models will be discussed. Studies on these models highlight the effects of CPE mutations in humans that can lead to CPE deficiency (see *Section VII*).

A. Diabetes in CPE-deficient mice

The CPE KO and $Cpe^{fat/fat}$ mice develop diabetes. Because of their different genetic backgrounds (*i.e.*, C57BKS for $Cpe^{fat/fat}$ and C57BKS/SV129 for the CPE KO mouse), slight differences in the progression of the disease were observed (6, 7, 163). However, in general, these mutants had higher glucose levels at 8–10 wk of age, which increased significantly soon after to peak at 17–20 wk. High glucose levels were maintained for approximately 2 months, after which levels began to decrease, suggestive of a reversible diabetic phenotype. The $Cpe^{fat/fat}$ females failed to develop the severe hyperglycemia of the $Cpe^{fat/fat}$ males (163). By comparison, CPE KO females not only developed hyperglycemia but also were more severely glucose intolerant than CPE KO males at similar ages. In addition, older females became insulin-resistant, whereas the males were less affected (7). The reversal of the diabetic phenotype seen for the male and female CPE KO mice and the $Cpe^{fat/fat}$ males represents an interesting observation. Concomitant with the development of hyperglycemia, fasting levels of plasma insulin-like immunoreactivity increased in parallel and were composed primarily of proinsulin (6, 7). The plasma levels of proinsulin in both animal models were exceptionally high (up to 100 ng/ml in the CPE KO mice), and it reached a plateau in the KO mice at approximately 30 wk. This is within the age range when the hyperglycemia began to revert toward

normal and suggests that the excessive levels of circulating proinsulin may have contributed to this reversal. This is not unexpected because proinsulin has insulin signaling activity, but at approximately 1% of that for mature insulin (164). The maintenance of elevated fasting glucose in the older CPE KO females (~1 yr old) was likely due to the insulin resistance as demonstrated in the fat cells of these mice (7).

Hyperproinsulinemia is a phenotype of the CPE-deficient animals, and this condition suggests a processing and/or secretory defect of proinsulin from the pancreatic islets. Indeed, IHC specifically for proinsulin in *Cpe*$^{fat/fat}$ islets showed significantly elevated staining in the pancreatic β-cells (163), and studies on isolated pancreatic islets showed stimulated secretion of primarily proinsulin (122). These findings were confirmed by transmission electron microscopy demonstrating the presence of granules without the characteristic electron-dense core seen in mature β-granules. Instead, granules were filled with an electron-lucent material consistent with noncrystallized proinsulin (6, 165).

B. Obesity in CPE-deficient mice

Besides being diabetic, the CPE KO and *Cpe*$^{fat/fat}$ mice are also obese (7, 163). The CPE KO mice are born as runts; by approximately 4 wk of age, they begin to gain weight, so by 8 wk they are heavier than their WT littermates. This weight gain continues into adulthood where at approximately 1 yr, both male and female CPE KO mice are two to three times heavier than their WT or heterozygote littermates. The weight gain is due almost exclusively to increased fat deposition, where approximately 40% and approximately 54% of the weight is contributed by fat in male and female CPE KO mice, respectively. Their obesity phenotype appears to be more severe than those of the *Cpe*$^{fat/fat}$ mice with weights reaching 70–80 g in the former. The onset of obesity in the CPE KO and *Cpe*$^{fat/fat}$ mice appears to be due to increased consumption of food (7, 166), although younger *Cpe*$^{fat/fat}$ have been reported to consume similar amounts of food as the WT controls (163). Additionally, the CPE KO mice have a decreased basal metabolic rate, reduced utilization of lipids for energy, and reduced spontaneous activity (7), all of which contribute to the obesity phenotype.

Eating and satiety are governed by multiple signals that involve the activation of both peripheral and central pathways, including leptin (167), cholecystokinin (CCK) (168), and glucagon-like peptide 1 (169) (Fig. 6). Leptin is a 16-kDa protein secreted from white fat cells in response to insulin (170) and is a key regulator of eating behavior (171). It activates receptors in the arcuate nucleus and ventromedial hypothalamus, which in turn signal hypothalamic POMC and cocaine- and amphetamine-regulated transcript (CART) neurons to generate α-MSH and mature CART. These latter peptides are strong anorexigenic peptides liberated by proteolytic processing of POMC and proCART, respectively (172, 173). At the same time, leptin also represses the signals from neuropeptide Y (NPY) and agouti-related peptide (AGRP) neurons that promote feeding. Although leptin levels in the CPE KO and *Cpe*$^{fat/fat}$ mice are elevated, they are not exceptionally high (7). It should be emphasized that a certain degree of leptin resistance occurs, resulting in the lack of signaling to the hypothalamic neurons involved in food satiety (163).

Recently, it has been shown that FOXO1, a transcription factor involved in the regulation of food intake, negatively controls the expression of CPE in hypothalamic POMC neurons (174). Ablation of FOXO1 specifically in hypothalamic POMC neurons results both in an increase in CPE and α-MSH expression, reducing food intake (174). Furthermore, in diet-induced obesity where CPE is normally decreased, ablation of FOXO1 in POMC neurons protected the animals from weight gain due to sustained expression of CPE and levels of α-MSH. Hence, CPE and its ability to generate α-MSH in these hypothalamic neurons plays a pivotal role in the regulation of food intake. It is not surprising therefore that in the hypothalamus of the CPE KO mice, the levels of mature α-MSH are reduced by approximately 94% compared with WT littermates (124). Parenthetically, these findings help to explain the CPE KO hyperphagic behavior where not only is leptin signaling blunted but also POMC processing to α-MSH is depressed. Other peptides involved in feeding behavior that have been analyzed from CPE KO or *Cpe*$^{fat/fat}$ mice include CART, NPY, and CCK (Fig. 6). Although overall levels of CART and NPY immunoreactivity in CPE KO hypothalamus are generally similar to WT controls, in both cases there is a marked lack of the mature bioactive peptides (124), suggesting the presence of precursor and intermediate forms of the peptides in this tissue. Reduced levels of CART peptide in humans with *CART* mutations (175, 176) and in the CART KO mice (177, 178) lead to an obesity phenotype. Hence, obesity is expected in the CPE KO mice. On the other hand, NPY is a powerful orexigenic peptide that stimulates feeding (179). Absence of mature NPY in the hypothalamus should result in reduced feeding and weight loss, as reported in the NPY KO mice (180). However, because obesity is seen in the CPE KO mice, it would appear that NPY is upstream of CART signaling. An additional contributor to control of feeding may be CCK8, a central peptide involved in food intake. Levels of CCK8 in the brains of *Cpe*$^{fat/fat}$ mice were re-

Figure 6.

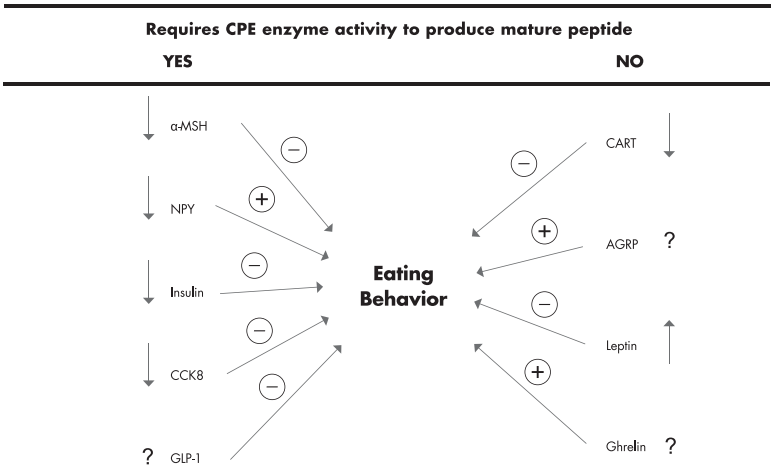

Figure 6. Summary of peptides involved in eating behavior. The control of eating behavior is complex and includes peptide signaling from peripheral and central sources. This is a short list of peptides that play a role in controlling this behavior. CART, α-MSH, insulin, glucagon-like peptide 1 (GLP-1), CCK8, and leptin all reduce eating behavior, indicated by the *minus sign*, whereas others like NPY, AGRP, and ghrelin stimulate eating, indicated by the *plus sign*. Peptides requiring CPE enzymatic activity are listed on the *left*, whereas those that do not require it are listed on the *right*. The *down and up arrows* indicate levels of the peptides in the CPE KO or $CPE^{fat/fat}$ mice as reduced or increased, respectively, compared to WT control mice. ?, Not determined.

duced by 74–90% with a concomitant increase in the precursor, CCK-Gly-Arg-Arg (181, 182).

Although the effect of CPE activity on feeding behavior is a prominent factor in the development of obesity, additional evidence suggests a role also for CPE in adipose tissue. For instance, CPE mRNA is abundant in sc and mesenteric fat (183). Interestingly, expression of CPE is at least 15-fold higher in visceral compared with sc fat along with thrombospondin-1 (82). Parenthetically, thrombospondin-1 is an extracellular matrix glycoprotein involved in forming multiprotein complexes with structural macromolecules that can interact with growth factors, cytokines, and proteases (184, 185). Because adipocytes do not contain a RSP (186) and because major peptide hormones (adiponectin, leptin, or resistin) produced by fat cells do not require processing by PC or CPE, the co-up-regulated expression of CPE with thrombospondin-1 suggests a functional role for CPE in this process that is distinct from peptide processing.

C. Bone metabolism in CPE-deficient mice

The regulation of bone metabolism is a complex balance between bone formation by osteoblasts and bone resorption by osteoclasts that dictate bone density (187, 188) (Fig. 7). In both male and female CPE KO mice, bone mineral density (BMD) is lower than WT littermates (124). In addition, levels of osteocalcin, a marker of osteoblast activity, and carboxy-terminal collagen crosslinks, a marker of osteoclast activity, were elevated, indicating that bone turnover was increased in the CPE KO mice (124). Osteocalcin is a peptide hormone produced by osteoblasts that acts on the pancreas to release insulin in addition to acting on fat cells to produce adiponectin that increases their sensitivity to insulin (189). The relationship of these two peptides to each other reflects the close connection between bone physiology and glucose homeostasis (Fig. 7).

The increased bone turnover in the CPE KO mice indicated a dysregulation in the peptides involved in governing this process, such as CART. Analysis of CART levels in the serum (7) and hypothalamus (124) of the CPE KO mice showed a virtual absence of the mature bioactive form. Thus, the inhibitory function of CART in bone resorption is absent in the CPE KO mice, which leads to a lower BMD. It is interesting to note that the lack of NPY and α-MSH, as mentioned above in the CPE KO hypothalamus, may be expected to increase the BMD, as it does in NPY KO mice (180) and NPY receptor Y2 KO mice (190), as well as in melanocortin 4 receptor (MC4R) KO mice (191). However, because BMD does not increase in the CPE KO mice, it appears that the involvement of CART in bone remodeling is downstream of NPY and α-MSH. Indeed, the increased bone mass associated with the MC4R KO mice has been attributed to increased CART expression because removing one allele of the *Cart* gene from mice heterozygous or homozygous for MC4R inactivation normalized bone parameters without changing energy metabolism (191).

A more direct role for CPE in bone was recently suggested for longitudinal bone growth. Microarray analysis of genes expressed in the perichondral and reserve growth plate zones identified *Cpe* as a highly expressed gene in both zones, although its role in this system is unknown (192). It is interesting to note that prohormones and/or proneuropeptides, in addition to the proteins involved in their processing, were not noted as highly or differentially expressed in this study, suggesting a dissociation between the enzyme function of CPE as a carboxypeptidase and the possibility of its functioning in an alternate capacity. *In situ* hybridization of *Cpe* mRNA during development also demonstrated a specific signal in the cartilage primordium of the ribs, suggesting involvement in rib formation during development (77).

Figure 7.

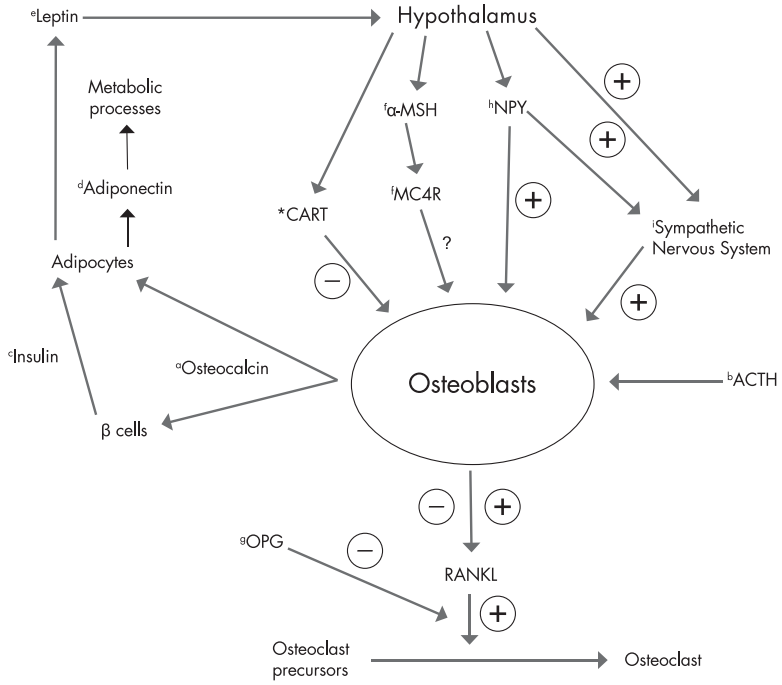

Figure 7. Schematic diagram showing the interplay of molecules involved in bone homeostasis. Peptides from the hypothalamus (CART, NPY, α-MSH), adipocytes (leptin) and osteoblasts (osteocalcin), and the sympathetic nervous system contribute to the regulation of bone remodeling. The *plus sign* indicates signaling in favor of osteoclastogenesis, and the *minus sign* indicates signaling that prevents it. a, Osteocalcin is released from osteoblasts and activates the pancreatic β-cell to release insulin and the adipocyte to release adiponectin (189, 330). b, ACTH plays a role in osteoblast proliferation (331, 332). c, Insulin acts on the adipocyte to release leptin (170). d, Adiponectin affects metabolic processes including insulin sensitivity (333). e, Leptin activates the hypothalamus to express the anorexigenic peptides, α-MSH and CART, and decrease the orexigenic peptides, NPY and AGRP, as well as to stimulate the sympathetic nervous system (334, 335). f, MC4R signaling is involved in bone metabolism, presumably through elevation of CART expression (191). g, Osteoprotegerin (OPG) regulates osteoblast differentiation (336). h, NPY plays a central role in bone remodeling (180). i, The sympathetic nervous system regulates bone remodeling (187). *, Reduced CART levels are associated with poor BMD and are downstream of α-MSH and NPY's role in bone metabolism (124).

D. Infertility and poor sexual performance in CPE-deficient mice

The obesity and diabetes in *Cpe*$^{fat/fat}$ and CPE KO mice develop after puberty, whereas the infertility has been reported anecdotally (7, 159, 163). Because obesity in mice can often affect reproductive performance (193), the infertility of *Cpe*$^{fat/fat}$ mice could be attributed to their obesity. Systematic investigations revealed that the fertilities of the WT and *Cpe*$^{+/fat}$ males and females were similar at approximately 90% (7, 194). However, before weight gain, only approximately 5% of the homozygous mutant matings from WT and *Cpe*$^{fat/fat}$ mice became pregnant. By comparison, when *Cpe*$^{fat/fat}$ males were mated with *Cpe*$^{+/fat}$ females at 45–50 d of age, fertilities were below 45%, and they declined dramatically as obesity developed. Fertilities of the CPE KO mice were even lower. Nevertheless, litter sizes were similar among all genotypes of WT and *Cpe*$^{fat/fat}$ mice.

Successful reproduction requires coordination and feedback among GnRH, the gonadotropins, and sex steroids in the hypothalamic-pituitary-gonadal axis. Of these hormones, only GnRH undergoes proteolytic processing, and it has been proposed that CPE is involved in this process (see Refs. 195 and 196). An examination of GnRH-like immunoreactivity from *Cpe*$^{fat/fat}$ and CPE KO hypothalami revealed that levels were reduced by up to 78% compared with their respective WT controls (7, 194). Combined HPLC and RIA analyses found that the molar percentages of the C-terminally extended GnRH intermediates were increased by 3- to 284-fold in male *Cpe*$^{fat/fat}$ hypothalami compared with their WT or *Cpe*$^{+/fat}$ controls (194). Moreover, levels of pro-GnRH were increased by approximately 2-fold. Additionally, the molar percentages of GnRH-[Gly^{11}], [hydroxy-Pro^{8}]GnRH, and fully processed GnRH were decreased by 2- to 4-fold, whereas the [Gln^{1}]GnRH intermediates were enhanced in the *Cpe*$^{fat/fat}$ males. Although the effect on pro-GnRH processing was expected because PC2 activity was reduced in *Cpe*$^{fat/fat}$ mice (160), the effects on the other processing steps were unanticipated.

Pituitary and gonadal functions were also assessed in *Cpe*$^{fat/fat}$ males (194). Although concentrations of basal LH and FSH were similar among genotypes across age, levels were reduced in older *Cpe*$^{fat/fat}$ mice. Interestingly, *in vitro* anterior pituitary responses to synthetic GnRH were enhanced in *Cpe*$^{fat/fat}$ pituitary cultures, whereas LH and FSH responses to Ca^{2+} ionophore were not distinguished by genotype. Hence, it appears that the GnRH receptor is up-regulated in the *Cpe*$^{fat/fat}$ pituitary, possibly due to decreased or altered release of GnRH.

Serum testosterone contents were similar among genotypes at 50 and 90 d of age but were decreased in *Cpe*$^{fat/fat}$ males at 200 d (194). Nonetheless, these latter concentrations were within the normal range for mice (197). Sperm counts and sperm motility were also depressed in the older mice.

Although the initial reduction in fertility in young *Cpe*$^{fat/fat}$ and CPE KO males was associated with the defect in hypothalamic GnRH processing, the rapid decline

after this age appeared to be due to other factors. For instance, vaginal plugs were readily observed when young *Cpe*$^{fat/fat}$ males were bred with heterozygous or WT females; they were rarely seen at older ages (194). These findings suggested that sexual behavior could be abnormal. Because sexual behavior in rodents is highly dependent upon intact olfaction (198), this sense was tested in *Cpe*$^{fat/fat}$ males at 90 d of age when fertility rates were rapidly declining. Olfaction was normal, and penile erections were evident when *Cpe*$^{fat/fat}$ males were paired with females. Thus, olfaction and physiological responses were intact. When an ovariectomized estrogen-progesterone-primed female was paired with either a WT or *Cpe*$^{fat/fat}$ male, both animals interacted with the female. Although *Cpe*$^{fat/fat}$ males spent more time with females than the WT controls, the latencies for full-mounting behavior were prolonged for *Cpe*$^{fat/fat}$ males, and their responses were either incomplete or inappropriate. Importantly, no instances of intromission or ejaculation were observed. Thus, whereas the *Cpe*$^{fat/fat}$ males show a high degree of sociability, their sexual behavior is aberrant. Because the *Cpe* mutation may affect the processing of many neuropeptides, future research should focus on identifying which peptides may be controlling sexual behavior, as well as what signaling pathways may be perturbed in the *Cpe*$^{fat/fat}$ males.

V. CPE in Neural Function and Behavior

CPE KO mice exhibit a number of behavioral anomalies, including deficient learning and memory (7, 199) and abnormal mood and emotional responses. These behavioral deficiencies are discussed in *Sections V.C and V.D*. As described above (*Sections II.D and III*), CPE is important in processing neuropeptides, sorting neuropeptides to the RSP, and transporting peptidergic vesicles to the membrane to facilitate peptidergic neurotransmission. Moreover, localization of SV to the preactive zone for exocytosis of classical neurotransmitters in hypothalamic neurons is also dependent on CPE. In *Section V.A*, we review an additional role of CPE in modeling the cytoarchitecture of neurons with respect to dendritic growth and pruning and the dendritic spine formation that can impact synaptogenesis and neural function.

Recent studies suggest that CPE plays an important role in neuroprotection during stress as reviewed in *Section V.B*. Absence of CPE in CPE KO mice leads to degeneration of hippocampal neurons during stress. Conversely, CPE expression in the brain is increased during different types of stress, possibly to protect neurons from degeneration. Indeed, *ex vivo* experiments in cultured neurons indicate that CPE is a neuroprotective protein (199, 200).

A. Aberrant neurotransmission and dendritic architecture in CPE KO mice

Abnormal neurotransmission has been reported in *Cpe*$^{fat/fat}$ and CPE KO mice specifically in the retina, hippocampus, and hypothalamus (141, 142, 199). The retina is one of the tissues with the highest expression of CPE in the mouse and rat (141, 201, 202). CPE is also highly expressed in the human ocular ciliary body (203) and the mouse retinal pigmented epithelia (RPE)/choroid in the eye (204). Immunohistochemical staining has localized CPE to photoreceptor synaptic terminals. With age, CPE KO and *Cpe*$^{fat/fat}$ mice showed progressively reduced electroretinography response sensitivity, decreased b-wave amplitude, and delayed implicit time with age, whereas maintaining a normal a-wave amplitude and normal morphology. This is indicative of a defect in synaptic transmission from rod and cone photoreceptors to bipolar cells. Electron microscopy of retinas from *Cpe*$^{fat/fat}$ mice has revealed significantly reduced spherule size, but normal synaptic ribbons and SV density, implicating a reduction in total number of vesicles per synapse in the photoreceptors of these mutants. This reduction in SV at the synaptic bouton (spherule) corroborates well with findings in peptidergic neurons in the hypothalamus that the CPE cytoplasmic tails on the SV are required for interaction with molecules to mediate localization of these vesicles to the preactive zone of the synapse for neurotransmitter release (*Section III.C*). In addition to impairment in glutamate release from CPE KO hypothalamic neurons (*Section III.C*), it has been reported that a mutation in the *Cpe* gene in *C. elegans* results in diminished acetylcholine release at the neuromuscular junctions (18). Together, these findings support an important role for CPE in facilitating localization of SV to the preactive zone and their tethering to the presynaptic membrane (see *Section III.C*).

The dendritic architecture and formation of specific types of dendritic spines also plays a central role in modulating neurotransmission (205). The pattern of dendritic growth and branching critically determines neuronal function (206). During brain development, neurons generally form elaborate dendritic arborizations that receive signals from axons. This stimulation is followed by modeling of the dendritic tree by pruning in the distal portion of the axon and in dendritic branches by retraction, degeneration, and dendritic shedding. Pruning is an essential part of the maintenance of the neuronal network (207).

Studies on 6-wk-old CPE KO mice revealed that the dendritic arborization in pyramidal layer V of cerebral cortex and hippocampal CA1 neurons was more complex

than in WT controls; more dendrites were observed just proximal to the soma and with more branch points (208). Additionally, there were age-dependent changes in arborization in cerebral cortical neurons that differed between the genotypes. Between 6 and 14 wk of age, WT cortex showed a significant decrease in dendritic arborization. Such a decrease is generally attributed to dendritic pruning, resulting in fewer dendritic arbors (209–212). In contrast, in CPE KO cortex, similar amounts of dendritic arborization were seen at 6 and 14 wk. These findings indicate that lack of CPE gives rise to aberrant patterns of dendritic growth, as well as inhibition of dendritic pruning in cerebral cortical and hippocampal neurons (199). Currently, the mechanism of CPE modulation of dendritic patterning is poorly understood.

Various reports have indicated that transcriptional factors such as Hamlet and Spineless in *Drosophila* (213), and Neurogenin 2 and Dlx homeobox in mammals (214–216) play a role in controlling dendritic arborization. Additionally, extracellular signaling molecules such as neurotrophins and their receptors have been implicated in mediating axonal pruning (207, 217–221). Recently, nitric oxide synthase 1 adaptor protein (NOS1AP), previously linked to schizophrenia in regulating dendrite morphology (222–226), has been shown to play a role in dendritic outgrowth through a CPE-mediated pathway in cultured hippocampal neurons (227). Overexpression and knockdown experiments showed a direct relationship between NOS1AP and dendritic number and branching in developing hippocampal neurons. Subsequently, yeast two-hybrid, coimmunoprecipitation and glutathione-S-transferase pull-down experiments identified CPE as a specific interacting protein with NOS1AP. Furthermore, short hairpin RNA knockdown of CPE in these neurons attenuated the reduction of dendrite branching elicited by the overexpression of NOS1AP, demonstrating that NOS1AP functions through CPE and that these two molecules operating together play a pivotal role in maintaining proper dendritic morphology and function of the nervous system (227).

The spines on the surface of dendrites are necessary for synapse formation. Dendritic spine numbers and structure change with synaptic activity and development (228–230). For example, the size of the head and length of the neck of the spine reflect its function. Spines with a larger head and a longer neck (M-type) will generally reflect more activity and show greater plasticity, as opposed to those that are small and stubby (D-type).

Studies on 14-wk-old CPE KO mice showed that the total number of dendritic spines on the basilar tree of cortical layer V and CA1 pyramidal neurons was not significantly different between CPE KO and WT neurons (199). Additionally, no differences in the percentage of M and N spines within the internal segments and the terminal tips of spines were observed in the CPE KO and WT animals. The number of D-type spines, however, which are characterized as not fully functional, was significantly higher in terminal tips of the cortical and hippocampal neurons of the CPE KO compared with the same neurons in the WT mice. An increase in the percentage of D-type spines in the CPE KO mice would lead to deficits in synaptogenesis, and this is exemplified by the long term potentiation (LTP) impairment in CA1 neurons observed in these mutants (199). Indeed, the aberrant pattern of dendritic arborization and spine morphology likely also contributes to the neuropsychiatric deficits, such as poor memory and learning, and other abnormal behaviors observed in the CPE KO mice (see *Sections V.C and V.D*).

B. Role of CPE in stress and neuroprotection

Several studies have shown that CPE expression in the brain is modulated during different types of stress. Jin *et al.* (231) showed that after transient global ischemia, there was a greater and more sustained increase in CPE expression in CA3 hippocampus, which correlated with survival of those neurons. By contrast, neurons in the CA1 region showed a more transient increase in CPE and were more vulnerable to degeneration. Subsequently, Zhou *et al.* (232) reported that after focal cerebral ischemia, there was an increase in CPE mRNA and an accumulation of proCPE in ischemic cortex compared with controls. Furthermore, in $Cpe^{fat/fat}$ mice, a sublethal episode of focal cerebral ischemia led to severe cell death in the ischemic cortex compared with WT mice. These findings suggest that after ischemic stress, neurons in the hippocampus and cortex up-regulate CPE expression, which is correlated with neuronal survival. However, the $Cpe^{fat/fat}$ mouse neurons undergo apoptosis after ischemic stress, suggesting that CPE may have a neuroprotective role.

Additional studies on the CPE KO mouse have provided more evidence that the lack of CPE results in hippocampal neuronal degeneration under stressful conditions. Initially at 6 wk of age, the CA3 pyramidal neurons of the CPE KO hippocampus were shown to be completely degenerated (199). Subsequently, the degeneration was correlated with weaning (maternal separation) at 3 wk of age combined with the stress caused by ear-tagging and tail-clipping. Delaying weaning, ear-tagging, and tail-clipping by 1 wk shifted the CA3 degeneration by 1 wk. Maternal separation alone at wk 3 only caused partial degeneration of the CA3 region. Thus, the complete CA3 degeneration in CPE KO mice is likely attributed to stress caused by maternal separation, ear-tagging, and tail-clipping (233).

Another form of stress is that imparted by restraining the animal. In one such study on C57BL/6 mice, acute restraint stress for 1 h resulted in decreased expression of the CPE mRNA in the hippocampus (234). Stress by social isolation of nonweaned piglets for 15 min also resulted in a decrease in CPE mRNA expression in the frontal lobe (235). However, in contrast to these acute restraint and short-term social isolation stresses, mild chronic restraint stress for 1 h/d for 7 d led to an increase in expression of the CPE mRNA and protein in the hippocampus. This regulation of CPE *in vivo* appears to be mediated by glucocorticoids, because treatment of rat hippocampal neurons in culture with dexamethasone produces an up-regulation of CPE mRNA—a result that is consistent with a bioinformatic search showing a glucocorticoid regulatory element in the promoter of the *Cpe* gene. Also, for fear response stress, a small but significant up-regulation of CPE mRNA in the amygdala of male rats subjected to cat (predator) odor was observed that correlated with avoidance and anxiety-like behaviors (59).

All these studies indicate that CPE is modulated during different kinds of stress, and those stressors such as ischemia, ear-tagging, tail-clipping, fear, as well as chronic restraint stress result in up-regulation of CPE in the cortex, amygdala, and hippocampus. Hence, in mice lacking CPE, these stressors appear to result in neuronal degeneration *in vivo*, at least in the context of the hippocampus and cortex of the CPE KO and $Cpe^{fat/fat}$ mice, respectively, and suggest that CPE may play a significant role in protecting neurons from a stressor. This is further indicated by *ex vivo* studies showing that cultured cerebellar neurons from $Cpe^{+/-}$ mice exhibited more cell death when exposed to low K^+ to induce apoptosis compared with neurons from control animals (200). Additionally, studies on cultured rat hippocampal neurons have demonstrated that oxidative stress-induced apoptosis by H_2O_2 treatment was prevented by overexpression of CPE (199). Moreover, overexpression of an enzymatically inactive mutant form of the CPE with a substitution of glutamate residue 300 acid to glutamine (236) was also effective in preventing H_2O_2-induced apoptosis, indicating that the neuroprotective action does not depend on an active enzyme (200).

The above examples suggest that the presence of CPE in a system is beneficial, and conversely its lack appears detrimental. It is therefore interesting to speculate whether CPE expression levels change with age, and if so, does it associate with disease. One such disease that has been studied is age-related macular degeneration that results in abnormal changes in the RPE and choroid and subsequent loss of RPE cells. In a study screening for changes in gene expression in the complex of RPE, Bruch's membrane, and choroid during aging, it was reported that 20-month-old mice showed a decrease in CPE mRNA compared with 2-month-old mice (204). Interestingly, CPE was only one of two genes down-regulated with age compared with 148 that were up-regulated. Further study of the potential role of CPE in age-related macular degeneration would be expected to shed light on the mechanism and etiology of this disease.

Because neurodegeneration is a hallmark of many age-related neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, it was not surprising to discover a human SNP in the CPE gene that was found in an Alzheimer's patient (see *Section VII*). This mutation in the *CPE* gene leads to neuronal degeneration when transfected into cultured hippocampal neurons (237). Given the apparent neuroprotective role of CPE, these observations would suggest that a decrease in CPE expression during aging or a specific mutation of the gene may contribute to neurodegenerative diseases such as macular degeneration and Alzheimer's disease seen in the aging human population.

In line with a role of CPE in cell survival/apoptosis, another observation involving CPE in apoptosis has been described for pancreatic β-cells (238, 239). It is known that elevated fatty acids reduce β-cell function and survival, linking hyperlipidemia to type 2 diabetes mellitus (T2DM) in obese patients. With palmitate treatment, human islets and MIN6 β-cells showed reduced CPE protein levels within 2 h of treatment, which preceded an ER stress response and ultimately cell death. The mechanism by which CPE is involved in this process is not fully understood. However, CPE translocation to the lysosome for degradation has been implicated. To address the issue of the specificity of palmitate action through CPE, short hairpin RNA was used to specifically reduce CPE by approximately 30%. This resulted in an increased ER stress response and increased apoptosis in islets. Conversely, overexpression of CPE partially rescued the β-cells from the palmitate-induced ER stress and apoptosis. Indeed, islets from $Cpe^{fat/fat}$ mice with low levels of CPE showed increased apoptosis *in vivo* and *in vitro*. Thus, in this system, CPE appears to have a protective function against palmitate-induced apoptosis in β-cells.

C. Regulation of mood and emotional responses in CPE mice

In studies with the $Cpe^{fat/fat}$ males, it appears that their reproductive dysfunction is due primarily to abnormalities in sexual behavior (194). Because anxiety and depression are comorbid with abnormal sexual behavior in humans (see Ref. 240), $Cpe^{fat/fat}$ mice were evaluated for appearance of these responses at 2 months, just before the onset of obesity at 3 months, and after obesity was evident at 5 months of age (241). At 2 months of age, anxiety-like

responses in the zero maze were similar between WT and *Cpe*$^{fat/fat}$ mice, indicating that the mutants were not anxious. However, by 3 and 5 months of age, these behaviors were evident in both male and female *Cpe*$^{fat/fat}$ animals, behaviors that could be alleviated by the anxiolytic drug, diazepam. Because selective serotonin reuptake inhibitors can be used to treat anxiety disorders in humans (242), behaviors were tested with fluoxetine. The anxiety-like responses of the *Cpe*$^{fat/fat}$ mice were normalized also with this drug. The responses to both drugs appear specific for reducing anxiety in the zero maze because diazepam increased locomotor activity in the open field, whereas fluoxetine reduced it for both genotypes. These findings indicate that anxiety-like responses are not apparent in young *Cpe*$^{fat/fat}$ mice but emerge before the onset of obesity. Notably, the anxiety-like behaviors are responsive to both diazepam and fluoxetine.

Because anxiety and depression may be comorbid in humans (243), *Cpe*$^{fat/fat}$ mice were evaluated for depression. Responses in the forced swim and tail suspension tests were analyzed at 2, 3, or 5 months of age. Relative to WT controls, mutants showed increased immobility times in both tests at all ages examined. Anhedonic responses to sucrose were reduced also in *Cpe*$^{fat/fat}$ mice. Hence, the mutants appear to display depressive-like behaviors. Interestingly, the depressive-like behaviors responded to acute treatment with a tricyclic antidepressant (imipramine) and a selective norepinephrine reuptake inhibitor (reboxetine), whereas the selective serotonin reuptake inhibitor (fluoxetine) or the atypical antidepressant (bupropion) was ineffective. However, when fluoxetine or bupropion was given for 14 consecutive days, the depressive-like behaviors were alleviated. Together, these findings indicate that *Cpe*$^{fat/fat}$ mice display depressive-like behaviors, and their responses can be normalized to those of WT controls with acute administration of imipramine or reboxetine or by 14 d of treatment with fluoxetine or bupropion.

The *Cpe*$^{fat/fat}$ model has a number of unique attributes for the study of anxiety and depression. First, the depressive-like behaviors appear to be preexisting in these mutants from an early age, whereas the anxiety-like responses emerge at a later time. This distinction is important because there is considerable controversy as to whether anxiety and depression are coexistent or whether one precedes the other. Second, the differential responses of anxiety- and depressive-like behaviors to fluoxetine are unique. Because acute fluoxetine treatment was successful in rescuing the anxiety-like behaviors whereas leaving the depressive-like responses intact, it appears that the underlying serotonergic mechanisms are different. The *Cpe*$^{fat/fat}$ mice are one of the first animal models to show this differentiation of serotonergic functions, and, as such, they should serve as a useful model to separate the serotonergic mechanisms that underlie anxiety from those that control depression. Finally, alleviation of depression in humans often requires prolonged therapy with antidepressants. The forced swim and tail suspension tests are used as acute screens for antidepressant action. Most animal models of depression respond to acute administration of antidepressants in these tests. Because at least 2 wk of therapy with fluoxetine or bupropion were required to alleviate the depressive-like behaviors in the *Cpe*$^{fat/fat}$ mice, these mutants may serve as an important model for human depression. An examination of this differential time-scale of response may reveal new mechanisms that are required for antidepressant efficacy.

D. Deficits in learning and memory in CPE KO mice

In previous work, it had been reported that after transient global ischemia, CPE expression was up-regulated and sustained in CA3 hippocampus, but transient in the CA1 region (231). These findings suggested that it might be important to examine hippocampal function in the CPE KO mice. The morphological and electrophysiological abnormalities have already been presented (*Sections V.A and V.B*). The behavioral functions of these mice were evaluated in tests of episodic and spatial memory, which require an intact hippocampus (see Refs. 244–246). In the novel object recognition task, individual WT and CPE KO mice were initially allowed to interact with two identical objects (199). The mice were then returned to their home-cage, and recognition memory was examined 20 min and 24 h later in a two-choice test where the now familiar and a novel object were presented. During both the 20-min and 24-h tests, WT mice showed marked preferences for the novel object. By comparison, CPE KO mice failed to demonstrate preference for either object during these times. Because no genotypic differences were observed in total object interaction time, these findings indicate that episodic short- and long-term recognition memory is impaired in the CPE KO animals. Similar conclusions were obtained in another test of episodic memory where the social transmission of food preference task was used (199).

The CPE KO mice were also evaluated for spatial memory in the Morris water maze (199). WT and CPE KO mice were first tested on the visible-platform version of the task. In this test, mice must learn to swim to a visible platform to escape the water. Because no genotype differences were observed on this task, general sensory and motor functions appear intact in both genotypes. On the hidden-platform version, mice must use and remember external cues in their

environment to guide them to the submerged platform. Over the first 3 d of acquisition training, swim latencies and swim distances to locate the platform were prolonged in the CPE KO mice compared with those of the WT controls. Due to these deficiencies and because visible-platform performance was normal, it appears that spatial memory is impaired in the CPE KO mice.

The learning and memory processes of the WT and CPE KO mice were assessed when the animals were approximately 2–3 months old, at an age when the body weights of the mice were just beginning to diverge. As such, the deficiencies in learning and memory of the CPE KO mice cannot be attributed to performance issues that may be influenced later by obesity. It is noteworthy that aside from deficits in hippocampally based learning and memory processes, the CPE KO mice were deficient also in hippocampal LTP (199). Morphological analysis of the hippocampus from the CPE KO mice revealed that the pyramidal neurons in the CA3 region had degenerated by 6 wk of age, providing an explanation for the hippocampal deficits observed in these mutant mice (199).

VI. CPE and Cancer in Humans

WT and the splice variant, CPE-ΔN, mRNA (see *Section II.A*) are expressed in neuroendocrine tumors such as pheochromocytomas (8). In epithelial-derived tumors, such as hepatocellular carcinoma (HCC) and colorectal carcinoma, only the splice variant is expressed (8). CPE-ΔN has been shown to induce proliferation and invasion in various tumor cell lines and to promote growth and metastasis of tumors in orthotopic models of nude mice. In *Section VI.A*, the mechanism of action of CPE-ΔN in promoting growth and metastasis will be discussed. Additionally, microarray studies of biopsies from many types of tumors, including those from Ewing sarcoma and cervical and kidney cancers, have suggested that metastatic tumors are correlated with elevated levels of CPE mRNA compared with that in normal tissue or benign tumor [for review, see Murthy *et al.* (247)], suggesting that CPE could be a biomarker for diagnosing metastatic disease. Although in those studies it is unclear whether the CPE mRNA detected is the WT and/or CPE-ΔN mRNA, recent clinical studies on HCC and pheochromocytoma indicate that CPE-ΔN is a potentially excellent biomarker for diagnosing and predicting future metastatic disease (8). This will be discussed in *Section VI.B*. The link of CPE to cancer is perhaps not surprising because it is located on chromosome 4q32.2, a locus that shows loss of heterozygosity in human glioblastoma (248).

A. Splice isoform of CPE (CPE-ΔN) promotes tumor growth and metastasis

Expression of the splice isoform of CPE (see *Section II.A*), CPE-ΔN, was found in different cell lines derived from liver, colon, breast, and head and neck cancers (8). Moreover, the expression of the CPE-ΔN mRNA was significantly elevated in highly metastatic *vs.* low metastatic isogenic cell lines from the same type of cancer. Furthermore, these epithelial-derived cancers did not express the WT-CPE. When expressed in low metastatic HCC cell lines, CPE-ΔN induced proliferation and invasion, and immunocytochemical studies revealed that CPE-ΔN was localized to the nucleus in highly metastatic HCC cells (8). These findings indicate that CPE-ΔN may act in the nucleus, and subsequent coimmunoprecipitation experiments have confirmed an interaction of CPE-ΔN with histone deacetylase 1 and 2 (8). CPE-ΔN mRNA when transfected into HCC cells increased the expression of the "neural precursor cell expressed developmentally down-regulated" gene (NEDD 9) (8), a metastatic gene (249), in a histone deacetylase 1 and 2 activity-dependent manner (8). Thus, these results indicate that CPE-ΔN drives invasion in tumor cells *ex vivo* and metastasis *in vivo*. The latter point was confirmed by two separate experiments. First, when highly metastatic HCC cells were treated with siRNA to down-regulate CPE-ΔN and then injected into the flank of nude mice, little tumor growth or metastasis occurred. Similarly, subsequent transplantation of the siRNA CPE-ΔN-treated tumor into the liver in nude mice also did not exhibit tumor growth or metastasis, compared with HCC cells treated with scrambled siRNA. These studies demonstrate that CPE-ΔN induces tumorigenesis and metastasis through up-regulation of the expression of *nedd9* gene.

B. CPE/CPE-ΔN as a diagnostic and prognostic biomarker for (neuro)endocrine and nonendocrine cancers

CPE protein and/or mRNA have been detected in (neuro)endocrine tumors such as insulinomas, pituitary tumors, neoplastic enterochromaffin-like cells, pheochromocytomas (PHEO)/paragangliomas (PGL) (247, 250–252), small-cell lung carcinomas (253, 254), and other cancerous cells infiltrating the lung in lung cancer patients (75). The abundance of CPE expression in these tumors is not surprising because CPE is required for biosynthesis of neuropeptides that serve as autocrine growth factors in these tumors (255). In a drug-resistant form of the small-cell lung carcinoma cell line (NCI H82), expression of both the 55- and approximately 50-kDa forms of CPE have been reported (253). In other endocrine tumors, significantly high levels of CPE expression were detected in some pituitary adenomas compared with normal pituitary. An oligonucleotide array analysis showed that CPE

is differentially expressed in four subtypes of adenomas (256). Prolactin-secreting pituitary adenomas showed no difference from normal pituitary in CPE expression, but in ACTH-secreting pituitary adenomas, expression was lower. However, *CPE* mRNA expression in GH-secreting pituitary adenomas was significantly higher than normal pituitary. In contrast, no CPE immunoreactivity was detected in another study specific to nonfunctioning pituitary macroadenoma (257). In endocrine tumors where protein analysis was carried out, WT-CPE protein was detected. In other studies where no CPE protein data were presented, the *CPE* mRNA detected in neuroendocrine tumors is likely to be that encoding the WT-CPE protein. However, because these studies predate the discovery of CPE-ΔN, it is not known whether (neuro)endocrine tumors also express CPE-ΔN. By comparison, CPE-ΔN mRNA has been investigated and detected in PHEO/PGL (8).

WT-CPE has been proposed as a prognostic biomarker in neuroendocrine tumors. Quantitative immunostaining of CPE has shown that elevated CPE expression is a statistically significant predictor of good prognosis in pulmonary neuroendocrine tumors such as typical carcinoids, small-cell lung cancers, and large-cell neuroendocrine carcinomas (254). However, it is unclear at this time how elevated levels of WT-CPE indicate good prognosis in these pulmonary carcinomas. The use of WT-CPE as a potential prognostic biomarker awaits further investigation.

PHEO and PGL are rare neuroendocrine tumors derived from chromaffin cells. PHEO are derived from chromaffin cells in the adrenal medulla, and closely related tumors of extraadrenal sympathetic and parasympathetic paraganglia are classified as PGL (258). PHEO and PGL occur most often during young-adult to mid-adult life with a slightly higher prevalence in females than males (259). Practically all PHEO and sympathetic PGL produce catecholamines (norepinephrine, epinephrine, or dopamine), but only about 70–80% of these tumors secrete these transmitters (260). Nevertheless, almost all catecholamine-producing PHEO and PGL metabolize the catecholamines to produce metanephrine, normetanephrine, or methoxytyramine (261–264). Thus, whereas catecholamine excess reflects various clinical presentations of these tumors, metanephrine and methoxytyramine excess are used in the biochemical diagnosis of these tumors (see Refs. 265–270, 365). Because catecholamine excess causes nonspecific symptoms (hypertension, tachycardia, anxiety, nervousness, sweating, etc.), approximately 50% of these patients are not diagnosed sufficiently early and often visit a physician too late, *i.e.*, when a tumor is large or, in about 10% of patients, when metastasis has occurred (259, 271–275). Parenthetically, larger and extraadrenal tumors have a higher incidence of becoming metastatic (276–279).

Most PHEO and PGL are sporadic; however, familial PHEO and PGL are found in more than one third of patients (280–284). At the present time, 10 PHEO or PGL susceptibility genes have been identified. Five of them represent major susceptibility genes (found in ~90% of PHEO and PGL) and include the *von Hippel-Lindau* (*VHL*) gene; the *RET* protooncogene, which causes multiple endocrine neoplasia type 2 (MEN2); the neurofibromatosis type I (*NF1*) gene, associated with von Recklinghausen's disease; and the genes encoding succinate dehydrogenase subunits D (*SDHD*) and B (*SDHB*), associated with familial nonsyndromic PHEO or PGL (for review, see Ref. 285). Minor susceptibility genes consist of the genes encoding *SDHA* and *SDHC*, which cause head and neck PGL or PHEO and PGL, respectively; *SDHA2*; *TMEM127*; and the recently discovered *MAX* gene (284, 286–291).

In contrast to small-cell lung carcinoma, WT-CPE levels in these PHEO/PGL showed no obvious correlation with survival (S. R. K. Murthy, Y. P. Loh, and K. Pacak, unpublished data). However, in a study of 14 PHEO/PGL patients having *SDHB/D* or MEN2 mutations, high copy numbers of CPE-ΔN mRNA have been correlated with poor prognosis (8). *SDHB* patients diagnosed with metastatic tumors at the time of surgery showed high CPE-ΔN mRNA copy numbers in their resected tumors that were 5- to 10-fold greater than those in benign tumors. In two *SDHB* patients that were diagnosed as having a benign tumor at the time of surgery, one had a low CPE-ΔN mRNA copy number and was disease-free 2.4 yr after the surgery, whereas the other with a high copy number developed metastatic disease within 2 yr. Similarly, in a group of MEN2 patients, all but one, who was diagnosed with benign tumors at the time of surgery, had low CPE-ΔN mRNA copy numbers and were disease-free for at least 6 yr after surgery. The MEN2 patient with high CPE-ΔN mRNA copy number developed recurrence within 8 yr. Another MEN2 patient who had a recurrent PHEO at the time of surgery and showed a high CPE-ΔN mRNA copy number developed recurrence within 4 yr. Although the number of patients in this study was small, the findings are very promising because no other predictive biomarker exists for these types of neuroendocrine tumors. Moreover, the use of CPE-ΔN as a biomarker for predicting future metastasis was superior to pathological analysis. Studies with more patients will further validate CPE-ΔN mRNA as a biomarker for diagnosis and predicting future metastatic disease in PHEO/PGL.

CPE-ΔN expression has also been analyzed in patients with HCC and colorectal cancer. In a cohort of 180 patients

with HCC, the ratio of CPE-ΔN mRNA or protein in tumor *vs.* normal surrounding tissue (T/N) predicted future recurrence (intrahepatic metastasis) with a sensitivity of 92% and a specificity of 76%. More importantly, stage 2 HCC patients who were given good prognosis but had a high T/N ratio developed recurrence within 2 yr, indicating again the value of CPE-ΔN as a biomarker for predicting future metastasis independent of pathological staging. High T/N (>2) values of CPE-ΔN were correlated with poor prognosis.

In a study of 68 colorectal cancer patients, 93.5% of those that did not have metastatic disease had tumor CPE-ΔN mRNA T/N ratios of 2 or less, whereas 86.5% of the patients that had lymph node or distant metastasis within 1 yr after surgery had tumor CPE-ΔN mRNA T/N ratios above 2 (8). These findings show that CPE-ΔN mRNA is also a good diagnostic biomarker for metastasis for colorectal cancer. Additionally, human oligodendrogliomas and astrocytomas also showed expression of CPE mRNA higher than in normal brain. Biopsies from patients with cervical cancer also indicated higher levels of *CPE* mRNA than in normal cervix (247). However, it has not been determined whether the mRNA in these cancers represents WT-CPE or CPE-ΔN mRNA. Future studies will indicate whether CPE or CPE-ΔN might also be a diagnostic and prognostic biomarker for metastasis in these types of tumors.

VII. Human CPE Genetic Mutations and Disease

Several mutations in the *CPE* gene have been identified and associated with diabetes and Alzheimer's disease (see *Section VII.A*). In addition, there are diseases such as hyperproinsulinemia and autoimmune diseases linked to *CPE* that are briefly reviewed in *Section VII.B*.

A. Single nucleotide polymorphisms in human CPE associated with disease

Transition or transversion of nucleotides in the genomes among individuals is referred to as SNP. There are three types of SNP. Nonsynonymous SNP lead to changes in the encoded amino acid, whereas synonymous SNP are silent and do not change the amino acid coding (292–295). The third type of SNP is present in the noncoding region, where they may influence promoter activity (gene expression) (296–298), mRNA conformation (*i.e.*, stability) (299–303), subcellular localization of mRNA and/or proteins (304–307), and microRNA binding (308, 309).

The human *CPE* gene is located on chromosome 4 at 4q32.3 (310). Its coding region spans approximately 120 kb and consists of nine exons. The *CPE* gene is often associated with T2DM. Elevated levels in proinsulin and/or the molar ratio of proinsulin to insulin are one of the features of T2DM, which indicates the contribution of some nonfunctional proinsulin-processing enzymes. Recently, several SNP have been identified in the *CPE* gene. The first study of SNP in the human *CPE* gene was by Utsunomiya *et al.* (311) (Table 3, items 1–3). In this study, they analyzed SNP in the promoter and entire coding region in the *CPE* gene in 269 Japanese subjects with non-insulin-dependent diabetes mellitus, 28 nondiabetic obese subjects, and 104 nonobese and nondiabetic controls. Their analysis revealed three nucleotide changes: a G-to-T substitution at nucleotide −53 (relative to start of transcription); a G-to-A substitution at nucleotide −144 in the pro-

TABLE 3. Various SNP in human CPE gene

Item no.	Chr. 4 position	mRNA position	dbSNP rs cluster id	Heterozygosity	Type of SNP	A inserts	Protein residue	Amino acid position	Enzymatic function	Disease associated	PMID reference
1	166416762	939			Missense	G-to-A	Asp-to-Asn	219	Active	None	9662053
2	166415972	144			5′ UTR	G-to-A	NA	NA	Active	None	9662053
3	166415881	53			5′ UTR	G-to-T	NA	NA	Active	None	9662053
4	166415969	132			5′ UTR	G-to-C	NA	NA	Active	None	11462236
5	166416905	1081			Missense	C-to-T	His-to-His	267	Active	None	11462236
6	166416952	1128			Missense	C-to-T	Arg-to-Trp	283	Less active	Increased risk of diabetes	11462236
7	166417373	1549			Missense	C-to-T	Ala-to-Val	423	Active	None	11462236
8	166403424	980	rs34516004	0.147	Missense	T-to-C	Trp-to-Arg	235	Not active	?	Unpublished data of the authors
9	166403445	1001	rs77809551	0.24	Missense	G-to-T	Val-to-Leu	242	?	?	
10	166405670	1164	rs116683740		Missense	A-to-G	Asn-to-Ser	296	?	?	
11	166416807	1587	rs75109518		Missense	T-to-G	Val-to-Gly	437	?	?	
12	166416318 166416338 166416346	495, 515, 523			Insert	A inserts	9 new amino acids	Amino acids change from 72 to 80	Not active	Possible Alzheimer's disease	EST evidence, unpublished data of the authors

SNP database (dbSNP) rs numbers are assigned to items 8 to 11; other SNP in the table are mined from the literature. QQ mutant (item 12) has been mined from EST database base (dbEST). SNP item 8 falls in the zinc peptidase domain of CPE protein and was found to be enzymatically inactive. Chr., Chromosome; NA, not applicable; ?, unknown.

moter region; and a silent G-to-A substitution in codon 219 in the coding region. However, none of these SNP correlated with non-insulin-dependent diabetes mellitus or obesity.

In another study conducted on a cohort of 272 Ashkenazi Jews with T2DM (312), four novel SNP in the transcript region and one at the promoter region of the *CPE* gene were identified (Table 3, items 4–7). At codon position 283, a C-to-T transition led to a nonsynonymous Arg-to-Trp (R283W) change at the protein level. The Arg283 residue is within the Zn-carboxypeptidase domain of the CPE protein and is conserved among CPE orthologs and most metallocarboxypeptidases that are enzymatically active, suggesting that this residue may contribute to the catalytic process. Indeed, the R283W CPE protein had a narrower pH optimum and was much less active at pH 6.0 to 6.5, indicating that the R283W CPE variant would be substantially less active than WT-CPE *in vivo*. In addition, the R283W CPE variant was less stable at higher temperatures than the WT enzyme. Of the T2DM cohort of Ashkenazi pedigrees, four specific families were found to segregate with the R283W mutation. Risk of early-onset T2DM was higher in patients within these four families who inherited one copy of this variant. Furthermore, the homozygous mutant is predicted to have a higher risk for the development of hyperproinsulinemia and diabetes.

A *CPE* SNP, rs34516004 (Table 3, item 8), has recently been analyzed (S. R. K. Murthy, unpublished data). This SNP consists of a T-to-C substitution that leads to a nonsynonymous Trp (W)-to-Arg (R) (W235R) change, and when expressed and assayed, this mutant was shown to be enzymatically inactive (N. X. Cawley, S. R. K. Murthy, and Y. P. Loh, unpublished data). This W235R mutant was also found in the AGI_ASP population, which is composed of African-Americans and Caucasians, with a heterozygosity value of 0.147, and as with the R283W mutation, people carrying this W235R mutation in the homozygous state could have higher risk of hyperproinsulinemia and diabetes.

An undocumented mutant was found in three EST from the Helix Research Institute, Japan, in Alzheimer's cortex, kidney tumor, and thalamus tissues (Table 3, item 12). This mutation contains three nucleotide insertions (A and/or C) that result in the substitution of amino acids after the prodomain of the CPE protein. The new amino acids in all three cases contain a common core sequence with two adjacent glutamine (Q) residues, hence termed QQ mutants. The mutation results in nine new amino acids replacing eight original amino acids at the N terminus in the mutant protein compared with the WT-CPE. The new amino acids have a combined isoelectric point that is two units lower than the WT sequence it replaces. The physicochemical properties of this mutant protein are predicted to be compromised due to the critical location of the mutation, *i.e.*, at the start of the first β-pleated sheet, which would be important in the structural integrity of the mutant protein. A complete analysis of this mutant is currently in progress (N. X. Cawley, Y. Cheng, T. Yanik, C. Liu, S. Young, S. R. K. Murthy, A. Papazian and Y. P. Loh, manuscript in preparation).

Studies from several groups have screened Chinese patients with symptoms of coronary atherosclerosis (313–315). In these studies, a number of point mutations were found in the *CPE* gene, specifically exons 4 and 5, that when present were genetically linked to an increased severity of the disease.

B. CPE-associated human disease

Familial hyperproinsulinemia (FH) is a human disease that results from specific mutations in the proinsulin gene that cause substantial increases in the levels of circulating proinsulin (316). One site where the mutations occur results in Arg65 of the proinsulin being changed to His, Pro, or Leu. Because Arg65 is part of the C peptide-A chain junction processing site, changes at this site result in poorer processing of the proinsulin to insulin. Another mutation occurs at His10Asp on the B-chain of insulin. This His10Asp proinsulin mutant protein has been shown to be secreted through the CSP at a much higher rate compared with WT proinsulin (317, 318). Hence, trafficking of the mutant proinsulin is impaired. Recall that CPE can bind to prohormone sorting signals that aid trafficking to the granules of the RSP. One such signal was described in (pro)insulin. Thus, proinsulin can bind to CPE (42). Mutation of this sorting signal in proinsulin resulted in impaired sorting of the mutant insulin to the RSP. Furthermore, some of the human mutants found in FH that were expressed in cells bound poorly to CPE in binding assays (121). These and other results suggest that in addition to the lack of processing the Arg65 mutants and the inability of the His10Asp mutant to hexamerize to form a higher order sorting signal, reduced binding of these mutants to CPE may play a role in their increased constitutive secretion and thereby contributes to FH.

Several other diseased states have been found to have elevated CPE levels. Insulinomas are primary tumors of insulin-producing cells of the islets, and they contain the complement of expected processing enzymes for this cell type (250). Insulinomas can lead to excessive insulin secretion and organic hypoglycemia. Analysis of insulinomas has shown that expression of CPE and the $G_{s\alpha}$ signaling subunit are often elevated (319). Thus, enhanced regulated secretion of insulin in the presence of elevated CPE may contribute to the hyperinsulinemia. Some sup-

port for this idea comes from cell culture experiments where overexpression of CPE in proinsulin-secreting cells has been shown to exert a marginal, but significant, increase in regulated insulin secretion (320).

The presence of CPE autoantibodies in the circulation has been studied extensively in cohorts of Chinese patients that exhibit latent autoimmune diabetes in adults. Here, there appears to be increased diagnostic value of analyzing CPE antibodies in this condition compared with the analysis of antibodies to glutamic acid decarboxylase (321) or SOX13 (322, 323). In an effort to identify latent autoimmune diabetes in adults within a phenotypic T2DM cohort, the prevalence of CPE antibodies was found to be 4.8%, suggesting that analysis of CPE antibodies may allow discrimination of a more latent subset of the adult-onset autoimmune diabetes compared with patients with antibody-negative T2DM (324).

Experimental autoimmune encephalomyelitis (EAE) is a mouse model for multiple sclerosis because it is a polygenic chronic inflammatory demyelinating disease of the nervous system. Oligonucleotide microarray analysis of inflamed spinal cord from EAE mice found that expression of *Cpe* was decreased by 1.5- to 4.1-fold as the severity of the disease progressed (325). Another study identifying trait loci for causes of EAE mapped one such loci to *Cpe* on chromosome 8 (326). Although presently it is unclear how CPE may be linked to this phenotype, the recent findings that Caspr2 and CPE can interact (140) suggests that CPE may be involved indirectly in myelination because contactin is implicated in neurite outgrowth, axon fasciculation, and myelin formation.

Finally, a deletion of both cathepsin B and L in mice results in early-onset neurodegeneration, a phenotype reminiscent of neuronal ceroid lipofuscinoses in humans (327). Interestingly, of the 19 proteins significantly increased, four were increased over 10-fold: Rab14, the Delta/Notch-like epidermal growth factor-related receptor, calcyon, and CPE. IHC showed swollen axons in the early developmental stages of the double KO mice, leading the investigators to suggest a role of cathepsin B and L in the recycling process during axon growth and synapse formation. Presently, it is unclear how CPE could compensate in this process.

VIII. Conclusions

In this review, we have emphasized new functions for an "old" protein, CPE. This protein has been well recognized as a peptide-processing enzyme for at least three decades. However, over the past 10 yr, evidence has been accumulating that CPE may serve additional roles as an interacting molecule or as mRNA that is highly expressed or suppressed in a variety of paradigms and diseases. In the past, much of the CPE data from screens were ignored due to a lack of understanding of why or how a prohormone-processing enzyme like CPE could be involved in cancer metastasis or interact with molecules found in the nucleus or cytoplasm when it is localized within secretory vesicles. Nevertheless, it has always been intriguing that CPE should be present at 10-fold greater amounts than the other prohormone-processing enzymes such as PC1 and PC2 in endocrine tissues and is far in excess of what is needed for the enzymatic reactions. Additionally, CPE is expressed in embryos before the development of the peptide hormone endocrine system, hinting at a trophic rather than an enzymatic role for CPE—perhaps in cell proliferation and/or differentiation. The present review has attempted to highlight the many studies on CPE and to emphasize some of its newer roles. These new roles include a number of features. 1) CPE appears to function at the cell-biological level as a sorting receptor for directing prohormones into the RSP and in the transport of peptidergic vescicles to their release sites. These attributes are mediated by different domains of the protein (cytoplasmic tail) that are not involved in its enzymatic activity. 2) CPE also seems to exert antiapoptotic/protective effects against stress in endocrine cells and neurons, as well as contributing to synaptic transmission and dendritic pruning. 3) New insights have been gained using the CPE KO and $Cpe^{fat/fat}$ mice. These functional roles include bone remodeling and effects on behavior that involve mood, learning, and memory. 4) The identification of the splice isoform, CPE-ΔN, has opened up new avenues of investigation, especially with regard to its possible role in tumorigenesis and metastatic disease. Importantly, CPE-ΔN also seems to have high potential as a prognostic biomarker for predicting future metastasis of some endocrine and nonendocrine tumors. 5) Finally, SNP in the human *CPE* gene have been linked to endocrinological and neurological diseases such as diabetes and Alzheimer's disease. In conclusion, CPE is a multifunctional protein that can subserve enzymatic as well as many nonenzymatic roles during development and in the adult endocrine and nervous systems. Mutations in the *CPE* gene are associated with a variety of disease states, and expression of its splice variant CPE-ΔN in tumors promotes metastatic disease in humans.

IX. Future Directions

Although the mechanisms of action of CPE in peptide processing are well-known and those mediating prohormone

sorting and vesicle transport for secretion and neurotransmission are becoming better understood, considerable work lies ahead in understanding the mechanisms underlying the roles of CPE in four major areas: 1) cell protection and survival; 2) embryonic development; 3) neuropsychiatric disorders; and 4) tumor metastasis. The present review of the literature suggests that CPE promotes endocrine and neuronal cell survival in adults and may influence cell growth and proliferation in developing cells in young animals and in embryos. Thus, it is possible that secreted CPE might serve as an autocrine/paracrine trophic factor to activate different signaling pathways that trigger transcription of specific genes to mediate these effects. This possibility needs to be explored in detail in the future. Some examples include how secreted CPE may protect endocrine cells and neurons from cell death under conditions of palmitate-induced ER stress in β-cells, as well as under oxidative stress and amyloid protein-induced toxicity. In embryonic development, the expression pattern of the CPE splice variants, especially CPE-ΔN, should be investigated because molecules expressed in tumors often are expressed only during embryogenesis and have a role in proliferation and differentiation. Moreover, CPE-ΔN has been shown to activate gene transcription, and therefore overexpression and analysis of all its downstream target genes in different cell types should provide new insights into its mechanism of action. Generating and studying mouse models bearing SNP of human *CPE* will also likely provide a better understanding of how these mutations cause disease states. Additionally, identification of both endogenous and environmental factors that can induce and regulate CPE expression and the splicing of the *CPE* gene to generate CPE-ΔN will greatly facilitate the understanding of how CPE can mediate abnormal behavioral responses and cause cancer and metastatic disease, respectively. Continued clinical studies on various endocrine and nonendocrine tumors should further validate CPE-ΔN as a promising multitumor prognostic biomarker for predicting future metastasis. One can envisage that future experiments will further reveal novel mechanisms of action for CPE and CPE-ΔN, especially as a prime regulator of many metabolic and endocrine functions, potentially as a survival and differentiating factor for neurons and endocrine cells, as well as a novel therapeutic target for cancer and degenerative diseases.

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