60 YEARS OF POMC

Biosynthesis, trafficking, and secretion of pro-opiomelanocortin-derived peptides

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Abstract

Pro-opiomelanocortin (POMC) is a prohormone that encodes multiple smaller peptide hormones within its structure. These peptide hormones can be generated by cleavage of POMC at basic residue cleavage sites by prohormone-converting enzymes in the regulated secretory pathway (RSP) of POMC-synthesizing endocrine cells and neurons. The peptides are stored inside the cells in dense-core secretory granules until released in a stimulus-dependent manner. The complexity of the regulation of the biosynthesis, trafficking, and secretion of POMC and its peptides reflects an impressive level of control over many factors involved in the ultimate role of POMC-expressing cells, that is, to produce a range of different biologically active peptide hormones ready for action when signaled by the body. From the discovery of POMC as the precursor to adrenocorticotropic hormone (ACTH) and β-lipotropin in the late 1970s to our current knowledge, the understanding of POMC physiology remains a monumental body of work that has provided insight into many aspects of molecular endocrinology. In this article, we describe the intracellular trafficking of POMC in endocrine cells, its sorting into dense-core secretory granules and transport of these granules to the RSP. Additionally, we review the enzymes involved in the maturation of POMC to its various peptides and the mechanisms involved in the differential processing of POMC in different cell types. Finally, we highlight studies pertaining to the regulation of ACTH secretion in the anterior and intermediate pituitary and POMC neurons of the hypothalamus.

Key Words

- hypothalamus-pituitary-adrenal axis
- POMC sorting
- POMC-derived peptide secretion
- vesicle trafficking
- prohormone processing enzymes

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Introduction

The birth of pro-opiomelanocortin (POMC) stemmed from the landmark work of Dr Choh Hao Li at the Hormone Research Laboratory at the University of California at Berkeley, where he first elucidated the chemistry of adrenocorticotropic hormone (ACTH) and subsequently β-lipotropin (LPH). Thereafter, an accumulation of peptide sequence studies from many laboratories led to the recognition that a number of biologically active peptides such as α-melanocyte-stimulating hormone (MSH) are derived from ACTH, and β-MSH and β-endorphin from β-LPH. Based on common amino acid sequences among these peptides such as α-MSH and β-MSH in ACTH and β-LPH, respectively, the hypothesis emerged that ACTH and β-LPH could be derived from a larger precursor consisting of both ACTH and β-LPH (for an historical perspective, see Lowry 2015). Subsequently, several groups, including

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Michel Chretien (Crine *et al.* 1979), Mains and Eipper (Mains & Eipper 1979) and ours (Loh 1979), employing pulse–chase experiments provided evidence that ACTH and β-LPH were derived from a larger common precursor, and that the sequential processing of this precursor led to the biosynthesis of the different biologically active peptides. At about the same time, cDNA cloning studies confirmed the existence of the common precursor for ACTH and β-LPH (Nakanishi *et al.* 1979). Hence, the name 'pro-opiomelanocortin' was coined by Michel Chretien for the ACTH–β-LPH precursor (Fig. 1) (Chretien & Mbikay 2016).

Intracellular organization of POMC maturation

Intracellular trafficking of POMC

POMC is synthesized in the corticotrophs and melanotrophs of the anterior (AL) and intermediate lobes (ILs) of the pituitary, respectively, as well as in peptidergic neurons in the arcuate nucleus of the hypothalamus. It is posttranslationally cleaved into peptide hormones that can include ACTH; β-endorphin; α-, β-, and γ-MSH; N-POMC(1–48); and β-LPH, in a tissue- and a cell-dependent manner. These peptides exhibit different physiological functions such as mitogenic activity N-POMC(1–48), steroidogenic activity (ACTH), satiety (α-MSH), and opiate-like activity (β-endorphin). After synthesis at the rough endoplasmic reticulum (ER) and folding in the ER, POMC is transported through the cell to end up ultimately in large dense-core secretory granules of the regulated secretory pathway (RSP). The route involves movement of the protein through the ER and Golgi to the *trans*-Golgi network (TGN), where it is sorted into nascent vesicles budding from the TGN that will mature into dense-core secretory granules as they are trafficked to the release sites close to the plasma membrane. During this movement within the cell, the prohormone is cleaved in a time- and compartment-specific way by prohormone convertases (PCs) to generate the peptide hormone complement, specific for that cell type. The peptides generated in the mature granules form an electron-dense core and are stored in these granules until secreted from the cell upon stimulation by a secretagogue. How POMC is transported through and processed in the endocrine cell from the site of synthesis to the dense-core secretory granules has been a long-standing question and one that has been studied by many investigators.

With the discovery of POMC as the precursor to ACTH and β-LPH (Mains & Eipper 1976, Mains *et al.* 1977, Crine *et al.* 1978), an explosion of work followed in the 1980s and 1990s addressing the question of cellular transport and processing of the prohormone. Ideal for studying these questions were the AtT20 cells, a mouse corticotroph cell line that normally expresses POMC and processes it into ACTH, β-LPH, and the 16-kDa N-POMC intermediate. Initial biochemical evidence demonstrated that the mature peptides, ACTH and β-LPH, were present in mature secretory granules of AtT20 cells purified by density gradient centrifugation on Ficoll (Gumbiner & Kelly 1981), leading to the idea that POMC must be processed in this compartment. It was subsequently found that POMC was also secreted through the constitutive secretory pathway (CSP) in these cells, that is, in an unstimulated manner, along with an endogeneous murine leukemia virus present in these cells (Gumbiner & Kelly 1982), demonstrating that the two secretory pathways were distinct in these cells, one being driven by bulk flow and tied to protein translation and the other requiring active sorting into storage granules and secretion triggered by external stimuli (Burgess & Kelly 1987). Indeed, at that time, transfection of VSVG

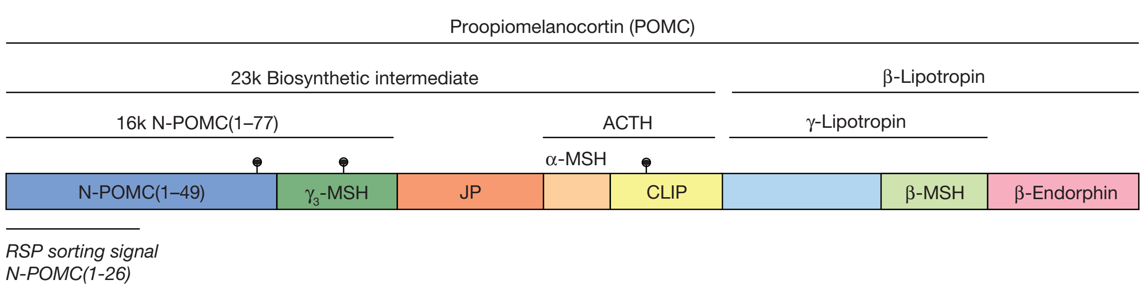

Figure 1
Schematic diagram of the bovine POMC protein. The prohormone encodes multiple peptides that can be cleaved by prohormone convertases in a cell- and a time-dependent manner. ACTH, adrenocorticotropin; MSH, melanocyte-stimulating hormone; JP, joining peptide; CLIP, corticotropin-like intermediate peptide; RSP, regulated secretory pathway. Lollipop symbols represent glycosylation sites.

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or human growth hormone (hGH) into AtT20 cells clearly demonstrated the two secretory pathways in these cells (Moore & Kelly 1985), because the vesicular stomatitis virus glycoprotein (VSVG) was secreted constitutively, whereas the hGH was secreted through the RSP. In support of this, electron microscopic (EM) and immunocytochemical analyses using an anti-ACTH antibody that could label ACTH and its precursor, POMC, showed ACTH-immunoreactivity (IR) in condensing vacuoles protruding from the *trans* most side of the Golgi apparatus (Tooze & Tooze 1986), demonstrating that granule cargo was sorted into these 'immature' granules. These condensing vacuoles and 25–30% of the mature dense-core granules were shown to contain unprocessed POMC, using an ACTH-β-LPH cleavage site-specific antibody, demonstrating that POMC was sorted into these immature granules where most of it was processed (Tooze *et al*. 1987*a*). Follow-on EM studies identified that the murine hepatitis virus shared this initial compartment with ACTH but diverged afterward (Tooze *et al*. 1987*b*), consistent with the virus being in the CSP. Analysis of newly synthesized proteins labeled with ^{35}S in methionine and sulfated proteoglycans identified several proteins in AtT20 cells that could be observed in the two distinct pathways, those that followed unprocessed POMC and those that followed ACTH (Moore *et al*. 1983*b*), leading to the conclusion that a common signal existed for proteins destined to be directed into the RSP. The idea that maybe these proteoglycans entering the RSP could participate in the sorting process was discounted when inhibitors of chrondroitin sulfate synthesis were used to reduce the levels of the proteoglycans and found no difference in the processing and secretion of ACTH (Burgess & Kelly 1984). However, similar to the hGH (Moore & Kelly 1985), exogeneously expressed proteins, including proinsulin (Moore *et al*. 1983*c*) and trypsinogen (Burgess *et al*. 1985), were also targeted to the RSP in AtT20 cells, suggesting that other prohormones and exocrine proteins contain a common signal recognized by the AtT20 cell machinery. More significantly, an important gain-of-function study using a fusion protein of VSVG coupled to the C-terminus of hGH demonstrated that the active sorting process of hGH could direct the constitutively secreted VSVG into the granules of the RSP in these cells (Moore & Kelly 1986), demonstrating that the sorting process for sorting into the RSP was dominant over the process of bulk flow transport through the CSP (Kelly 1985). This sorting event was believed at that time to be similar to that of the lysosomal enzymes that use the mannose-6-phosphate receptor (Sly & Fischer 1982). Support for this idea came from observations that, similar to a pH-dependent sorting and recycling of lysosomal enzymes, sorting of POMC to the RSP was prevented in the presence of chloroquine (Moore *et al*. 1983*a*), a compound that neutralizes acidic compartments. In the presence of chloroquine, reduced production of newly synthesized ACTH in the mature granules was observed with an increase in constitutive secretion indicative of mis-sorting. A similar observation was made later, when ammonium ions, which have the same pH neutralizing effect on acidic compartments, were used (Dyken & Sambanis 1994), supporting the role of pH as a very important component of the sorting process.

Sorting of POMC to dense-core secretory granules

POMC is a 31-kDa protein that contains 38 positively charged (arginine and lysine) residues throughout the sequence (without the signal peptide), many of which are PC cleavage sites. Interestingly, these positively charged residues appear to be physiologically balanced by 39 negatively charged (glutamate and aspartate) residues in the POMC protein. Thus, almost one-third of the POMC protein is composed of arginine/lysine and glutamate/aspartate amino acids. Hence, POMC is a highly charged protein and very soluble in aqueous solution. Although POMC is a highly charged protein, it was found to bind tightly to membranes from enriched preparations of secretory granules derived from mouse or frog neuro-IL (NIL) of the pituitary, suggesting that it was interacting with a receptor (Loh & Tam 1985). In further studies on POMC, limiting domain transfer (gain of function) (Tam *et al*. 1993, Cool & Loh 1994) and deletion or mutation (loss of function) experiments (Cool *et al*. 1995) demonstrated that the N-terminus of POMC, specifically N-POMC(1–26), contained information that was sufficient and necessary for sorting POMC to the RSP in AtT20 cells and Neuro2a cells, respectively. Molecular modeling of N-POMC(1–26), made possible by earlier structural analyses of N-POMC(1–26) that solved the di-sulfide bond pairs in this domain (Bennett 1984, Bennett *et al*. 1986), identified a 3D motif (Fig. 2) containing two acidic (Asp^{10} and Glu^{14}) and two aliphatic hydrophobic (Leu^{11} and Leu^{18}) residues that were highly conserved (Cool *et al*. 1995) and predicted to be a consensus sorting signal motif. This motif was subsequently found in monomeric and hexameric proinsulin (Dhanvantari *et al*. 2003), brain-derived neurotrophic factor but not nerve growth factor (Lou *et al*. 2005), and proenkephalin (Normant & Loh 1998, Loh *et al*. 2002), and predicted to bind to a prohormone sorting receptor.

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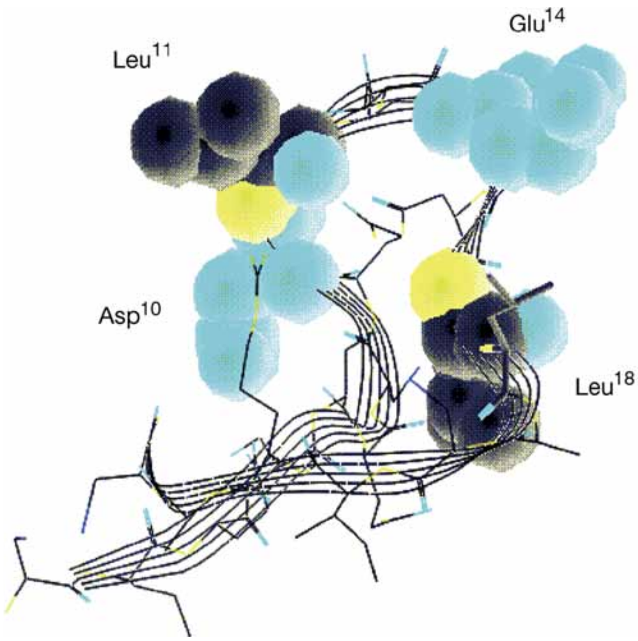

Figure 2
An NMR confomer of the N-POMC(1–26) peptide encoding the RSP sorting signal. Note the two acidic residues, Asp[10] and Glu[14], and the two hydrophobic residues, Leu[11] and Leu[18], comprising the sorting signal motif.

In follow-up studies, Loh and colleagues coupled the N-POMC(1–26) peptide, containing the sorting signal motif of POMC, to beads and used it in affinity chromatography using NIL Golgi-derived membranes, a putative source of a prohormone sorting receptor. Solubilized membranes were applied to the column under acidic conditions (pH 5.5) and bound proteins were eluted at pH 7.4. Using this approach, a candidate sorting receptor for POMC was identified as carboxypeptidase E (CPE) (Cool *et al.* 1997) because it was the major protein in the eluate identified by amino acid sequencing. CPE was classically known since the early 1980s as an enzyme involved in the maturation of peptide hormones by removing lysine and arginine amino acids from the C-termini of peptide hormone intermediates (see the ‘Exopeptidases in POMC peptide processing’ section (Fricker & Snyder 1982, Hook *et al.* 1982*a*)). Subsequent cross-linking and binding studies confirmed its identity and characterized it with low-affinity first-order binding kinetics (K_D = 6 μM) (Cool & Loh 1998). In addition, binding of N-POMC(1–26) to CPE did not require the active site of CPE, as CPE with its active site mutated bound the ligand to the same extent as WT CPE (Zhang *et al.* 1999). Also, addition of guanidinoethylmercaptosuccinic acid (GEMSA), a potent inhibitor of CPE, did not prevent binding (Loh *et al.* 1997), demonstrating that binding of POMC to CPE did not depend on the carboxypeptidase enzymatic activity of CPE. Indeed, molecular modeling of CPE identified a putative sorting signal binding site in Arg[255] and Lys[260] of CPE (Zhang *et al.* 1999) (Fig. 3), further demonstrating that the binding of N-POMC(1–26) was independent of the active site. Support for CPE as a sorting receptor for POMC came from studies on the *Cpe*$^{fat/fat}$ mouse (Naggert *et al.* 1995) where the mutant CPE contains a Ser202Pro mutation rendering the protein unstable and subject to degradation (Varlamov *et al.* 1997, Cawley *et al.* 2003). Hence, the *Cpe*$^{fat/fat}$ mouse was viewed as a CPE-deficient mouse. Secretion studies from NIL and AL pituitary primary cells (Cool *et al.* 1997, Shen *et al.* 1999) of these mice suggested defective sorting of POMC, indicative of the lack of a sorting receptor. This support comes from the analysis of the CPE knockout (KO) mouse (Cawley *et al.* 2010). In the complete absence of CPE, POMC processing to α-MSH in the NIL and hypothalamus of the pituitary is reduced by 81–94%, respectively, and there is an ~10-fold increase in the tissue levels of POMC and its 23-kDa biosynthetic intermediate in the NIL resulting in serum levels of POMC/23-kDa intermediate almost eight-fold higher in the CPE KO mice compared to WT mice (Cawley *et al.* 2010). This suggests a trafficking

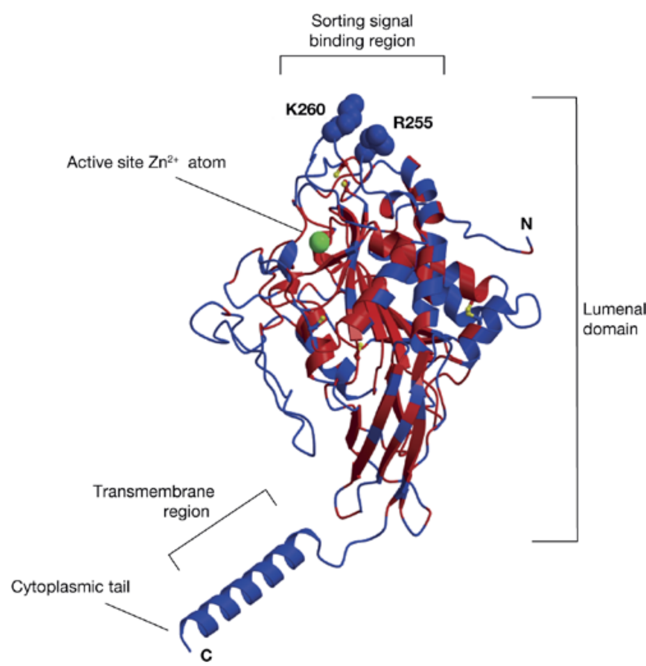

Figure 3
Molecular model of CPE based on the crystal structure of CPD. The red areas indicate the similarity of CPE and CPD, whereas the blue areas indicate the unique areas to CPE. Note the N-POMC(1–26) sorting signal binding site composed of Arg[255] and Lys[260].

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defect of POMC and accumulation in the TGN with increased constitutive secretion, although a processing defect and accumulation of the POMC likely contributes to the phenotype. In the AL of the pituitary, processing of POMC to ACTH is significantly reduced; however, in contrast to the NIL, only a small increase in the tissue content of POMC was observed. Pulse–chase experiments on primary cultures of AL cells found significantly reduced ACTH production and secretion as well as a small but significant increase in the stimulated secretion of POMC, demonstrating that some POMC was sorted into the RSP but its processing to ACTH was reduced (unpublished data of the authors). Later studies in the *Cpe*$^{fat/fat}$ mice suggested that compensation by another potential sorting receptor, secretogranin III (SgIII), was possible, because ACTH was released from the AL of the pituitary from these mice in a CRH-dependent manner, and SgIII, a known sorting receptor for chromogranin A (Hosaka *et al.* 2002), was upregulated in the pituitary of these *Cpe*$^{fat/fat}$ mice (Hosaka *et al.* 2005). Interestingly, our pulse–chase experiments also showed that ~42% of the newly synthesized POMC was unaccounted for in the cells from the CPE KO mouse compared to WT cells (Fig. 4, unpublished data of the authors), indicative of degradation. This suggests that the corticotrophs may compensate for the poor trafficking and accumulation of POMC in the absence of CPE, by directing it to the lysosomes for degradation *in vivo*.

A major constraint in the idea of one protein being a receptor for all the prohormones in the cell was that the

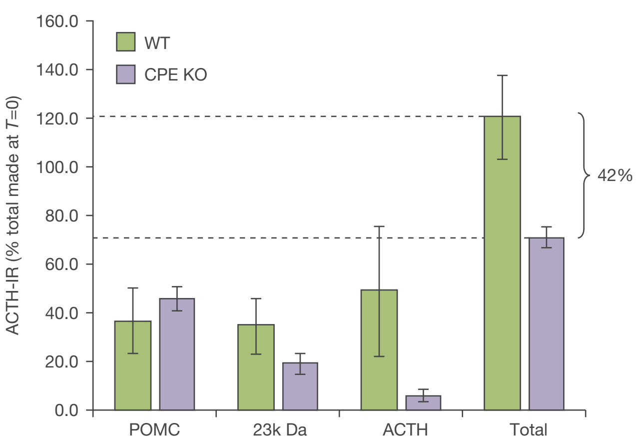

Figure 4
Pulse–chase studies of POMC in mouse AL cells. Pituitary AL cells from WT and CPE KO mice were cultured and metabolically labeled with ^{35}S-Met for 30 min and chased for 2 h. Immunoreactive ACTH molecules were analyzed by immunoprecipitation and quantified. Note the reduced levels of ACTH made in the CPE KO cells. The overall recovery of total ACTH-IR was less in the CPE KO cells compared to the WT cells indicative of degradation (unpublished data of the authors).

stoichiometry did not favor it. It was therefore proposed that homotypic and even heterotypic oligomerization of prohormones may allow concentration of the cargo followed by binding to a receptor. This phenomenon early in the sorting process would be distinguished from peptide hormone aggregation and condensation in the maturing secretory granules. In support of this, evidence suggested that POMC can loosely aggregate homotypically and heterotypically with proenkephalin to form dimers and multimers (Cawley *et al.* 2000), and this aggregation is enhanced in the presence of increased calcium and reduced pH, conditions expected in the TGN (Chandra *et al.* 1991, Seksek *et al.* 1995). More importantly, the N-POMC(1–26) domain was not required for aggregation, thus allowing it to act as a bridge to connect the aggregated cargo with the membrane form of CPE to initiate sorting into the granules of the RSP. Aggregation-induced sorting into the granules of the RSP is one hypothesis proposed to answer how prohormones are sorted at the TGN away from constitutively secreted proteins, because it has been shown that many RSP cargo proteins aggregate under these mild acidic and high-calcium conditions (Chanat & Huttner 1991, Colomer *et al.* 1996, Jain *et al.* 2002), including CPE (Rindler 1998).

RNA interference technology is a powerful tool to specifically reduce the expression of a target gene. Using siRNA to reduce CPE in Neuro2a cells, transfected POMC was secreted constitutively and no punctate staining for ACTH-IR by immunocytochemistry was seen in the knocked down cells, supporting the findings from the *Cpe*$^{fat/fat}$ mouse (Normant & Loh 1998). Later studies show conflicting results in AtT20 cells with respect to ACTH secretion. In both cases, POMC secretion through the CSP was significantly elevated when CPE was knocked down indicative of inefficient sorting to the RSP; however, in one case, ACTH secretion was normal (Kemppainen & Behrend 2010) and in the other, it was not (Cawley *et al.* 2015). Notably, knockdown of SgIII, previously shown to bind POMC (Hosaka *et al.* 2005) and also known as a sorting receptor for chromogranin A (Hosaka *et al.* 2002, 2004), also caused a significant increase in constitutive secretion of POMC and a reduction in ACTH secretion via the RSP (Cawley *et al.* 2015). These results suggest a mechanism involving several membrane-bound proteins that can possibly interact with each other, such as CPE and SgIII (Hosaka *et al.* 2005), or interchange, so that the ultimate important cellular process of the endocrine cell can be carried out, that is, to provide the peptide hormone ready for secretion upon stimulation of the mature secretory granule. Hence, sorting of POMC to the granules of the RSP likely requires interaction with multiple membrane-associated molecules, of which CPE and SgIII are primary candidates, in addition to SgII (Sun *et al.* 2013), at the lumenal side of the TGN during the initial budding, and this interaction results in the active sorting and retention of the prohormone as the immature granule forms and matures.

Transport and exocytosis of POMC secretory vesicles

At the TGN, EM studies showed that POMC was sorted into condensing vacuoles that were seen to contain a clathrin coat (Tooze & Tooze 1986), which was removed during the granule maturation process as no visible structures indicative of the clathrin triskelion were found on the mature granule. Indeed, the vesicle coat contains many proteins involved in the maturation of the vesicle and storage, and then fusion with the plasma membrane upon stimulation (reviewed elsewhere; Kogel & Gerdes 2010, Bonnemaison *et al.* 2013). How POMC vesicles are transported from the TGN to their release site was an interesting question and has recently been studied using live cell imaging in AtT20 cells.

In the AtT20 cells, after the initial site of budding at the TGN, the ACTH vesicles must be transported to the ends of the processes close to the plasma membrane, where they are stored until released. It was seen early on that during cell division, the mature dense-core granules containing ACTH redistribute in the cell, from being localized at the Golgi and tips of the processes in interphase, randomly distributed during metaphase and anaphase but then align at the midbody as it develops during cytokinesis during telophase, a process dependent on the microtubules (Tooze & Burke 1987). Other studies using acridine orange and enhanced fluorescence microscopy demonstrated the saltatory movement of the ACTH-containing vesicles mostly in the anterograde direction and some in the retrograde direction, and the movement, reported at a rate of 3–5 µm/s, was dependent on microtubules (Kreis *et al.* 1989). Transport of POMC vesicles along microtubules has not been studied in detail until recently. Previous work by Loh and colleagues identified that the C-terminus of some CPE could traverse the granule membrane and interact with Arf6, a small cytosolic GTPase involved in clathrin-independent endocytosis (Arnaoutova *et al.* 2003). Subsequent yeast two-hybrid studies showed that the C-terminus of CPE interacted with dynactin (unpublished data). Confirmation of this interaction came from biochemical pulldown and coprecipitation experiments, which showed that the C-terminus of CPE specifically bound to dynactin from AtT20 cell lysates (Park *et al.* 2008). The complex contained kinesins 2 and 3 as well as dynein; microtubule dependent motor proteins for anterograde and retrograde transport, respectively. Interestingly, kinesin 2 transports vesicles at a rate of ~0.5 µm/s (Scholey 2013), a speed observed by live cell imaging of POMC-RFP-containing vesicles in AtT20 cells that was eliminated when the C-terminus of CPE was constitutively overexpressed in the cytosol to act as a dominant negative molecule to inhibit endogeneous CPE C-tail interaction with dynactin (Park *et al.* 2008). These results demonstrated that POMC vesicles were anterogradedly transported along microtubules by the motor proteins, kinesins 2 and 3, and the vesicle anchor was through the C-terminus of CPE. An additional interacting protein elucidated from the yeast-two-hybrid studies was identified as γ-adducin (Lou *et al.* 2010), a protein involved in the cortical actin assembly just under the plasma membrane. It was proposed that as the granule arrives at the end of the microtubules, the CPE C-terminus can interact with γ-adducin to establish a storage zone for the mature granules. Overexpression of a C-terminal (CT) tail region of γ-adducin also caused an accumulation of POMC vesicles at the TGN in AtT20 cells suggestive of a role in POMC vesicle budding from the TGN through interaction with peri-Golgi F-actins (Lou *et al.* 2013).

Processing of POMC

Processing of POMC involves many enzymes, including endoproteases, exopetidases, acetylation, and amidation enzymes, to generate the POMC peptides such as ACTH, α-MSH (Ac-ACTH(1–13)-NH_2), β-LPH, and β-endorphin (Fig. 5). In this section, we describe the sequential processing steps and the enzymes involved in the maturation of POMC and its derived peptides.

POMC processing at paired basic residue-specific sites

Prohormone convertases Processing of POMC can begin at the TGN, although the primary site of proteolytic cleavages occurs within the immature secretory granule. In the TGN, the pH is ~6.8 (Seksek *et al.* 1995), whereas the secretory granule pH is between 4.5 and 5.5 (Loh *et al.* 1984). Hence, the processing enzymes that include various endoproteases and exopeptidases involved in the maturation of POMC have to function within this pH range. The first step in POMC processing is endoproteolytic cleavage at signature pairs of basic residues (Fig. 5).

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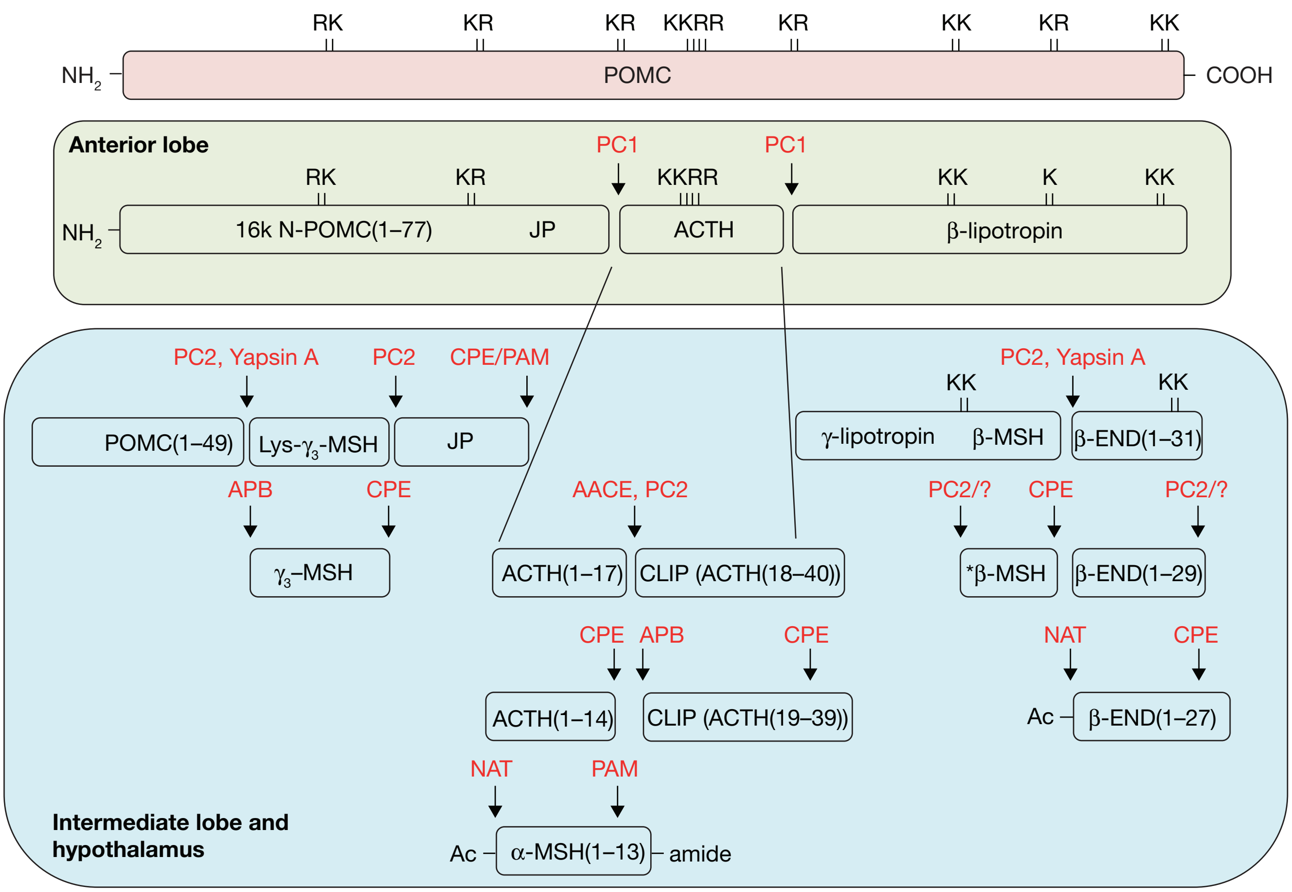

Figure 5

General schematic depicting the processing of bovine POMC. In the AL, PC1/3 is the primary convertase involved in the generation of 16-kDa N-POMC, ACTH, and β-LPH. A more comprehensive processing pattern is seen in the IL and hypothalamus, yielding α-MSH and β-endorphin. APB, aminopeptidase B-like; AACE, acidic ACTH-converting enzyme; CPE, carboxypeptidase E; NAT, *N*-acetyltransferase; PAM, peptidylglycine α-amidating monooxygenase; -END, -endorphin; JP, joining peptide; -MSH, -melanocyte-stimulating hormone; PC, prohormone convertase; Ac, acetyl; K, lysine; R, arginine. *α-MSH in humans may not occur naturally (Scott & Lowry 1974).

The major endoproteolytic enzymes for these cleavages are PC1/3 and PC2. These PCs are subtilisin-like enzymes related to yeast kexin and were cloned in 1991 and shown to cleave POMC (Thomas *et al*. 1991) and other prohormones at specific paired basic residues. (For a review of the historical discovery of the PCs, see Seidah 2011, Chretien & Mbikay 2016).

Both PC1/3 and PC2 enzymes are synthesized as a pro-form and are specifically trafficked to the secretory granules of the RSP where the majority of POMC processing occurs. The mature forms of these enzymes function at an acidic pH (Zhou & Lindberg 1993, Friedman *et al*. 1994, Lindberg *et al*. 1995); hence, they are ideal to work in the mature granule where prohormones are fully processed. The activation of PC1/3 begins early in a pre-Golgi compartment suggestive of an autocatalytic activation on the carboxyl side of the RSKR motif in its pro-segment cleavage site (Benjannet *et al*. 1993, Zhou & Lindberg 1993, Goodman & Gorman 1994). Because the ~87-kDa form of PC1/3 is active and present in the Golgi, it can begin to act on the first cleavages of POMC to yield 16-kDa N-POMC, ACTH, and β-LPH (Fig. 5) in this compartment, although the major cleavage occurs within the immature secretory granules. PC2 is activated later in the immature secretory granule and found to be responsible for the cleavage of 16-kDa N-POMC, ACTH, and β-LPH to yield N-POMC(1–77), α-MSH, and β-endorphin, respectively (Zhou *et al*. 1993). Interestingly, the activity of both these PCs appears to be under the control of endogeneous inhibitors/chaperones. PC1/3 pro-peptide expressed in *trans* is able to act as an endogeneous inhibitor of PC1/3 (Lee *et al*. 2004). Additionally, proSAAS (Fricker *et al*. 2000) has been found to be processed by PC1/3 to yield a polypeptide that acts as an inhibitor for PC1/3 (Qian *et al*. 2000), and therefore regulates its activity on other substrates. In AtT20 cells, proSAAS

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expression has been found to inhibit the processing of POMC under pulse/chase conditions (Lee *et al*. 2004). In the case of pro-PC2, a protein named 7B2, is required as a chaperone to help with its transport to the Golgi and its activation (Muller *et al*. 1997). Pro-PC2 forms a complex with 7B2 in the ER and is then trafficked to the Golgi where 7B2 is cleaved by furin to generate a 31-amino acid CT peptide. The CT peptide then binds to pro-PC2 and acts as a potent inhibitor. Pro-PC2 is then sorted into the immature secretory granule where it is autocatalytically processed into PC2 within the acidic environment of immature secretory granules. PC2 in turn cleaves the CT peptide at VVAKK^{189}↓SVP, followed by removal of the basic residues KK by the exopeptidase, CPE, to generate an inactive form of the CT peptide that liberates the active PC2 enzyme (Fortenberry *et al*. 1999, Mbikay *et al*. 2001). The importance of 7B2 in POMC processing is seen in the 7B2 KO mouse. These mice, unlike PC2 KO mice, die early due to Cushing's disease because of excessive secretion of ACTH from the IL of the pituitary due to the lack of processing by an active PC2 enzyme that would normally produce α-MSH in this tissue. This indicates a role of 7B2 not only in POMC processing indirectly, but also in secretion of its derived peptides (Mbikay *et al*. 2001). Indeed, stimulated secretion of ACTH from AtT20 cells is negatively correlated to cellular levels of 7B2, also reflecting a possible role in POMC processing and secretion in these cells (Bergeron *et al*. 2002).

The physiological importance of PC1/3 in POMC processing came from several lines of evidence. This included *in situ* hybridization studies, revealing that PC1/3 mRNA was expressed primarily in anterior pituitary corticotrophs that synthesize ACTH, whereas it was colocalized with PC2 in the intermediate pituitary that synthesizes α-MSH (Seidah *et al*. 1991). This led to further understanding of the previous *in vitro* pulse–chase studies using AtT20 and primary cultures of anterior and intermediate pituitary cells and hypothalamic neurons (Loh 1979, Liotta *et al*. 1980, Mains & Eipper 1981*b*) attributing cleavage of POMC at paired basic residues to generate N-POMC (16 kDa), ACTH, and β-LPH in anterior pituitary corticotrophs to PC1/3, whereas PC2 cleaved 16-kDa N-POMC to yield N-POMC(1–77), ACTH to yield α-MSH, and β-LPH to yield β-endorphin in melanotrophs in the intermediate pituitary and hypothalamic neurons (Fig. 5): observations subsequently affirmed by additional experiments (Zhou *et al*. 1993, Friedman *et al*. 1994, 1996, Paquet *et al*. 1996). *In vivo* studies using gene KO in mice for PC1/3 and PC2 (Furuta *et al*. 1997, Zhu *et al*. 2002) and the finding of two human patients with defects in PC1/3 (Jackson *et al*. 1997, Farooqi *et al*. 2007) showed that both these enzymes are not essential for life. Moreover, PC1/3 null mice process POMC poorly to ACTH (Zhu *et al*. 2002) yet have normal levels of circulating corticosterone. They also exhibit retarded growth and developmental defects because the enzyme is responsible for processing other prohormones. PC2 null mice look normal at birth but show retarded growth, and they do not fully process POMC-derived peptides (Furuta *et al*. 1997). Peptidomic analyses of PC1/3 (Wardman *et al*. 2010) and PC2 (Zhang *et al*. 2010) KO mice revealed that the loss of PC1/3 is often compensated for by PC2, but the reverse is not always true. This corroborates with *in vitro* studies in GH3 cells that express PC2 and not PC1/3, showing that exogeneously expressed POMC was completely processed to ACTH-related peptides (ACTH(1–14), ACTH(1–15), and ACTH(1–17)) as well as β-endorphin and Lys-γ-MSH (Friedman *et al*. 1996). A female patient deficient in PC1/3 protein due to both splicing and nonsynonymous mutation in the *PC1/3* gene showed low expression levels of the enzyme and high circulating levels of several forms of partially processed POMC intermediate ACTH products (Jackson *et al*. 1997). The patient was obese and had poor glucose homeostasis. Although this patient differs from PC1/3 null mice that are not obese (Zhu *et al*. 2002), the current finding points to an important role of PC1/3 *in vivo* in POMC processing.

Yapsin A In addition to PC1/3 and PC2, another enzyme known as Yapsin A (or POMC-converting enzyme), an aspartic protease, has been purified to apparent homogeneity from bovine pituitary IL and neural lobe secretory granules as well as from adrenal chromaffin granules. It has been shown to process POMC at paired basic residues to yield N-POMC(1–77), ACTH, β-LPH, and β-endorphin (Fig. 5; Loh *et al*. 1985, Azaryan *et al*. 1995). This enzyme is related to Yapsin 1 or yeast aspartic protease 3, a gene product of the *yps1* gene in yeast that has also been shown to process pro-α-mating factor at paired basic residues, similar to the yeast kex-2 enzyme, (Egel-Mitani *et al*. 1990). Yapsin 1 is able to cleave POMC at paired basic residues as well (Azaryan *et al*. 1993). Yapsin A has been characterized as an ~70-kDa enzyme that has a pH optimum of 4.0–5.0. An antibody generated against Yapsin 1 has been used to immunologically identify mammalian yapsin 1-like proteins in bovine and mouse endocrine and neuroendocrine tissues (Cawley *et al*. 1996). Yapsin 1-like

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IR has also been found exclusively in human pancreatic islet α-cells, and purified yapsin 1 can generate glucagon by processing proglucagon *in vitro* (Cawley *et al.* 2011). Although Yapsin A has not been cloned, current studies suggest that a mammalian aspartic protease present in endocrine tissue, similar to Yapsin 1 in yeast, may play a role in processing of POMC and other prohormones *in vivo*. Additional 'backup' enzymes could be important in ensuring that prohormone processing is maintained, such that genetic defects in the PCs may not necessarily produce a phenotype.

Tetrabasic residue-specific enzymes A calcium-activated serine protease, named acidic ACTH-converting enzyme (AACE), with a pH optimum of 5.0–6.0 and being highly specific for tetrabasic residues, has been reported to be present in bovine IL secretory granules (Estivariz *et al.* 1992). AACE-cleaved ACTH(1–39) at the tetrabasic residues of the Arg^{17}–Arg^{18} bond to yield ACTH(1–17) and corticotropin-like intermediate peptide (CLIP) but did not cleave the paired basic residues of POMC. The enzyme has not been cloned, but AACE could play a role in the processing of ACTH to α-MSH besides PC2.

Exopeptidases in POMC peptide processing

Carboxypeptidase E/H Subsequent to endoproteolytic cleavage of POMC at paired basic residues, an exopeptidase is required to remove the CT basic residues to yield the biologically active peptides. CPE or carboxypeptidase H (initially known as enkephalin convertase or carboxypeptidase B-like enzyme) was first discovered in 1982 as an enzyme capable of removing C-terminally extended lysine and arginine residues from enkephalin peptides (Fricker & Snyder 1982, Hook *et al.* 1982*a*). CPE is a metalloprotease with Zn bound at the active site. It has a pH optimum of 5.5 that is stimulated by Co^{2+} and specifically inhibited by GEMSA. It was also shown to remove the basic residues from ACTH(1–17), a peptide liberated by PC1/3 from POMC, to generate ACTH(1–16), ACTH(1–15), and ACTH(1–14) (Hook & Loh 1984). At that time, other peptides with C-terminally extended lysine and arginine residues such as vasopressin and oxytocin were also shown to be removed by CPE (Hook & Loh 1984, Kanmera & Chaiken 1985). Because of its localization and optimum activity in the acidic environment of secretory granules, where peptide hormone intermediates are processed, and because of its specificity for C-terminally extended lysine and arginine residues, CPE was considered to be the primary carboxypeptidase for most if not all peptide hormone intermediates, including those derived from POMC. Indeed, proteomic analysis of pituitaries from the CPE-deficient mouse, *Cpe*$^{fat/fat}$, showed a significant accumulation of the CT basic residue-extended POMC-derived peptides, compared with WT pituitary, indicating the role of CPE in the normal processing of these peptides *in vivo* (Che *et al.* 2005).

There are two forms of CPE: a soluble form that is enzymatically much more active than a membrane form (Hook 1985). Some of the membrane forms can assume a transmembrane orientation in the secretory granule membrane giving rise to a cytoplasmic tail (Dhanvantari *et al.* 2002, Zhang *et al.* 2003). The membrane form can act as a sorting receptor for prohormones at the TGN, and the cytoplasmic tail is involved in secretory granule transport by associating with microtubule motors (see the 'Transport and exocytosis of POMC secretory vesicles' section). CPE, synthesized as a precursor (pro-CPE, ~55 kDa in size), is trafficked to the TGN where it associates with the membrane through interaction with lipid rafts and is subsequently sorted into immature secretory granules after budding. Some of the CPE is then processed to the mature soluble form (molecular weight ~50 kDa) within the secretory granule, where it can act enzymatically to cleave basic residues from peptide products liberated from POMC. CPE is secreted, and several studies indicate that it plays other important nonenzymatic roles as a signaling molecule acting extracellularly in neuroprotection and prevention of stress-induced depression (for a review, see Cawley *et al.* 2012).

The physiological importance of CPE as a processing enzyme and a sorting receptor for prohormones was evident from several studies. A mutation in the *Cpe* gene was found in the *Cpe*$^{fat/fat}$ mouse that presented with severe obesity, diabetes, and infertility (Naggert *et al.* 1995). In these *Cpe*$^{fat/fat}$ mice, it was reported that POMC was accumulated 24-fold above normal animals in the anterior pituitary, and it was poorly processed to ACTH, although larger 24-kDa form of ACTH was present (Shen & Loh 1997). Furthermore, POMC was secreted constitutively at high levels, showing no response to stimulation by corticotropin-releasing hormone (Shen & Loh 1997), a finding not reproduced later by others (Hosaka *et al.* 2005), possibly reflecting a change in the mice within the intervening years. POMC levels were elevated in the circulation of *Cpe*$^{fat/fat}$ mice versus normal mice. This poor maturation of POMC could be a result of inefficient sorting of POMC into the granules of the RSP

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for full processing because CPE acts as a sorting receptor, resulting in constitutive secretion of partially processed POMC products that accrued in the Golgi (Shen & Loh 1997). These mice also had hyperproinsulinemia (Naggert *et al.* 1995) and GnRH peptides with extended basic residues that were inactive, resulting in the infertility phenotype in these animals (Srinivasan *et al.* 2004).

As indicated previously, obesity was also an observed phenotype in the *Cpe*$^{fat/fat}$ and CPE KO mice, contributed in part by autophagy due to a disruption in the hypothalamic circuitry that controls satiety (Cawley *et al.* 2004). In both cases, defective processing of hypothalamic POMC to α-MSH, a major anorexigenic peptide that controls satiety in this tissue, resulted in increased food intake and obesity, demonstrating that CPE played a key role in appetite regulation and energy balance. In support of this were the observations that ablation of Forkhead box protein O1 (FOXO1) in POMC neurons (POMC-FOXO1$^{-/-}$) reduced food intake without affecting energy expenditure. The study showed that FOXO1 is a corepressor of CPE expression at the promoter level. Consequently, increased levels of the hypothalamic neuropeptides, α-MSH and β-endorphin, were observed in the POMC-FOXO1$^{-/-}$ mice. FOXO1 deletion therefore protected the mice against weight gain, in a diet-induced obesity paradigm, by increasing the satiation POMC peptide, α-MSH (Plum *et al.* 2009). Hence, in this POMC-FOXO1$^{-/-}$ model, deletion of FOXO1 allowed increased expression of CPE in the POMC hypothalamic neurons that subsequently affected the levels of active PC2 by inactivation of the CT inhibitor peptide of 7B2. Concomitantly, the CPE can process the acetylated ACTH(1–16) intermediate to α-MSH (see the 'Prohormone convertases' section on PC2; Zhu *et al.* 1996). This study further demonstrates the important physiological function of CPE in obesity. Corroborating these mouse studies is a recent description of the first human with a truncating homozygous null mutation for CPE, which showed the patient presented with obesity, type 2 diabetes, as well as intellectual disability (Alsters *et al.* 2015), further emphasizing the critical role CPE plays in prohormone processing and sorting.

Aminopeptidases Although PC1/3 and PC2 generally cleave POMC and derived peptides on the carboxyl side of paired basic residues, the cleavage of ACTH at the tetrabasic residues by PC2 to release CLIP (Fig. 5) with an N-terminal arginine indicates that certain cleavages occurs between two basic residues. Cleaving of POMC in between basic residue doublets by Yapsin A has also been reported. Thus, there is a need for an aminopeptidase B-like enzyme to remove the N-terminal basic residue. An aminopeptidase B-like enzyme has been found in bovine pituitary IL and NL secretory granules (Gainer *et al.* 1984). The enzymatic activity is found as both a soluble and a membrane form, has a pH optimum of 6.0, is stimulated by Co^{2+} and Zn^{2+}, and cleaves Arg preferentially over Lys. However, it will not cleave an N-terminal Arg if it is followed by a proline such as in CLIP. Characterization of the enzyme indicates that it is an ~70-kDa glycoprotein and is coordinately secreted with α-MSH, indicating its colocalization in the same secretory granules (Castro *et al.* 1989).

Acetylation and amidation of POMC-derived peptides

Acetylation of POMC peptides N-acetylation of peptide hormones may serve to increase the stability of the peptide by protecting them against the action of aminopeptidases and enhance their half-life in the circulation. Acetylation also has profound biological effects on the POMC peptides. Although the melanotropic activity of α-MSH (Guttmann & Boissonnas 1961) is potentiated and its half-life increased by N-acetylation, acetylation of β-endorphin completely abolishes its opiate activity (Deakin *et al.* 1980). Because these two peptides are derived from POMC, acetylation could be used to regulate the relative amounts of melanotropic and opiate activities. While it has been reported that in mammals, α-MSH is the predominant form, diacetylated and deacetylated forms are also present in the pituitary IL, although the existence of these latter forms do vary among species. *N*-Ac-β-endorphin has been found in both the AL and the IL from postnatal day 1 (P1) through adulthood. In the IL, the level increases to 90% of the endorphins present by P14, but in the AL, *N*-Ac-β-endorphin drops dramatically to <5% in adult rats (Alessi *et al.* 1983).

An *N*-acetyltransferase enzymatic activity has been found in bovine and rat intermediate pituitary secretory granules that could acetylate both ACTH(1–14) and β-endorphin (Chappell *et al.* 1982, Glembotski 1982, Gibson & Glembotski 1985). Competition studies (Glembotski 1982) using fragments of ACTH and β-endorphin peptides and acetylation enzymatic activity from bovine secretory granules, as well as comparative studies of ACTH and β-endorphin acetylation enzymatic activities (Chappell *et al.* 1982) from rat IL secretory granules indicate that the same acetylation enzyme is responsible for acetylating the N-terminus of both ACTH(1–14) and β-endorphin to yield *N*-Ac-ACTH(1–14) and Ac-β-endorphin, respectively. This enzymatic

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activity specifically localized to the secretory granules of rat intermediate pituitary has been referred to as opiomelanotropin acetyltransferase (OMAT) (Chappell *et al.* 1982). Unlike other acetylation enzymatic activities in the pituitary, OMAT has a pH optimum of 6.0–6.6 and is inhibited by detergents. Because the secretory granule acetylation enzymes in the bovine and rat intermediate pituitary have not been cloned, it remains to be determined if they are similar or identical molecules.

N-acetyltransferase activity and POMC expression have been shown to be coregulated in the intermediate pituitary (Millington *et al.* 1986). Secretion of POMC peptides from the intermediate pituitary is under inhibitory control by dopamine (Fischer & Moriarty 1977). It was found that haloperidol, a dopamine antagonist, coordinately increased the acetyltransferase activity, POMC mRNA levels, and POMC peptides, whereas bromocryptine, a dopamine agonist, had opposite effects (Millington *et al.* 1986). Only the acetyltransferase activity from secretory granules was affected by these pro- and anti-secretogogue activities but not the acetylation activity in the RER or Golgi. Additionally, changes in acetylation of β-endorphin have been demonstrated in rat IL cells in culture when exposed to dopamine agonists and antagonists (Ham & Smyth 1984). These studies suggest that acetylation of these peptides may be modulated by their secretory activity. It has been reported that repeated stress selectively increased the biosynthesis and release of *N*-Ac-β-endorphin(1–31) from the IL of rats and is the major form in plasma (Akil *et al.* 1985). By contrast, in the anterior pituitary, after repeated stress, the major form released is β-endorphin(1–31), rather than β-LPH, normally released under nonstressed conditions. Thus, the acetylation of POMC-derived peptides contributes another mechanism for the regulation of their hormonal activity.

Amidation of POMC peptides Synthesis of α-MSH involves the amidation of *N*-Ac-ACTH(1–14). In addition, another POMC-derived peptide, joining peptide (Fig. 5) is also amidated. The enzyme involved in the amidation reaction is peptidylglycine α-amidating monooxygenase (PAM). Studies on the amidation of POMC peptides and PAM are reviewed by Mains and Eipper (Kumar *et al.* 2015) in this book issue.

Regulation of POMC processing

POMC is differentially processed endoproteolytically in the anterior and intermediate pituitary, and the arcuate nuclei of the hypothalamus to generate different end products (Fig. 5). A number of factors can dictate the differential processing.

Tissue-specific glycosylation of a residue close to the cleavage active site can influence the ability of the enzyme to cleave the site. Indeed, the fate of N-POMC(1–77) exemplifies such a mechanism because only ~50% of this POMC intermediate was cleaved in pituitary tissue into N-POMC(1–49) and Lys-γ_3-MSH (Seger & Bennett 1986). Metabolic labeling studies and POMC fragment analysis identified that the O-linked glycosylation of Thr-45 regulated the processing of N-POMC(1–77) into N-POMC(1–49) (Bennett 1986), suggesting that stearic hindrance by the sugar moiety could prevent the processing. This has important physiological significance because the regulation of processing that this site produces controls the level of N-POMC(1–49) and the mitogenic activity it exhibits (Pepper & Bicknell 2009).

Another mechanism for tissue-specific processing of POMC in the AL and IL is dictated by the presence of different processing enzymes as reviewed previously. Whereas the intermediate pituitary expresses PC1/3 and PC2 resulting in the processing of ACTH to α-MSH and β-LPH to β-endorphin, the anterior pituitary expresses primarily PC1/3 that does not catalyze theses cleavages *in vivo*. Additionally, the tetrabasic residue-specific enzyme AACE is present in much higher amounts in the IL than in the AL of the pituitary (Estivariz *et al.* 1992), and this contributes to the differences in processing of ACTH and β-LPH in these two lobes. The presence of PC2 in the hypothalamic POMC neurons could also account for the processing of ACTH to α-MSH in theses neurons (Zheng *et al.* 1994, Joshi *et al.* 1995).

Secretion of POMC-derived peptides

After posttranslational processing via cleavage by prohormone convertases, POMC-derived peptides are packaged and stored as electron-dense cores in secretory granules and secreted in response to simulation by secretagogues. POMC-derived peptides are mainly secreted from corticotrophs and melanotrophs of the AL and IL of the pituitary gland, respectively, as well as from peptidergic neurons of the arcuate nucleus of the hypothalamus. For example, ACTH, β-LPH, and some β-endorphin are secreted by corticotrophs, whereas α-MSH and β-endorphin are primarily secreted by melanotrophs and from the hypothalamus. The secretion of POMC-derived peptides is regulated by various secretagogues as discussed next. This section is not intended as a comprehensive list

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of effectors of POMC-derived peptide secretion, as that is exceedingly complex, but more as a summary of several pathways that play roles in this process.

Regulation of secretion of POMC-derived peptides from the AL of the pituitary The pituitary gland is composed of the AL (adenohypophysis) and the posterior lobe (neurohypophysis, or neural lobe (NL)) with an IL present between the AL and the NL, and is considered by many to be the master endocrine gland, although the neural lobe is technically considered an extension of the hypothalamus (Fig. 6). It is the middle component of the hypothalamic–pituitary–adrenal axis and is involved with multiple endocrine functions such as growth, stress, and reproduction. Nerve fibers from the hypothalamus extend through the median eminence and infundibular stem to the pituitary through the pituitary stalk. Axons containing arginine vasopressin (AVP) and oxytocin from the magnocellular neurons of the hypothalamus (supraoptic nucleus (SON) and paraventricular nucleus (PVN)) innervate the capillary bed of the posterior lobe and are released to the circulation through the hypophyseal vein. Other axons from the PVN terminate earlier at the capillary network in the lower infundibular stem close to the AL and release neurotransmitters and peptide hormones into the portal network, which in turn regulates the secretion of peptide hormones from cells of the AL. Because the IL is in close proximity to the AL, molecules secreted into and through the AL affect secretion from the IL too.

With respect to POMC, *in vivo*, in response to short-term or long-term stress, the neurons in the hypothalamic PVN secrete corticotroph-releasing factor/hormone (CRF, also known as CRH) into the hypophyseal portal system (Swanson *et al.* 1983), which then stimulates the secretion of POMC-related peptides from the corticotrophs of the AL, including primarily ACTH (Chan *et al.* 1982). The ACTH exits the pituitary via the hypophyseal vein, which in turn activates the adrenal cortex to produce glucocorticoids during times of stress. In addition, β-LPH/β-endorphin is released to activate opioid pathways in the body in response to pain. Among their many physiological roles, glucocorticoids in turn exhibit a negative feedback inhibition on the corticotrophs in the AL and the hypothalamic neurons to keep the levels of circulating ACTH under control. Hence, the AL is primarily under the positive regulation of CRF and the negative regulation of glucocorticoids, although there are many other hormones and compounds that contribute to the net secretion of POMC-derived peptides from the corticotrophs. Indeed, dexamethasone, a glucocorticoid homolog, was shown to reduce the serotonergic-induced secretion of β-LPH *in vivo*, suggesting that serotonin neurons may regulate and contribute to the release of

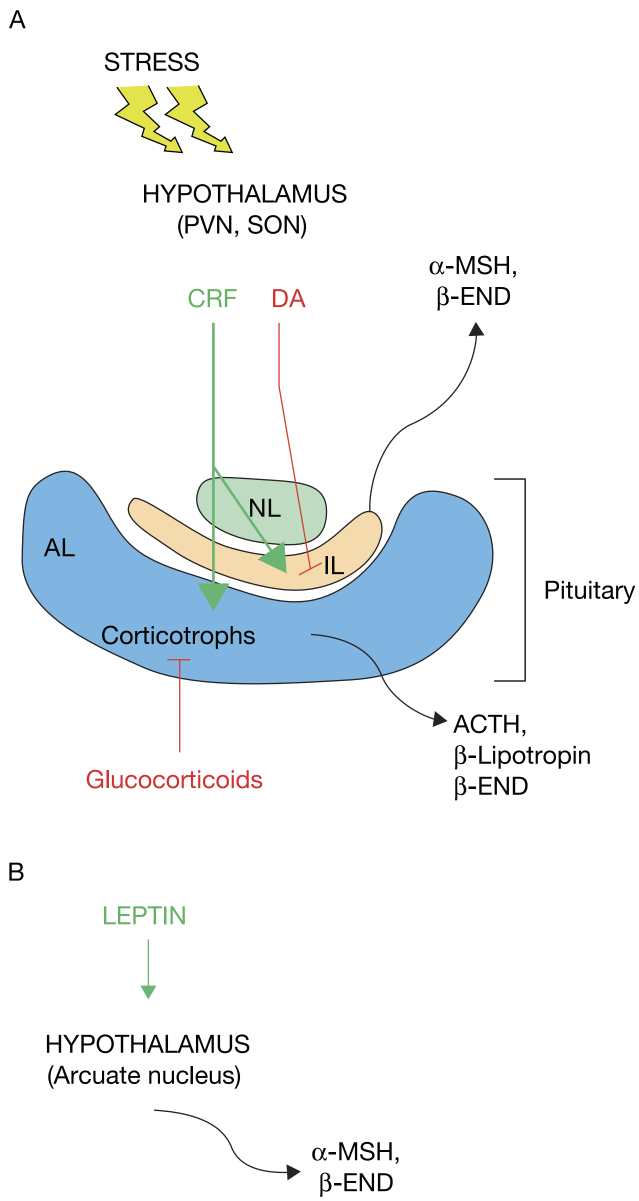

Figure 6
Simplified schematic of the hypothalamic–pituitary axis. (A) Corticotrophs in the AL are under positive regulation by CRF released from the hypothalamus during times of stress. These cells release ACTH that causes the secretion of glucocorticoids from the adrenal cortex. Glucocorticoids then inhibit ACTH, β-LPH, and β-endorphin release in a negative feedback manner. Dopamine (DA) inhibits and CRF increases the secretion of α-MSH and β-endorphin from the melanotrophs of the IL. (B) Leptin secreted from adipocytes activates POMC neurons in the arcuate nucleus of the hypothalamus to release α-MSH and β-endorphin. See main text for other neurotransmitters and peptide hormones that help regulate the secretion of POMC-derived peptides from these tissues.

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β-LPH (and by association ACTH) from anterior pituitary corticotrophs *in vivo* (Sapun-Malcolm *et al.* 1983).

In addition to glucocorticoids, other factors, such as atrial natriuretic factor (ANF) (Shibasaki *et al.* 1986) and somatostatin (Invitti *et al.* 1991), have been shown to inhibit POMC-derived peptide secretion by affecting CRF function. For ANF, it had been proposed that its inhibition was through cGMP signaling; however, later studies did not support this (Bowman *et al.* 1997). For somatostatin, the selective inhibition of CRF-induced secretion of β-endorphin *in vivo*, and not ACTH and β-LPH, was confounding; however, it was proposed that treatment with somatostatin possibly reduced the processing of POMC to β-endorphin in corticotrophs to account for the reduced levels. Alternatively, the effect could be through regulation on pituicytes or other nonpituitary tissue specifically expressing β-endorphin and not corticotrophs. This was considered an example of dissociated secretion of POMC-derived peptides. Gamma-aminobutyric acid (GABA) can also inhibit β-endorphin secretion, possibly through an interaction between GABAergic neurons and CRF neurons, and exerts a tonic inhibitory role on the CRF-regulated corticotroph secretion (Petraglia *et al.* 1986).

In contrast to those molecules involved in the negative regulation of POMC-derived peptides secretion, many other factors have been shown to stimulate their secretion via effects on CRF, e.g. clusterin, a peptide secreted from the pituitary and hypothalamus after stress, increased basal and CRF-stimulated POMC promoter activities, and intracellular cAMP levels, thus augmenting CRF-stimulated ACTH production and secretion (Shin *et al.* 2013). Indeed the regulation of expression and synthesis of POMC by secretagogues (Aoki *et al.* 1997) affects the cellular content and hence secretion profiles, e.g. melatonin and bone morphogenetic protein 4 (Tsukamoto *et al.* 2010, 2013). Some hormones or compounds also appear to stimulate secretion of POMC-related peptides from the anterior pituitary via diverse mechanisms. For example, Melittin, the major peptide component of bee venom and a powerful stimulator of phospholipase A2, generated a signal in corticotrophs of rat AL resulting in the stimulated secretion of ACTH and β-endorphin, although the mechanism appeared to be independent of the phospholipase A2 activation (Knepel & Gerhards 1987). Interleukin-1, a cytokine released from cells of the immune system in response to infection, enhanced secretion of β-endorphin by inducing protein kinase C (Fagarasan *et al.* 1989). In addition, the renin–angiotensin system increased the secretion of ACTH, β-LPH, and β-endorphin by stimulating the secretion of AVP from neurons of the SONs and PVNs of the hypothalamus to exert its stimulatory effect on the AL, thus increasing the secretion of β-endorphin (Beuers *et al.* 1982). AVP had been shown to induce the secretion of POMC-derived peptides from primary cultures of human anterior pituitary cells; however, it was not as potent as CRF (Chan *et al.* 1982).

Small molecule studies have also identified further levels of control on the secretion of POMC-derived peptides from the AL. It was observed that the calcium antagonist, nimodipine, increased β-endorphin secretion through an action on the adrenal glands. It was proposed that, because glucocorticoids exhibit feedback inhibition on the regulation of biosynthesis and secretion of POMC, nimodipine, which reduced adrenal gland responsiveness to ACTH, might increase β-endorphin release from the anterior pituitary gland by reducing glucocorticoid secretion from the adrenal cortex (Costa *et al.* 1984). Hence, regulation of adrenal responsiveness to ACTH affects corticotroph behavior. Other small molecules such as cyclosporin A and tacrolimus (FK506), immunosuppressant drugs, stimulated POMC-derived peptide secretion and potentiated phorbol ester- and CRF-stimulated secretion (Sheppard 1995), demonstrating that these immunosuppressant drugs act at a common point in these pathways.

As indicated in the 'Intracellular organization of POMC maturation' section, AtT20 cells have been used extensively in the study of POMC biosynthesis and trafficking as well as in the regulation of secretion of its POMC-derived peptides. These cells, as noted previously, store and secrete ACTH- and β-endorphin-related peptides in a calcium-dependent manner in response to secretagogues or by membrane depolarization with high levels of K^+. Membrane depolarization by action potentials and calcium influx was shown to be closely linked to the regulated secretion of the mature granules in AtT20 cells (Surprenant 1982). Using isoproterenol, a nonselective β-adrenergic agonist, or raising the external calcium concentrations increased both action potential frequency and ACTH/β-endorphin-like peptide secretion in AtT20 cells. However, a complete blockade of action potential activity had no effect on basal hormone secretion in these cells, indicating that the mechanisms underlying stimulated hormone secretion were different from those responsible for basal secretory activity. Indeed, norepinephrine, a member of the catecholamine family, stimulated the release of ACTH, β-endorphin, β-LPH, and 16-kDa N-POMC from AtT20 cells, an effect that was fully

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blocked by cobalt, demonstrating that the stimulated secretion was calcium dependent, the hallmark of regulated secretion (Mains & Eipper 1981*a*). Also in AtT20 cells, phorbol ester, vasoactive intestinal peptide, forskolin, β-adrenergic agonist, as well as the calcium ionophore stimulated the secretion of a dynorphin-converting enzyme found in these cells, in parallel with CPE and ACTH, demonstrating the wide responsiveness of the granules containing ACTH in these cells to many secretagogues (Devi 1992).

As with the feedback inhibition by glucocorticoids on the corticotrophs *in vivo*, glucocorticoids rapidly inhibit the secretion of these peptides from these cells *in vitro* by increasing transcription and translation of proteins that inhibit synthesis or increase the catabolism of the peptides (Sabol 1980). The fast inhibitory effect may partly be due to a glucocorticoid-dependent reduction in CRF stimulation by blocking the CRF-dependent calcium signaling (Antoni 1996). Other studies in AtT20 cells showed that CRF at concentrations that stimulated ACTH secretion also increased phospholipid methylation. These effects were blocked not only by dexamethasone, a synthetic glucocorticoid that selectively inhibits corticotroph secretion *in vitro*, but also by the phospholipid methyltransferase inhibitors, 3-deazaadenosine and L-homocysteine thiolactone, suggesting that phospholipid methylation might be a CRF receptor-mediated event associated with ACTH secretion (Hook *et al*. 1982*b*).

In contrast to the negative regulation by glucocorticoids on the AtT20 cells, no evidence for autoinhibition of secretion by accumulated secreted peptides (i.e., ultrashort feedback) was found. Furthermore, synthetic human ACTH and synthetic camel β-endorphin did not alter secretion of peptides when added to the culture medium at up to 10,000 times above physiological levels (Mains & Eipper 1981*a*).

Regulation of secretion of POMC-derived peptides from the IL of the pituitary Mammals and lower vertebrates have a well-developed IL in which α-MSH is released into the blood stream through the hypophyseal vein to affect the regulation of pigmentation by melanocytes. However, in humans, the IL is only present in the fetus as a distinct area but in adults is reduced to a thin layer of cells between the AL and the posterior lobe of the pituitary, or it is entirely absent (McNicol 1986). As such, the relevance to human physiology is limited because most work on the IL has been done in tissue obtained from a variety of animals, including frog, mouse, rat, and dogs. As mentioned previously, the IL is in close proximity to neurotransmitters and peptide hormones released from the hypothalamic neurons in the capillary network of the hypophyseal artery in addition to being at the interface of the neurohypophysis. Because the IL is composed primarily of a homogeneous population of melanotrophs, its main function is to provide MSH-like peptides, in addition to β-endorphin, to the circulation in response to hypothalamic signals.

Early on, dopamine was shown to effectively inhibit the release of ACTH-like material from isolated rat NIL, whereas other compounds were reported as potent secretagogues such as acetylcholine, serotonin, and AVP (Fischer & Moriarty 1977), demonstrating that the net secretion was likely a balance between stimulatory and inhibitory signaling. Indeed, release of ACTH and MSH from dog IL was inhibited by dopamine, somatostatin, norepinephrine, and epinephrine; the last two however were blocked by haloperidol (a dopamine D_2 receptor antagonist), suggesting signaling through the dopamine receptor (Kemppainen *et al*. 1989). In addition, haloperidol increased POMC mRNA expression in rat IL (Hollt & Bergmann 1982) but decreased the CRF receptor expression (Shiver *et al*. 1992), whereas bromocriptine, a dopamine receptor agonist, did the opposite. Indeed, CRF receptors are present on both lobes of the pituitary (Aguilera *et al*. 1987) but show different levels of expression in response to dopamine agonists and antagonists (Shiver *et al*. 1992). Notably, these two compounds did not affect CRF receptor levels in the AL and demonstrated a tight regulation of POMC expression and secretion of POMC-derived peptides from the IL by specific tonic inhibition of dopamine D_2 receptors and subsequent regulation of CRF receptor expression (Beaulieu *et al*. 1984), although other neurotransmitters, for example, GABA, also participate (Tomiko *et al*. 1983). Similar to the regulation of corticotrophs in the AL, release of POMC-derived peptides from the IL is stimulated by CRF and CRF-like peptides, for example, urotensin I and sauvagine (Tran *et al*. 1990). Hence, CRF stimulates corticotrophs to secrete ACTH and β-LPH and melanotrophs to secrete α-MSH and β-endorphin.

Further studies in perifused rat IL demonstrated that the amounts of spontaneously secreted ACTH- and LPH-related peptides were proportional to the amounts in which these peptides were found in extracts of IL (Tilders *et al*. 1981). This high basal rate of secretion was presumably due to the lack of tonic inhibition by dopamine in this *in vitro* system. However, isoproterenol

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could stimulate the release of various peptides, including α-MSH, ACTH, and β-endorphin-like peptides above this baseline (Tilders *et al*. 1981). Studies in bovine IL indicated that 8-bromo-cAMP significantly increased and bromocriptine significantly reduced the secretion of α-MSH (Castro *et al*. 1989).

There are many studies investigating the regulation of secretion of peptide hormones, specifically POMC-derived peptides, from the IL; too many to adequately describe here, however, it can be seen from this section that the interplay between signaling molecules from the hypothalamus and peripheral tissues results in controlling the levels of POMC peptides delivered to the circulation. Taken together, the regulation of secretion of POMC-derived peptides from the AL and IL of the pituitary gland involves many factors with the final outcome depending on the competing action of these stimulatory and inhibitory factors.

Regulation of secretion of POMC-derived peptides from the arcuate nucleus of the hypothalamus The arcuate nucleus is the third major site for POMC expression; other tissues have also been identified in which POMC is expressed, for example, the skin and the placenta (for review and references within, see Stevens & White 2010). As already stated, the final peptides produced in the hypothalamus are similar to those from the IL: α-MSH and β-endorphin. These neurons are responsive to leptin and insulin, and other neural and humeral signals, as indicators of peripheral energy stores *in vivo* and are primarily known to centrally regulate food intake through α-MSH action on the melanocortin 4 receptors (MC4Rs) in other hypothalamic areas and in areas of the brain stem important in regulating the energy balance (Cowley *et al*. 2001, Lin & Salton 2013). Mutations in MC4R were first reported to be associated with inherited human obesity in 2008 (Loos *et al*. 2008), demonstrating the importance of this signaling pathway in human health. Indeed, defective POMC processing in humans and mouse models leads to severe obesity, as well as ACTH deficiency and hypopigmentation (Jackson *et al*. 1997, Krude *et al*. 1998, Jackson *et al*. 1999, Yaswen *et al*. 1999). In this regard, POMC is a particularly interesting molecule in the homeostatic regulation of appetite and obesity. For a more comprehensive review on POMC neurons in the arcuate nucleus and their regulation, readers are directed to a recent review by Sharon Wardlaw (Wardlaw 2011) and another review published in this special issue of *Journal of Molecular Endocrinology* by Roger Cone (Anderson *et al*. 2016).

Secretion of ACTH in tumors When secretion of POMC-derived peptides is abnormal, a disease state can occur. For instance, positive regulation of ACTH secretion by CRF, vasopressin as well as other factors leads to excessive levels of ACTH when the feedback inhibition of glucocorticoids is faulty (Imura 1985). This leads to excessive glucocorticoids (cortisol) in circulation, leading to Cushing's syndrome. A similar dysregulation may occur with an ACTH-producing pituitary adenoma giving rise to Cushing's disease. Another example is Nelson's syndrome, which results from a rapid enlargement of a preexisting ACTH-secreting pituitary adenoma that occurs after the removal of the adrenal glands, thus lacking the negative feedback of cortisol on the production of ACTH (Biller *et al*. 2008). A study on the cultured pituitary adenoma from a patient with Nelson's syndrome showed that somatostatin-14 and somatostatin-28 suppressed the secretion of POMC-derived peptides; however, other neuropeptides such as arginine vasopressin, vasoactive intestinal polypeptide, and oxytocin stimulated the secretion of POMC-derived peptides. Substance P, thyrotropin-releasing factor, Met-enkephalin, and Leu-enkephalin were also found to modulate the secretion of POMC-derived peptides, suggesting that adenomas of this nature may have multiple receptors to various neuropeptides (Shibasaki & Masui 1982) that could regulate POMC peptide secretion *in vivo*.

Summary

POMC is a multivalent prohormone capable of producing at least seven peptide hormones depending on its processing by prohormone-converting enzymes. The prohormone is sorted into the nascent immature granules of the RSP at the TGN through interaction with at least two membrane-associated proteins, CPE and SgIII. As the granules mature, POMC is cleaved into its complement of peptide hormone intermediates, which are further processed by exopeptidases and amidating and acetylating enzymes to produce the bioactive peptide hormones. The granules containing POMC are transported in a microtubule-dependent anterograde manner through interaction of the CPE cytoplasmic tail with dynactin and the motor proteins, kinesins 2 and 3. The mature granules are stored close to the plasma membrane and are released in a secretagogue-dependent manner that depends on calcium. Stimulated release of the mature granules is regulated by multiple contributing factors;

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primarily among them is corticotrophin-releasing factor acting on the corticotrophs, which in turn are inhibited by glucocorticoids. Melanotrophs are under the tonic inhibition of dopamine, whereas POMC neurons in the hypothalamus respond to leptin. The subject of the biosynthesis, sorting, trafficking, and secretion of POMC and its bioactive peptides has been studied extensively and represents an area of physiology, which now has a broad and substantial foundation in knowledge, thanks to the pioneers in this field. This strong base allows future investigators to ask more stringent questions about the generation, roles, and regulation of these peptides in normal and diseased states *in vivo*.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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