1. Introduction

1.1 POMC discovery, distribution and function

Proopiomelanocortin (POMC) is a precursor giving rise to a variety of peptides including corticotropin (ACTH), lipotropin β (β-LPH), the corticotropin-like intermediary lobe peptide (CLIP), melanocyte stimulating hormones (α-, β-, and γ-MSH), and the opioid peptide β-endorphin$_{1-31}$ (END) (Fig. 1.1). Evidence that these peptides are produced from precursor molecules has mainly been provided in the late 1970's by Mains and Eipper as well as by Roberts and Herbert. Biochemical studies of Mains and Eipper in mouse pituitary tumor cells (AtT-20) revealed that low and higher molecular weight ACTH (4.5, 13, and 23 kDa) derive from high molecular weight ACTH glycoproteins of 28.5 and 31 kDa. These findings were obtained from the kinetic analysis of steady and pulse-labeled glycoproteins with radioactive amino acids (Mains and Eipper 1976). These authors have further shown that high molecular weight ACTH glycoproteins (28.5 and 31 kDA) from AtT-20 cells contain the same methionine, lysine, and phenylalanine peptides (Eipper et al. 1976). The high molecular weight ACTH glycoprotein of 28.5 kDA was likewise identified by Roberts and Herbert (1977a) who investigated AtT-20 mRNA translation products synthesized in a cell-free protein synthesizing systems. The tryptic analysis of this peptide revealed that higher molecular weight ACTH glycoproteins contain only a single copy of ACTH$_{1-39}$, the majority of the precursor amino acids remained unidentified in that study. At the same time pituitary β-LPH was identified as the precursor of END (Li and Chung 1976; Li et al. 1976). Other observations indicated that ACTH, β-MSH, END, and β-LPH might relate to each other. For example, ACTH and β-MSH (amino acids 41-58 of β-LPH) were detected in the same pituitary cells (Orth et al. 1973; Phifer et al. 1974). Other studies showed that plasma β-MSH and ACTH determined by radioimmunoassay (RIA) in tumors from patients with ectopic ACTH syndrome rise and fall together (Abe et al. 1969). Such findings prompted Mains and Eipper as well as Roberts and Herbert to study the simultaneous presence of ACTH and END in radioactively labeled AtT-20 products (Mains et al. 1977; Roberts and Herbert 1977b). In both studies the labeled products were immunoprecipitated using antibodies directed against ACTH and END. Immunoprecipitates were analyzed by sodium dodecyl sulfate/polyacrylamide gel

electrophoresis, paper chromatography, and digestion with trypsin. The result of both studies was that ACTH and END share the 31 kDa precursor molecule. Similarly, another group that investigated non-pituitary cells suggested that ACTH, lipotropins, and END were derived from a common, high molecular weight precursor (Bertagna et al. 1978). These authors analyzed the secretion of ACTH, β-LPH, and END from human pulmonary small cell carcinoma cells by gel filtration of the culture medium and immune affinity chromatography. Referring to its end products the precursor was named pro-opiomelanocortin (POMC) (Rubinstein et al. 1978).
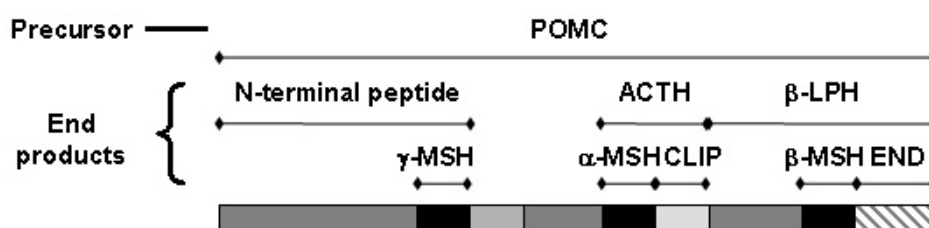

Fig. 1.1 Scheme of the superstructure of the POMC precursor molecule that contains the amino acid sequences of various biologically active peptides such as corticotropin$_{1-39}$ (ACTH) and β-endorphin$_{1-31}$ (END). Modified from Drouin et al. (1985).

POMC is mainly expressed in corticotroph cells of the anterior pituitary lobe and in melanotroph cells of the intermediate lobe (Eipper and Mains 1978), the latter is absent in adult humans. In addition, several non-pituitary tissues express POMC and related peptides. In the brain, POMC-expressing neurons predominate in the arcuate nucleus located in the mediobasal hypothalamus (Abrams et al. 1980; Gee et al. 1983). Several tissues for reproduction such as testis, ovary (Bardin et al. 1987; DeBold et al. 1988a; DeBold et al. 1988b), and placenta (Rees et al. 1975; Grigorakis et al. 2000) express POMC peptides. Moreover, ACTH, β-LPH, γ-MSH, and END were detected in adrenal gland, kidney, lung, thyroid gland, liver, colon, and duodenum (DeBold et al. 1988a; DeBold et al. 1988b). More recent studies showed that POMC, β-LPH, different MSHs, and END are produced in skin cells like keratinocytes, melanocytes, and dermal microvascular endothelial cells (Schauer et al. 1994; Wintzen et al. 1996; Scholzen et al. 2000). Furthermore, POMC-related peptides have been demonstrated in immune tissues/cells such as spleen (Lolait et al. 1984; Mechanick et al. 1992; Lyons and Blalock 1995), lymph nodes (Cabot et al. 1997; Mousa et al. 2000), and circulating blood leukocytes (Smith and Blalock 1981; Harbour et al. 1991).

The POMC peptides have several physiological functions. Plasma levels of POMC peptides are usually related to the pituitary which is the major source of these peptides. The anterior pituitary peptides regulate several homeostatic body functions such as temperature, gonad activity, blood flow, and food and water intake via the hypothalamic-pituitary-adrenal-axis. The hypothalamus releases regulating factors such as corticotropin-releasing hormone (CRH) and vasopressin into the portal blood vessel system which is located between the median eminence and the anterior pituitary (Fig. 1.2). CRH induces the release of ACTH from the pituitary which then binds to melanocortin type 2 receptors on adrenal membranes. This ACTH binding induces steroidgenesis. Glucocorticoids, androgenic steroids, and mineralocorticoids are subsequently released from the adrenal gland and, by a negative feedback on the hypothalamus, decrease CRH release (Fig. 1.2). Another regulatory circuit involves the arcuate nucleus POMC neurons (Fig. 1.2) (Cowley et al. 1999). These neurons express POMC when increasing levels of leptin are released from white fat tissues (Mizuno et al. 1998). Appetite and energy expenditure are subsequently regulated by the release of α-MSH (Cowley et al. 1999). Disruption of α-MSH signaling leads to adiposity and linear growth as in humans or mice lacking melanocortin type 4 receptors (Butler and Cone 2003). Plasma ACTH and β- or γ-LPH induce eumelanin production in melanocytes (Fig. 1.2) leading to a brown coloration of the skin. In the absence of these peptides or of melanocortin type 1 receptors pheomelanin is produced leading to red pigmentation (Schioth et al. 1999; Ringholm et al. 2004). In human dermal microvascular endothelial cells the expression of POMC was demonstrated to be regulated by ultraviolet light (Fig. 1.2) (Scholzen et al. 2000). This indicates that POMC peptides produced in skin cells could also be involved in pigmentation. Humans that have mutations which lead to the complete loss of the POMC gene function show severe early-onset obesity, adrenal insufficiency, and red hair (Krude et al. 1998; Krude et al. 2003). Similar symptoms are present in POMC-deficient mice: the animals are obese, show adrenal insufficiency, and skin pigmentation is more yellowish (Yaswen et al. 1999).

While these ACTH-, MSH-, and LPH-related effects are mediated via melanocortin receptors, the target receptors of END are opioid receptors in the central nervous system involved in the inhibition of pain (analgesia/antinociception) (Fig. 1.2), in the regulation of body temperature, feeding, and emotional states. The opiate activity of END was detected by Li and Chung (1976) using competitive

binding assays on rat brain homogenates in comparison to [^{3}H]etorphine, a synthetic opioid receptor agonist. The antinociceptive properties of END were subsequently demonstrated *in vivo* (Loh et al. 1976). After intracerebral injection of END in mice and rats different tests were applied to record pain behaviors to different stimuli: to heat (tail-flick and hot plate test), to chemicals (acetic acid-induced writhing method), and to cold (ice water-induced wet shake responses). In all four tests END showed central antinociceptive activity and effects were reversed by the opioid receptor antagonist naloxone. However, human POMC-deficient subjects showed no obvious deficits in pain perception, specific investigations for ethical reasons were not performed in these children (Krude et al. 2003). But there is evidence that endogenous antinociception is impaired in mice lacking END (Rubinstein et al. 1996). Other studies provided evidence that END is involved in the modulation of the immune response. For example END stimulated human mononuclear cell chemotaxis measured *in vitro* (van Epps and Saland 1984). This effect was blocked by pretreatment with naloxone. In the same study the *in vivo* infusion of END into the cerebral ventricle of rats resulted in the immigration of macrophage-like cells. But most opioid receptor-mediated effects of END in animals seem to suppress immune reactions (Fig. 1.2) like natural killer cell activity or cell proliferation (Panerai and Sacerdote 1997). This has been shown for example by Panerai and co-workers (1995) who investigated the effect of END on cytotoxicity and proliferation of mitogen-activated splenocytes. The intracerebroventricular administration of END induced a significant inhibition of both splenocyte cytotoxicity and proliferation. The opposite effect was observed after intraperitoneal administration of naloxone or when antibodies against END were administered intracerebroventricular or peripherally: the two immune responses increased significantly. However, the activity of END is not restricted to opioid receptors, END can also act via non-opioid receptors in nervous and immune system (Wollemann and Benyhe 2004).

Fig. 1.2 (next page) Variety of the physiologic functions of pituitary and hypothalamic POMC peptides. Pituitary POMC peptides act in the periphery: they are released into the peripheral blood system and exert their hormonal function for example via melanocortin receptors (MCR) on the adrenal gland and on skin melanocytes or via opioid receptors (OR) on immune cells. Hypothalamic POMC peptides are neurotransmitters acting on MCRs and ORs in the brain. Modified from Schiebler and Schmidt (1999) and Raffin-Sanson et al. (2003).
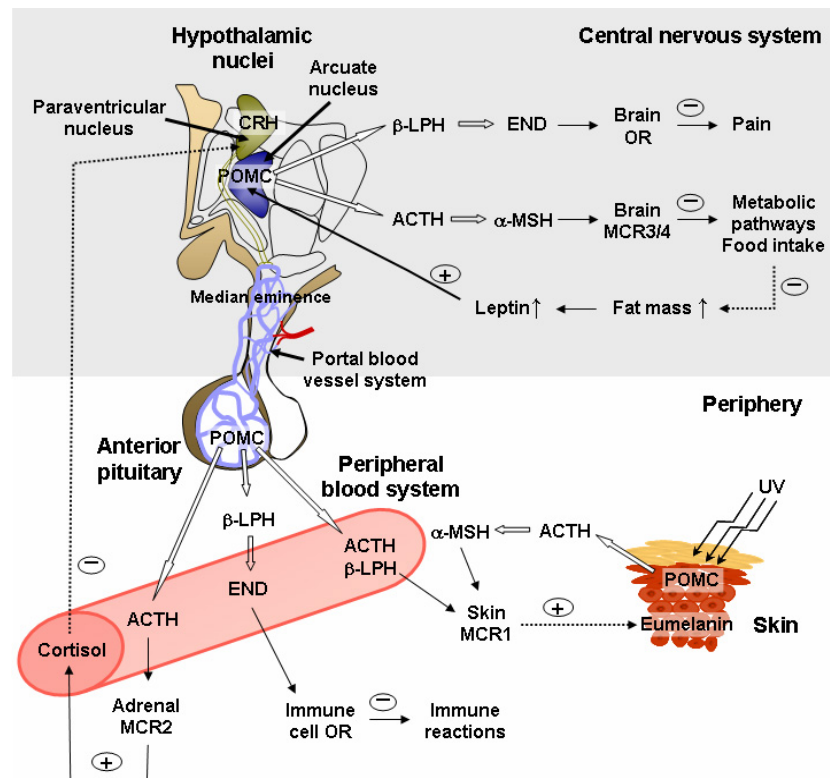
1.2 POMC mRNA expression in pituitary

The sequence of bovine pituitary POMC mRNA was unraveled first (Kita et al. 1979; Nakanishi et al. 1979), followed by the mapping of the human POMC gene (Takahashi et al. 1983) and that of rats (Oates and Herbert 1984; Drouin et al. 1985). There is a single POMC gene located on chromosome 2 (location: 2p23.3) in humans and on chromosome 6 (location: 6q14) in rats. The POMC gene contains three exons and two introns (Fig. 1.3) (Drouin et al. 1985). Common promoter binding motives such as the TATA- and two CAAT-boxes are present in exon 1, about 30 and 70 bp upstream of the transcription initiation site, respectively. The first exon, comprising untranslated regions such as the 5´-end cap structure of the mRNA (Fig. 1.3), spans 86 bp in humans (NIH accession no. V01510) and 97 bp in rats (NIH accession no. J00759). The second exon is 132 bp in humans and rats. It contains the 78 bp spanning signal sequence (Fig. 1.3) encoding the signal peptide in both species. Peptides such as ACTH, α-, β-, and γ-MSH, and END are encoded on the third exon (833 bp in humans and 688 bp in rats). The first and second exons are separated by an intron of 3.7 kb in humans and 3.1 kb in rats (intron A, Fig. 1.3). The second and third exons are separated by an intron of 2.9 kb in humans and 1.8 kb in rats (intron B, Fig. 1.3). A comparative analysis of the POMC mRNA structure of eight animal species including rat, mouse and human sequences showed that the peptide

encoding regions for ACTH, β-MSH, and END are relatively conserved while the N-terminal region of β-LPH is more variable (Mertvetsov et al. 1991).

POMC gene transcription can be regulated by several different factors. The T box factor Tpit is specifically expressed in corticotroph and melanotroph pituitary cells (Lamolet et al. 2001). POMC gene transcription by Tpit requires cooperation with another transcription factor called Pitx1 previously described by the same group (Lamonerie et al. 1996). Moreover, POMC transcription is sensitive to stress hormones such as CRH (Jin et al. 1994). Other studies within AtT-20 cells showed that the POMC promoter activity can be stimulated by inflammatory cytokines like the interleukins IL-1β, IL-6, and tumor necrosis factor α (TNFα) (Katahira et al. 1998).

Subsequent to transcription, a series of modifications generate mRNA from primary or *heterogeneous nuclear* RNA (*hn*RNA, Fig. 1.3) (Lodish et al. 2001). First, both introns are spliced out at highly conserved intronic sequences (GpT at the 5' splice site and ApG at the 3' splice site) (Rogers and Wall 1980). The splicing is followed by the ligation of the exon sites. In addition, exon 2 can be spliced 30 bp downstream of intron A both in humans and rats (Drouin et al. 1985; Lacaze-Masmonteil et al. 1987). After capping of the 5´-end and polyadenylation of the 3`-end, mature or full-length POMC mRNAs of about 1200 bp in humans and 1100 bp in rats are formed (Fig. 1.3).
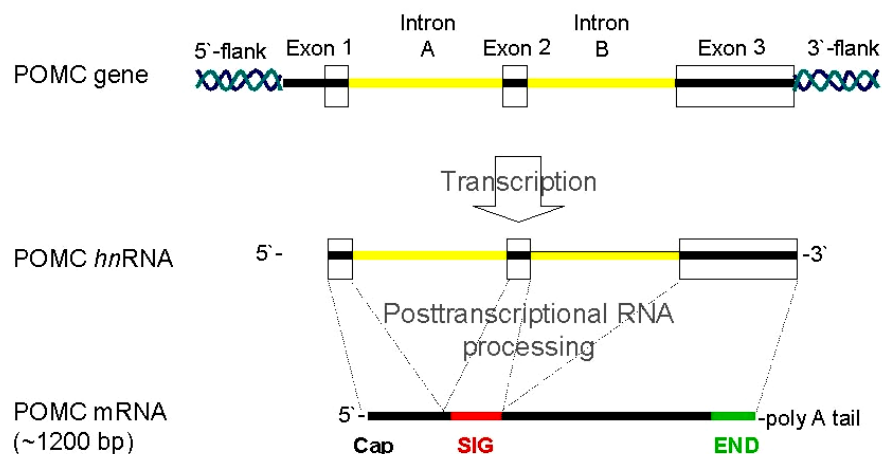

Fig. 1.3 Scheme of POMC transcription in pituitary cells. The transcription of POMC reveals an intermediate product containing the three exons as well as two intron sites (*hn*RNA). Further posttranscriptional modifications process *hn*RNA into mRNA: the introns are removed by splicing and re-ligation of the exon sites, exon 1 residues form a 5`-cap, and the 3`-end is polyadenylated. Adapted from Lyons and Blalock (1997).

1.3 POMC peptide processing in pituitary

Extensive studies have unraveled the classical posttranslational processing of POMC as schematically shown in Fig. 1.4 A. Posttranslational POMC processing begins as the nascent polypeptide chain (pre-POMC) enters the endoplasmic reticulum (ER) directed by the 26 amino acid signal peptide (Cool and Loh 1994; Loh et al. 2002). It was shown that the deletion of amino acids 2 to 26 prevented sorting of POMC to the regulated secretory pathway and obliterated the regulated secretion of POMC-derived peptides in neuronal cells (Cool and Loh 1994). Pre-POMC is 267 and 235 amino acids in humans (NIH accession no. AAH65832) and rats (NIH accession no. AAH58443), respectively; the molecular weight is approximately 31 kilo Dalton (kDa). Within the ER the 26 amino acid signal peptide is removed from the N-terminus revealing the mature POMC of 241 amino acids in humans and 209 in rats (Fig. 1.4 B). Then POMC binds to a membrane-bound sorting receptor of the trans-Golgi network, identified as carboxypeptidase E (CPE), that further directs the prohormone towards the regulated secretory pathway (Cool et al. 1997). Here the cleavage of POMC starts and proceeds within the secretory granules (Tanaka et al. 1991; Tanaka and Kurosumi 1992). POMC contains eight pairs and one quadruplet of basic amino acids predominantly of the Lys-Arg type that are potential cleavage sites. Cleavage is performed by two prohormone convertases, PC1 (also called PC3) and PC2, belonging to the subtilisin-like superfamily of enzymes (Fig. 1.4 A). These prohormone convertases are located within secretory vesicles (Christie et al. 1991; Bennett et al. 1992). PC2 was discovered first (Smeekens and Steiner 1990) and PC1/3 shortly thereafter (Seidah et al. 1991). Both PCs exhibit sequence specificity (Benjannet et al. 1991; Zhou et al. 1993; Friedman et al. 1994). PC1/3 and PC2 cleave the sites Glu-Gly-Lys-Arg, Glu-Phe-Lys-Arg, and Lys-Asp-Lys-Arg. In addition, PC2 cleaves Ala-Gln-Arg-Arg and Gly-Lys-Lys-Arg motives. However, both enzymes are not POMC-specific and also cleave other peptide precursors like pro-enkephalin (Benjannet et al. 1991; Korner et al. 1991). In the pituitary, corticotroph and melanotroph cells exert alternate prohormone convertase expression (Day et al. 1992). Corticotroph cells express only PC1 and its proteolytic action is limited to four POMC cleavage sites, thereby ACTH, β-LPH, the joining peptide (JP), and big γ-MSH are major end-products (Fig. 1.4 B), while only little amounts of END are processed. In melanotroph cells, predominantly PC2 and to a much lesser extend PC1/3 are expressed, the predominant end-products are α-MSH, corticotropin-like intermediate

product (CLIP), β-MSH and END (Fig. 1.4 B). If both enzymes are present PC1/3 mediates the initial cleavage step of POMC into the ACTH-biosynthetic intermediate molecule and β-LPH (Fig. 1.4 B) (Benjannet et al. 1991; Seidah et al. 1999; Tanaka 2003). Thereafter, PC2 converts β-LPH into β-MSH and END (Fig. 1.4 B) (Marcinkiewicz et al. 1993). This second step depends on the activation of pro-PC2, which is bound to a chaperone-like binding protein called 7B2. After transport of this complex from the ER to later compartments of the secretory pathway, pro-PC2 matures to active PC2 after the full dissociation of all 7B2 domains (Mbikay et al. 2001). Pro-PC2 has to encounter 7B2 intracellularly, otherwise it cannot generate a catalytically active mature species, but the mechanism is unknown (Muller and Lindberg 1999). The activity of these prohormone convertases was found to increase with decreasing pH which correlates with the acidification of maturating secretory granules. For PC1/3 optimal pH ranges from 5.0 – 6.5, PC2 is most active at pH 5.0 (Zhou and Lindberg 1993; Lamango et al. 1996). After cleavage of POMC by PC1/3 and PC2 the end products have two basic pair residues at their C-terminus that are removed by CPE (Douglass et al. 1984; Che et al. 2001). Further on, the biological activity of the POMC peptides can be altered by chemical modifications such as glycosylation, amidation, phosphorylation, acetylation, and sulphation. For example, the N-terminal acetylation of END eliminates the opioid activity of this peptide (Smyth et al. 1979; Deakin et al. 1980). Deakin and colleagues (1980) performed binding studies and found that N-terminal acetylation as well as the deletion of amino acids 1 - 8 or 28 - 31 of END strongly decreased its binding to brain opioid receptors. The N-terminus of END was found to provide the predominant binding site for opioid receptors, whereas the C-terminus enhanced the affinity for the receptor. The same study investigated the analgesic effects of intraventricular administered END peptides determined by tail-flick latencies of rats in response to heat. N-acetylation of END abolished and C-terminal amino acid depletion attenuated the analgesic effect of END (Deakin et al. 1980). Thus, posttranslational modifications contribute to the overall diversity and activity of POMC products. END and other POMC peptides are subsequently released into the extracellular space from secretory granules deriving from the Golgi network (Fig. 1.4 A). This vesicular release is regulated by releasing factors such as arginine vasopressin and CRH (Gumbiner and Kelly 1982; Castro et al. 1989; Cool and Loh 1994).
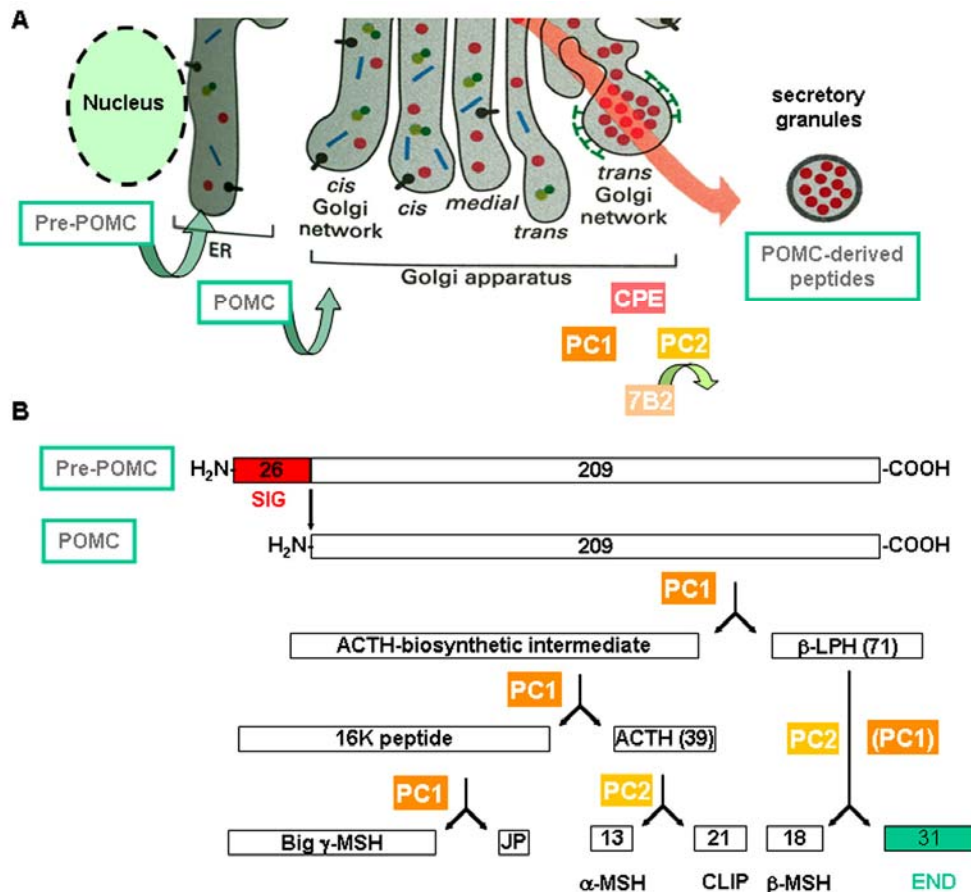
Fig. 1.4 Scheme of POMC processing in rat neuroendocrine cells. **A.** The nascent polypeptide chain (Pre-POMC) of 235 AA lengths comprises POMC and the N-terminal signal peptide. The signal peptide (SIG) is removed before the mature POMC polypeptide translocates towards the Golgi network. At the *trans*-Golgi-network membrane-bound prohormone convertases 1 and 2 (PC1, PC2) cleave POMC into biologically active peptides. The chaperone-like protein 7B2 is important to activate PC2. Carboxypeptidase E (CPE) is important for the sorting of the peptides to be packed into secretory granules and cleaves the paired amino acids that reside after PC 1 and 2 cleavage. Secretory granules bud off from the trans-Golgi site to travel towards the plasma membrane. **B.** POMC-cleavage sites of PC1 and PC2. In pituitary cells PC1 cleaves POMC into the ACTH-biosynthetic intermediate and β-LPH. END is mainly produced by PC2 that cleaves β-LPH. Numbers indicate amino acids of the different molecules. Adapted fromTanaka (2003).

1.4 POMC – relevance in opioid-mediated antinociception

POMC-derived END is the most familiar endogenous opioid peptide. So far three other groups are known, the enkephalins, dynorphins, and endomorphins. While the first two like END derive from large precursor molecules, i.e. pro-enkephalin and pro-dynorphin, respectively, the origin of the last, endomorhins, remains unclear. All these opioid peptides except endomorphins share the N-terminal amino acid sequence Tyr-Gly-Gly-Phe-Met/Leu by that they can bind to opioid

receptors and, thereby, attenuate pain. To mimic clinical inflammatory pain, many animal studies investigated opioid-mediated antinociception in rats with Complete Freund's Adjuvant (CFA)-induced inflammation. CFA is a suspension of oil and heat-inactivated mycobacteria (*Mycobacterium butyricum, M. tuberculosum).* A local subcutaneous unilateral application of CFA into the hindpaw of rats induces nociceptive behavior (Stein et al. 1988). These animals show reduced locomotor activity, avoid to put weight on the paw, lift the affected limb from the ground, and flex the knee joint permanently, indicating spontaneous pain at rest. Nociceptive thresholds to thermal or mechanical stimuli are decreased leading to the withdrawal of the paw when heat or pressure are applied (thermal and mechanical hyperalgesia). The paw tissue becomes swollen, red, and hot, but the inflammation remains confined to the treated side for about three weeks (Stein et al. 1988). When such animals were exposed to mild stress (cold water swim stress), paw pressure thresholds of the inflamed paw were transiently elevated indicating decreased nociception (Parsons et al. 1990; Stein et al. 1990a; Stein et al. 1990b). This antinociception could be antagonized by the systemic administration of naloxone and naltrexone methobromide. Since the latter cannot cross the blood-brain barrier to reach central opioid receptors, these findings suggest that antinociception was mediated by opioid receptors in the periphery. When antibodies against END or the opioid receptor antagonists were administered locally (into the inflamed tissue) both treatments lead to a reversal of stress-induced antinociception (Stein et al. 1990a; Stein et al. 1990b). These findings were subsequently confirmed in humans. In patients undergoing knee surgery the local but not systemic administration of naloxone increased pain (Stein et al. 1993). Thus, in addition to their actions in the central nervous system, opioid peptides can inhibit peripheral inflammatory pain by binding to opioid receptors (μ-, δ-, and κ) on peripheral sensory neurons as reviewed by Stein et al. (2003).

As described above, the most extensively characterized source of END is the pituitary gland (Guillemin et al. 1977; Smith and Funder 1988). Whether pituitary END contributes to peripheral opioid-mediated antinociception was analyzed in surgically hypophysectomized rats (Parsons et al. 1990). This study showed that the removal of the pituitary did not abolish opioid-mediated antinociception, suggesting that besides the pituitary another source of opioid peptides is involved. In inflamed tissue opioid peptides are found in immune cells (Sibinga and Goldstein 1988). The presence of

opioid peptide-containing immune cells in peripheral inflamed rat paw tissue was shown in several studies (Stein et al. 1990b; Przewlocki et al. 1992; Mousa et al. 2000; Mousa et al. 2001; Rittner et al. 2001). Consistently, the histological analysis of synovial cellular infiltration in humans showed opioid peptide-containing cells (Stein et al. 1996). Moreover, inflammatory cells were found to express POMC mRNA as detected with a oligonucleotide probe against POMC exon 3 using *in situ* hybridization (Przewlocki et al. 1992). These findings indicated that immune cells produce opioid peptides at the site of inflammation. However, it remained unclear whether full-length POMC mRNA was expressed.

The functional relevance of immune cell-derived END in peripheral antinociception was investigated in immunosuppressed animals. Whole body irradiation or cyclosporin A treatment abolished swim stress-induced antinociception in rats (Stein et al. 1990b; Przewlocki et al. 1992). Hermanussen and colleagues (2004) showed that cyclosporin A treatment of animals with CFA-induced hindpaw inflammation resulted in decreased numbers of $CD4^+END^+$ lymphocytes infiltrating the paw which was accompanied by increased hyperalgesia. This effect was reversed by the intravenous donation of concanavalin A-activated but not of naïve lymphocytes. Moreover, opioid-mediated antinociception in inflamed tissue produced by stress could be abolished by blocking adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and selectins (Machelska et al. 1998; Machelska et al. 2002; Machelska et al. 2004) that are involved in the migration of opioid containing cells (Fig. 1.5). Conversely, the efficacy of swim stress-induced antinociception increased with the duration of inflammation and with rising numbers of infiltrating immune cells containing opioid peptides (Rittner et al. 2001).

It was further analyzed which opioid peptides and releasing agents contribute to this stress-induced antinociception. END, met-enkephalin, and dynorphin were found to be involved in early peripheral opioid-mediated antinociception while later effects seemed to be evoked mainly by END (Stein et al. 1990b; Machelska et al. 2003). Opioid peptide release was triggered by environmental stimuli such as stress (Parsons et al. 1990; Stein et al. 1990a; Stein et al. 1990b) and by releasing factors such as chemokines (Rittner et al. 2006), catecholamines (Binder et al. 2004; Mousa et al. 2004) or CRH (Fig. 1.5) (Schäfer et al. 1994; Cabot et al. 1997). Stress-induced antinociception seemed to be mediated by endogenous CRH. For example, the local, but not systemic administration of a CRH receptor antagonist (α-helical CRH) dose-

dependently attenuated stress-induced antinociception (Schäfer et al. 1996; Machelska et al. 2003), suggesting a peripheral receptor specific action of endogenous CRH. In addition, antinociception was reduced when animals were pretreated locally with CRH antisense oligonucleotides that inhibited CRH mRNA translation (Schäfer et al. 1996).

Opioid peptides are mainly delivered to the site of inflammation by granulocytes during early stages (2 - 48 h), whereas macrophages become the predominant opioid peptide containing cell type at later stages after CFA-inoculation of rat hindpaws (Rittner et al. 2001; Brack et al. 2004). Subsequently, T and B lymphocytes infiltrate this site starting about 96 h post CFA-inoculation (Przewlocki et al. 1992; Cabot et al. 1997; Mousa et al. 2001; Rittner et al. 2001; Brack et al. 2004). Unlike monocytes/macrophages and granulocytes, lymphocytes usually do not enter non-lymphoid peripheral tissue without being previously activated by antigen-presenting cells within secondary lymphoid tissues such as lymph nodes (LN) (Butcher and Picker 1996; Fabbri et al. 1999). Activation is a crucial step for lymphocyte maturation and modification of several surface molecules involved in adhesion, which allows such reprogrammed lymphocytes to escape the normal recirculation between blood and lymph and to enter inflamed or injured tissue (Butcher and Picker 1996; Tietz and Hamann 1997). Lymphocytes within inflamed paw tissue already contain END at their arrival (Przewlocki et al. 1992; Mousa et al. 2004), indicating that END synthesis may precede or take place during migration. Previous studies have shown that END containing leukocytes within inflamed paw tissue were $CD4^+CD45RC^-$, suggesting that this opioid is mainly present in activated memory cells (Cabot et al. 1997; Mousa et al. 2001). In addition, the amount of POMC exon 2 mRNA transcripts was higher in LN-derived lymphocytes draining inflamed paws than in LN cells draining normal tissue, but there was no difference in END contents (Cabot et al. 1997). In circulating lymphocytes, END but not POMC exon 2 mRNA levels were higher in animals with 96 h hindpaw inflammation than in controls. These findings raised the question whether the draining LN was a site of enhanced POMC gene transcription during painful inflammation. This prompted a detailed study on full-length POMC mRNA expression and END production in lymphocytes under inflammatory conditions.
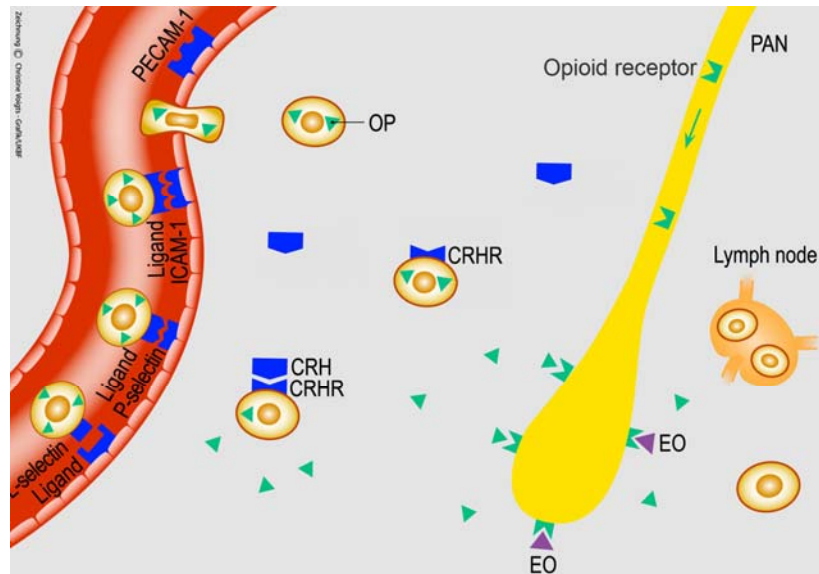
Fig. 1.5 Migration of opioid-containing cells and opioid peptide secretion within inflamed tissue. During inflammation P-selectin, ICAM-1 and PECAM-1 are upregulated on vascular endothelium. Opioid peptide (OP) producing immune cells co-express L-selectin. These adhesion molecules interact with their respective ligands. Thereby, L- and P-selectin mediate rolling of the cells along the vessel walls and ICAM-1 mediates their firm adhesion and diapedesis. In response to stress or to the releasing agent CRH, CRH receptors (CRHR) are activated and OP release is elicited. OP or exogenous opioids (EO) bind to opioid receptors on primary afferent neurons (PAN), leading to analgesia/antinociception. Opioid-depleted lymphocytes may home to regional lymph nodes. Adapted from Stein et al. (2003).

1.5 POMC in immune cells

POMC-related peptides such as ACTH and END have been identified in immune cells (e.g. splenic macrophages, peripheral blood lymphocytes) in the early 1980s (Smith and Blalock 1981; Lolait et al. 1984). The presence of full-length POMC mRNA was demonstrated in immune cells a few years later (Lolait et al. 1986; Westly et al. 1986). Lolait and co-workers used a 550 bp cDNA probe encoding the 3'-terminal regions of the rat POMC gene, including the ACTH and END coding sequences. Their Northern Blot analysis revealed that the 1.1 kb POMC mRNA was expressed in splenic macrophages from rats and mice. They further characterized the translation products by a combination of RIA and High Performance Liquid Chromatography (HPLC) and found that macrophages predominantly produced unacetylated, opioid-active END while N-acetylated END was present in lower amounts, as in pituitary. At the same time other studies revealed that the production of END in human peripheral leukocytes can be induced by CRH and suppressed by

the glucocorticoid analogue dexamethasone (Smith et al. 1986), suggesting that the POMC gene may be expressed and similarly controlled in leukocytes as in pituitary cells. However, the extensive analysis of several non-pituitary tissues demonstrated that these tissues predominantly express shorter POMC mRNA transcripts than found in the pituitary (Bardin et al. 1987). These truncated POMC transcripts comprise about 800 bp. The analysis of such truncated transcripts by RNase H digestion demonstrated that the size heterogeneity of POMC mRNAs observed in various tissues in comaprison to pituitary POMC transcripts was not due to differences in the lengths of the poly(A) tail (Chen et al. 1986). By using S1 nuclease mapping analysis these authors moreover showed that the POMC mRNAs isolated from pituitary, testis or ovary share the nucleotide sequences coding for ACTH, β-LPH, and the 3'-untranslated region. Then other studies showed that truncated POMC mRNAs contained no signal sequence and were lacking the entire exons 1 and 2 (Jeannotte et al. 1987; Lacaze-Masmonteil et al. 1987). Such truncated transcripts may accumulate during the degradation of full-length POMC mRNA. Another possible origin of truncated POMC transcripts was suggested by Lacaze-Masmonteil and colleagues (1987). They reported on GC-rich areas in the human intron B sequence that are similar to GC-box motives including the sequence 5'-GGGCGG-3' and may serve as transcription initiation sites as in housekeeping genes. The initiation of POMC transcription under control of such GC-box promoters would allow the transcription of truncated mRNA molecules of variable length, all lacking the signal sequence (Fig. 1.6). Despite the conservation of the third POMC exon more recently published transfection studies provided evidence that translation products of truncated POMC transcripts that lack the signal sequence are neither processed to authentic POMC peptides nor secreted (Clark et al. 1990; Cool and Loh 1994). To date, the role of these truncated transcripts remains unclear and their occurrence has stimulated a controversy about the capability of non-pituitary cells to produce full-length POMC mRNA and about its processing to secretable products. The frequent detection of truncated POMC transcripts in leukocytes (Lacaze-Masmonteil et al. 1987; DeBold et al. 1988b; Oates et al. 1988; Buzzetti et al. 1989; Galin et al. 1991; van Woudenberg et al. 1993; Maier and Blalock 1994) raised doubts about a functional POMC system in immune cells. However, subsequent research confirmed the findings of Lolait and colleagues by showing that normal and stimulated macrophages expressed full-length POMC mRNA albeit to a much lower

extent than the pituitary (Mechanick et al. 1992; Lyons and Blalock 1997). The presence of the full POMC mRNA sequence in nonstimulated lymphocytes was further on questioned (Lolait et al. 1986; Westly et al. 1986; Mechanick et al. 1992; van Woudenberg et al. 1993; Lyons and Blalock 1997), with the exception of one study (Stephanou et al. 1991).

On the other hand, there is evidence that full-length POMC may be expressed in lymphocytes under pathological conditions (Fig. 1.6). In a case report of a patient with ectopic ACTH syndrome lymphocytes in thymic hyperplasia were the source of high ACTH production (Ohta et al. 2000). Northern blotting and *in situ* hybridization of these thymic lymphocytes revealed that full-length POMC mRNA was present. In another study, Buzzetti and colleagues (1989) detected full-length POMC mRNA in a CD4-positive, HIV-infected T lymphoma cell line. This POMC gene expression was accompanied by higher ACTH levels in the lymphoma cells than detected in normal human peripheral blood mononuclear cells not expressing the full POMC mRNA transcript. In addition, lymphocytes were shown to express full-length POMC mRNA after stimulation with mitogenic substances such as concanavalin A (Lyons and Blalock 1997) or after infection with pathogens such as the Newcastle disease virus (Westly et al. 1986). However, other studies showed that the stimulation of immune cells with concanavalin A or CRH lead to an enhanced expression of truncated POMC mRNA (van Woudenberg et al. 1993; Maier and Blalock 1994). These findings raised the question whether the lymphocytic expression of POMC mRNA and the processing of its translation products may be different in normal versus pathological states. Recently, our group showed that POMC protein and END co-localized with POMC processing enzymes in peripheral blood-derived monocytes and lymphocytes from normal rats and from rats with inflamed paws (Mousa et al. 2004). This may indicate that processing could occur under both normal and inflammatory conditions. Like in the pituitary, the release of POMC-derived peptides from lymphocytes seems to depend on releasing factors and the presence of the corresponding receptors. For example, our group analyzed the release of END *ex vivo* after CRH and IL-1β treatment of normal and inflamed lymph node (LN)-derived cells from rats (Schäfer et al. 1994; Cabot et al. 1997). IL-1β and CRH receptors were present on cells located within the lymph follicles (B-cells) and were upregulated during inflammation (Mousa et al. 1996). Accordingly, both IL-1β and CRH induced release of END in cell suspensions prepared from inflamed LN (Schäfer et al. 1994; Cabot et al. 1997).

These effects were inhibited by IL-1 and CRH receptor antagonists, respectively, and by the removal of intracellular calcium. Furthermore, increasing potassium concentrations evoked the release of END (Cabot et al. 1997). Moreover, it was shown that the chemokine macrophage inflammatory protein-2 (CXCL2/3) can selectively induce END release from polymorphonuclear cells *in vivo* (Rittner et al. 2006). This release was dependent on intracellular calcium. Together, these findings indicate a vesicular release of POMC peptides from immune cells, as previously described in neuronal cells (Gumbiner and Kelly 1982; Castro et al. 1989; Cool and Loh 1994). In addition, the ultrastructural analysis of macrophages and lymphocytes in inflamed paw tissue revealed that END was located within vesicular structures (Mousa et al. 2004). These studies raised the question whether immune cells are indeed capable of expressing POMC derivates containing the signal peptide, an essential missing link in the pathway leading to regulated END secretion.
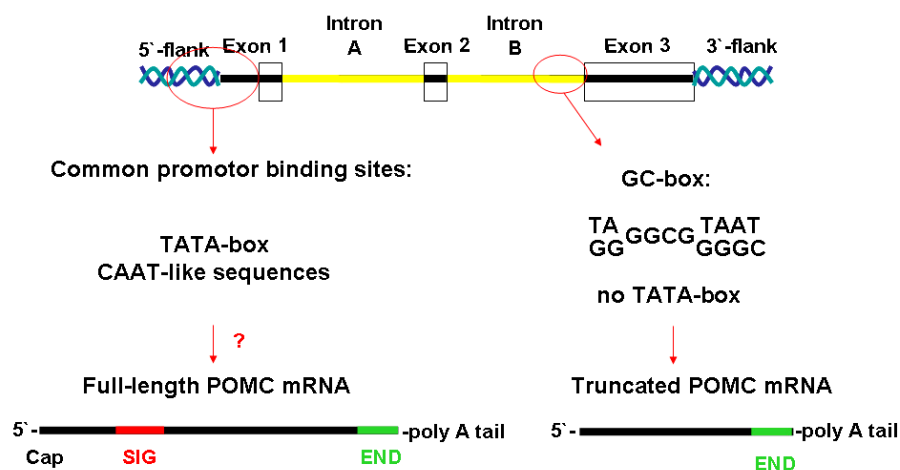

Fig. 1.6 Scheme of possible alternative transcription initiation of POMC in lymphocytes. The transcription of full-length POMC mRNA in immune cells is disputed but may be enhanced under pathological situations as indicated by the question mark. Alternative transcription initiation sites (GC-boxes) located in intron B may explain the occurrence of truncated POMC mRNAs lacking exon 1 and 2-related sequences such as the 5'-end cap and the signal sequence, respectively.