

INVESTIGATION OF CELLULAR MECHANISMS OF SER9LEU
PROOPIOMELANOCORTIN (POMC) MUTATION IN NEURONAL CELLS

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PROOPIOMELANOCORTIN (POMC) MUTATION IN NEURONAL CELLS**

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ABSTRACT

INVESTIGATION OF CELLULAR MECHANISMS OF SER9LEU PROOPIOMELANOCORTIN (POMC) MUTATION IN NEURONAL CELLS

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Pro-opiomelanocortin (POMC) is a precursor protein and synthesized in many different tissues of the body. It is proteolytically cleaved into different biologically active peptides which have different physiological functions based on the tissues they are found. Some of these peptides act in the brain to reduce food intake.

The main regulator of the food intake and energy homeostasis is the arcuate nucleus (ARC) within the hypothalamus, and hypothalamic neurons expressing POMC have important roles in the regulation of body weight and energy homeostasis. These neurons are stimulated by the signals from extracellular matrix (ECM) to release the contents of mature POMC vesicles. The active peptides of POMC are released into the ECM and lead to anorexigenic response which suppress the food intake.

Due to the important role of POMC in the regulation of body weight, many studies have focused on mutations in *POMC* of obese people. One of these mutations is Ser9Leu POMC found among children having early-onset obesity. It is located in the signal peptide of *POMC* which is removed after the co-translation of the protein in the normal state of the cell.

In this study, the cellular mechanisms of the Ser9Leu POMC mutation were investigated. It was hypothesized that Ser9Leu POMC inhibits the translocation of POMC into the endoplasmic reticulum (ER) and leads to the accumulation of mutant proteins which may be secreted in the constitutive secretory pathway. Therefore, mouse neuroblastoma cells (N2a) were transfected with wild-type and Ser9Leu POMC DNAs, and secreted proteins from N2a cells were analyzed with the Western blot method using anti-ACTH (adrenocorticotrophic hormone). Results revealed that the Ser9Leu POMC mutation did not cause a different effect on the cellular mechanism for its secretion from the neuronal cells.

Keywords: Pro-opiomelanocortin; Arcuate nucleus; Obesity; Secretory pathways;

ÖZ

NÖRON HÜCRELERİNDE SER9LEU PROOPİOMELANOKORTİN (POMC) MUTASYONUNUN HÜCRESEL MEKANİZMALARININ İNCELENMESİ

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Pro-opiomelanokortin (POMC) bir prekürsör proteindir ve vücudun birçok farklı dokusunda sentezlenir. Proteolitik kesilmelerle biyolojik aktif peptitler oluşturur ve bu peptitler, bulundukları dokulara göre farklı fizyolojik fonksiyonlara sahiptir. Bu peptitlerin bazıları, besin alımını azaltmak için beyinde görev alır.

Besin alımı ve enerji homeostazisi ağırlıklı olarak hipotalamus içindeki arkuat nükleusun (ARC) kontrolünde olup, POMC'yi ekspres eden hipotalamik nöronlar vücut ağırlığı ve enerji metabolizmasının düzenlenmesinde etkin role sahiptirler. Hücre dışı sinyaller, bu nöronları uyarak olgun granüllerin içindekileri salgılamasını sağlar. Böylece, aktif POMC peptitleri salgılanarak, besin alımını baskılayan anoreksijenik etkilere yol açar.

POMC'nin vücut ağırlığı regülasyonundaki önemli rolü nedeniyle, birçok çalışma obez insanların POMC genindeki mutasyonlara odaklanmıştır. Bu mutasyonlardan biri, erken başlangıçlı obeziteye sahip çocuklarda bulunan Ser9Leu POMC'dir. Ser9Leu POMC mutasyonu, proteinin translasyonundan sonra kesilerek çıkarılan sinyal peptidinde bulunur.

Bu alıřmada, Ser9Leu POMC mutasyonunun hücresel mekanizmaları arařtırılmıřtır. Ser9Leu POMC'nin, POMC'nin endoplazmik retikuluma (ER) translokasyonunu inhibe ettiėi ve konstittif salgı yolunda salgılanacak mutant proteinlerin birikmesine yol atıėı hipotezi ne srlmřtr. Bu nedenle, fare nroblastoma hücreleri (N2a) yabarııl tr ve Ser9Leu POMC DNA'ları ile transfekte edildi ve N2a hücrelerinden salgılanan proteinler ACTH antikoru kullanılarak Western blot yntemi ile analiz edildi. alıřma sonuları, Ser9Leu POMC mutasyonunun, proteinin nron hücrelerinden salgılanmasına neden olan hücresel mekanizması zerinde farklı bir etkiye neden olmadıėını ortaya koymuřtur.

Anahtar Kelimeler: Pro-opiomelanokortin; Arkuat nukleus; Obezite; Salgı yolları.

Dedicated to my love and my family

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LIST OF ABBREVIATIONS

α -, β - and γ -MSH	α -, β - and γ -melanocyte-stimulating hormone
β -END	β -endorphin
β -LPH	β -lipotropin
ACTH	Adrenocorticotropin
ARC	Arcuate nucleus
AtT-20	Anterior pituitary tumor cells of mouse
CNS	Central nervous system
CPE	Carboxypeptidase E
CgA	Chromogranin A
CLIP	Corticotropin like intermediate peptide
ECM	Extra cellular matrix
ER	Endoplasmic Reticulum
ISG	Immature secretory granules
LDCGs	Large dense-core secretory granules
MSG	Mature secretory granules
N-AT	N-acetyltransferase
N2a	Neuro-2A cells, mouse neuroblastoma cells
PAM	Peptidyl-glycine α -amidating monooxygenase
PHM	Peptidylglycine α -hydroxylating monooxygenase
PAL	Peptidyl- α -hydroxyglycine α -amidating lyase
PC	Protein convertase
POMC	Pro-opiomelanocortin
RSP	Regulated secretory pathway
SDS	Sodium dodecyl sulfate
TGN	Trans-Golgi network
VAMP-4	Vesicle-associated membrane protein-4

CHAPTER 1

INTRODUCTION

1.1. PRO-OPIOMELANOCORTIN (POMC)

Human pro-opiomelanocortin (POMC) is a complex archetypal protein which is a precursor of hormones and neuropeptide, encoded by the POMC gene. It is cleaved into multiple smaller peptide hormones which have several different roles in the body. The phenomena of POMC as a precursor peptide emerged over time with the studies on adrenocorticotrophic hormone (ACTH) as increases in researches on sequences of POMC (Harno et al., 2018).

1.1.1. The History of POMC

POMC was cloned more than 30 years ago (Takahashi et al., 1981). During the 1950s to 1970s, two groups of peptides were identified. One group consists of ACTH, corticotropin-like intermediate peptide (CLIP) and α -MSH (α -melanocyte-stimulating hormone) and the second group consists of β -lipotropin (β -LPH), β -MSH, and β -END (β -endorphin). Due to similarity in their sequences, this emerged a precursor paradigm in which there was a single precursor peptide being processed into multiple biologically active components (Takahashi, 2016). After the isolation of the gene encoding ACTH- β -LPH, this hypothesis was confirmed by the mid-1970s (Clark, 2016). In 1977, the common precursor of those hormones was found in anterior pituitary tumor cells of mouse (AtT-20), and the following year it was found in human nonpituitary tumor cell line (Catania et al., 2004). A 31-kDa peptide was recognized by antibodies to both ACTH and β -LPH, and identified by using the radiolabeled amino acids in the cells by performing immunoprecipitation and SDS gel

electrophoresis (Mains and Eipper, 1979). Then researchers analyzed for the first time the nucleotide sequence of the cloned DNA for the ACTH- β -LPH precursor, and confirmed the existence of a large common precursor protein in 1979 (Clark, 2016). Hence the name of ‘proopiomelanocortin’ was first proposed in 1979 and has been continued to be used hereafter (Chrétien et al., 1979).

1.1.2. POMC and Gene Expression

After the cloning of full-length *POMC* cDNA, many studies have been carried out to identify the complete gene structure of other species such as mouse, rat, and pig. In all species, the overall gene sequence is highly conserved. However, differences seen in the length of peptides and has led to a confusion of nomenclatures of peptide numbering the amino acids of POMC in species (Harno et al., 2018).

The human *POMC* is 8kb long and encodes 266 amino acids and consists of 3 exons separated by 2 large introns (Bekdash et al. 2014). As shown in Figure 1, it is located on the short arm of chromosome 2 at position 23.3 (2p23.3) (Krude et al., 2003).

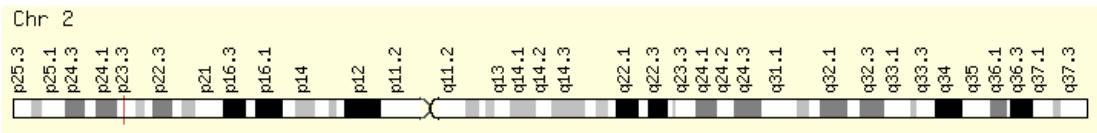


Figure 1. Genomic location of *POMC*. In humans, *POMC* is located on the short arm of chromosome 2 at position 23.3, shown by red line (Retrieved from NCBI).

In humans, exon 1 of *POMC* has a length of 87 bp and does not have a translation product since it spliced before translation of the gene (Figure 2). An intron next to exon 1 has a 4 kb length and is followed by short second exon (152 bp). Exon 2 contains the N-terminal region of POMC protein and the signal sequence to encode a

signal peptide which helps the localization of the nascent peptide into the endoplasmic reticulum (ER). It is followed by the second intron (2.2kb) upstream of largest exon 3 (835 bp¹). Most of the translated mRNA is encoded by exon 3, except for the hydrophobic signal peptide and the N-terminal glycopeptide consisting of 18 amino acids. Since exon 3 contains the majority of the coding regions, it also encodes the termination codon and the signal for addition of the polyA tail (Cawley et al., 2016; Raffin-Sanson et al., 2003; Catania et al., 2004).

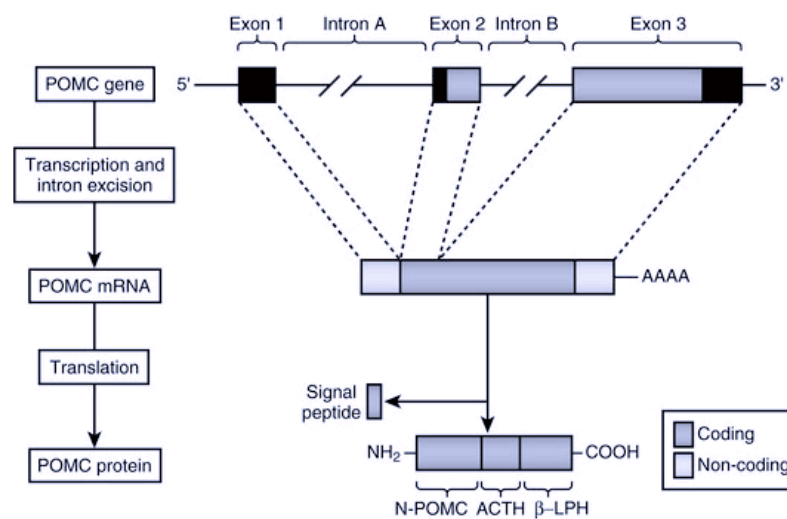


Figure 2. Schematic representations of the genomic structure of *POMC*. *POMC* consists of 3 exons and 2 introns and is transcribed into the mRNA with polyA tail. After translation, the POMC precursor consists of a 26-amino acid signal sequence, and cleaved into the mature peptide hormones after processing of POMC (Stevens and White, 2009).

POMC has 3 different promoters that have a role in the regulation of differential transcription of *POMC* in different tissues (Bekdash et al., 2014). The three promoter regions which have a role in the controlling of transcription are named promoter 1 (P1), promoter 2 (P2), and promoter 3 (P3). P1 is upstream of exon 1 in the 5' *POMC*

¹ It is stated to be 833 bp in an other review (Lim et al., 2000)

promoter region and contains a CpG-rich island; it has a predominant role in some cancers, which control an epigenetic mechanism of *POMC* by hypermethylation (Böhm and Grässel, 2012); P2 in the normal pituitary gland has a role in controlling the transcription; and P3 has weak activity in several peripheral tissues (Catania et al., 2004). In the normal non-expressing tissues, these promoters containing CpG islands are methylated, which lead to silencing of its expression. In tissues which express *POMC*, the promoters are not methylated, allowing the essential transcription factors to bind (Bekdash et al., 2014).

Human POMC polypeptide is 31-kDa and consists of 38 positively charged (arginine and lysine) residues and 39 negatively charged (glutamate and aspartate) residues, making it physiologically balanced, without the signal peptide among the 241 amino acid residues (Coll, 2007; Cawley et al., 2016). Thus, almost more than one-third of the POMC protein consists arginine/lysine and glutamate/aspartate residues. This makes POMC as highly charged protein and very soluble in aqueous solution (Cawley et al., 2016). Eight pair and one quadruplet group of lysine and arginine residues resides on the cleavage sites of POMC (Figure 3).

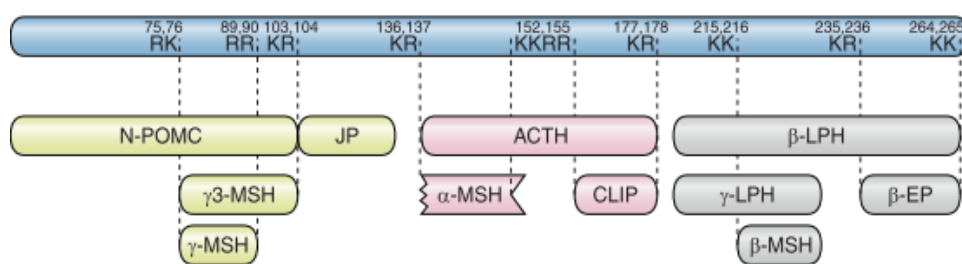


Figure 3. Schematic diagram of POMC processing with cleavages sites. POMC contains pairs of Arginine (R)/Lysine(K) residues which are located on the cleavage site of peptides. Enzymatic cleavage occurs at these specific di-basic amino acid residues and produces smaller peptides (Harno et al. 2018).

Even though POMC is not processed in all body tissues, it was found that *POMC* is widely expressed in many tissues such as testis, ovary, placenta, liver, heart, kidney, thyroid, colon and adrenal gland. However, primary regions are hypothalamus, the pituitary gland, medulla and several peripheral tissues (Harno et al., 2018; Pritchard and White, 2007). Although *POMC* expression and its derived peptides were originally found in the pituitary, its expression and processing were also found in the nervous system and peripheral tissues (Catania et al., 2004). In the brain, the arcuate nucleus (ARC) of the hypothalamus contains high amounts of POMC cell bodies (Cawley et al., 2016), but POMC expression was also found in the other areas of brain such as amygdala, hippocampus, the cortex, and the nucleus tractus solitaries of brainstem to lesser amount (Bekdash et al., 2014), while in the spinal cord and dorsal root ganglion, POMC mRNA was found only in detectable level (Catania et al., 2004).

1.1.3. Vesicular Trafficking of POMC

Within the cell, POMC moves through the ER and Golgi to the Trans-Golgi Network (TGN). At the end of this route, POMC is sorted and packaged into immature vesicles budding from the TGN to be processed, then within the vesicles, it moves to the release sites of the cell membrane (Cawley et al., 2016). A lot of data from studies suggested that there are at least two distinct pathways that POMC peptides follow. POMC is either secreted by the constitutive secretory pathway which protein is stored in immature secretory granules and released constitutively, or secreted by regulated secretory pathway (RSP) by being processed and stored in mature secretory granules upon a secretagogue (Figure 4) (Harno et al., 2018). After the transcription of *POMC*, POMC mRNA is translated to pro-protein at the rough ER and then folded in ER (Millington, 2007). When protein meets ER quality control which is required for further processing of protein, POMC is transported through the Golgi stacks into the nascent vesicles, which are then being released from the secretory granules of the RSP or transported to out of cell by the constitutive secretory pathway (Figure 4) (Kim et al., 2018).

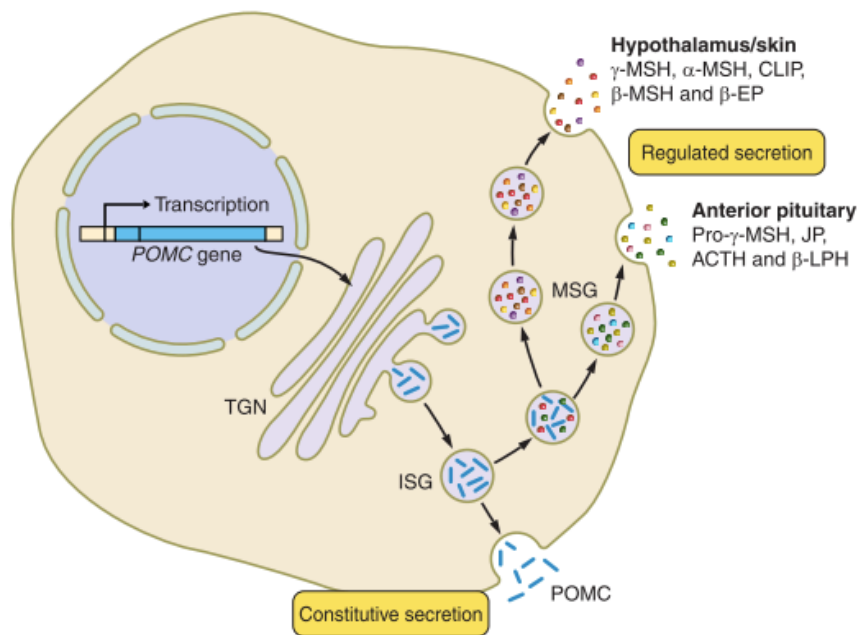


Figure 4. Constitutive and regulated secretory pathway (RSP) of POMC precursor. After the transcription of POMC in the nucleus, it is moved to the ER and subsequently the TGN. Then it is sorted to vesicles budding from TGN. POMC is either stored in immature secretory granules (ISG) without being processed to be secreted constitutively, or it is stored in the mature secretory granules (MSG) where all the cleavage events occur, and secreted in the RSP (Harno et al. 2018).

In the Golgi apparatus, pro-hormone POMC moves through the end of the cisterna, where membrane blebbing appears to generate secretory granules. There are two different data which some researchers showed that processing of POMC primarily occurs in secretory vesicles (Fernandez et al., 1997; Tanaka et al., 1997), whereas other evidences showed that it may begin in the TGN (Schnabel et al., 1989; Milgram and Mains, 1994). However, it was shown that many of enzymatic cleavages take place in the Golgi apparatus and secretory vesicles. Thus, initial phases of processing which is the cleavage of POMC at the C- terminal of ACTH begin in Golgi apparatus, and further modifications occur in the secretory granules (Pritchard et al. 2002).

The sorting of pro-hormones in RSP depends on pH of TGN and the secretory granules. pH of TGN is 6.8 (Seksek et al., 1995) and changes to 4.5-5.5 (Loh et al., 1984) into granules, as POMC moves. This is the optimal conditions of environment where pro-hormone convertases can be activated to start initial phase of POMC processing (Harno et al., 2018).

1.1.3.1. Signal and Sorting Motif

The early structural analysis of the N-terminus of POMC resulted in the identification of the signal peptide of POMC (Figure 5). Studies demonstrated that specifically the 26 amino acids of the N-terminal sequence, comprising the signal sequence of the prohormone, provides the information that is necessary and sufficient for the translocation of the nascent protein through the membrane of the rough ER (RER) (Harno et al. 2018; Yang et al. 2016). The signal peptide anchors the protein to the ER membrane. After the translocation to the ER, this signal peptide of POMC is removed by a signalase enzyme within the ER (Eipper and Mains, 1980). The signal peptide has three consensus domains; the first is the amino-terminal positively charged region (n-region), the second is a central, hydrophobic part (h-region), which contains strings of leucine residues, and the last is a more polar carboxy-terminal domain (von Heijne, 1990). The h-region plays a role in the insertion of the protein into the ER, while the positively charged residues in the N-tail are important for the orientation of this insertion (Del Giudice et al., 2001).

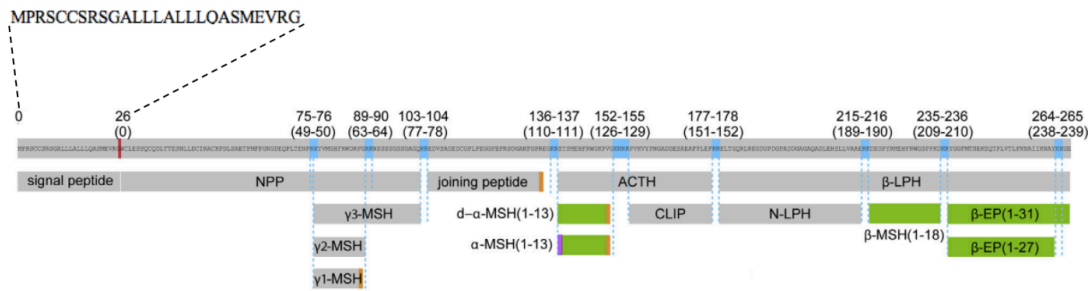


Figure 5. Whole amino acid sequence of the POMC. The recognition site at the end of the N-terminal of POMC contains a 26 amino acids signal peptide, which enables the protein to translocate to the ER and is removed after translocation of the protein. The remaining N-terminal sequence beginning from 0 (red highlighted) contains the sorting signal, which is necessary for sorting by RSP (Kirwan et al., 2018).

After the removal of the signal peptide within the ER, the remaining N-terminal sequence starts with the sorting signal which is required for the proteins to be sorted by RSP. Sorting signals of several secreted proteins have been identified and found to have a consensus motif which contains an amphipathic loop with hydrophobic and charged amino acid residues exposed on the surface of the loop (Shen and Loh, 1997). Sorting signals of proteins occur by the formation of two disulfide bonds forming Cys28/Cys50 and Cys34/Cys46, resulting in a specific 'hairpin' conformation (Seeger and Bennett, 1986). The deletion of these disulfide bridges results in the secretion of mutant POMC by the constitutive secretion pathway in Neuro-2a cells (N2a) (Cool and Loh, 1994), which provides evidence that this sequence is necessary for the sorting by RSP.

Analysis of a molecular model of N-POMC comprising the sorting signal also shows that it contains two acidic (Asp¹⁰ and Glu¹⁴) and two amphipathic (Leu¹¹ and Leu¹⁸) amino acids which are on the amphipathic loop of POMC and are highly conserved, and act as a consensus sorting signal motif (Figure 6) (Cawley et al., 2016).

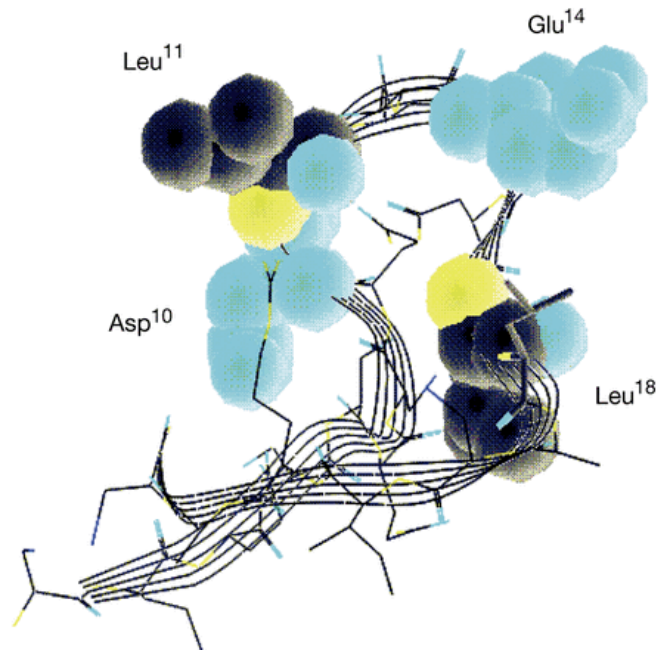


Figure 6. A molecular model of the N-POMC peptide. N-POMC comprises the RSP sorting signal. The sorting signal motif contains two acidic residues and two hydrophobic residues, which are Asp10 and Glu14, Leu11 and Leu18 (Cawley et al., 2016).

1.1.3.2. Sorting Receptor and Granins

The sorting of proteins to the RSP cannot be ensured just by the sorting signal. There should be other proteins that link the neuropeptides to the membrane of the granules budding from the TGN in order to inhibit the entry of other proteins into the same granules (Orci et al., 1987). A proposed mechanism for sorting of the neuropeptide precursor is the binding of this consensus signal motif to the membrane-associated receptor, and subsequent packaging within the immature secretory granules budding from TGN (Cool et al. 1997). The disulfide stabilized loop structure of the amino terminus of POMC interacts with carboxypeptidase E (CPE), known as the sorting receptor of POMC. CPE is known since the 1980s and is synthesized as a 55-kDa protein. There are two forms of CPE; one is soluble form and the other is membrane

bound (Hook, 1985). The soluble form has a role within the secretory granules in enzymatic cleavage of lysine/arginine amino acids of the C-terminus of peptides derived from POMC (Fricker and Snyder, 1983). The membrane form of CPE acts as a sorting receptor. The sorting signal of POMC interacts with the ligand binding domain on CPE composed of Arg²⁵⁵ and Lys²⁶⁰ residues (Figure 7), which are distinct from the enzyme active site and also recognizes other proteins such as pro-insulin and pro-enkephalin (Zhang et al., 1999; Cawley et al., 2016). CPE interacts with the membrane via lipid rafts containing glycosphingolipids and cholesterol, and this association is necessary for sorting pro-hormones to the RSP (Dhanvantari and Loh, 2000). Studies demonstrated that binding requires an optimal pH environment as the luminal pH of TGN (~6.2), and the presence of calcium in the lumen of the TGN. However, calcium is required for the aggregation and processing of prohormones only, not for the binding of CPE. Therefore, the binding of CPE to POMC under optimal conditions facilitates the trafficking of POMC to the granules of the RSP, so that POMC can be processed further (Cool et al., 1997).

Although there is much known on the binding of CPE to POMC, it is still not clear at which stage POMC binds to CPE. From ER to the TGN, it could bind in the ER to move within TGN. However, CPE leads POMC to be selected for immature granules that bud off the TGN. The relative importance of CPE in the sorting of POMC has not been clearly understood, but the studies using Cpe-deficient mice demonstrated that POMC is secreted constitutively in large amounts, but not targeted to mature secretory granules (MSG) of RSP and processed (Cool et al. 1997). These findings for CPE require more research to identify other molecules that play a role in the process of sorting.

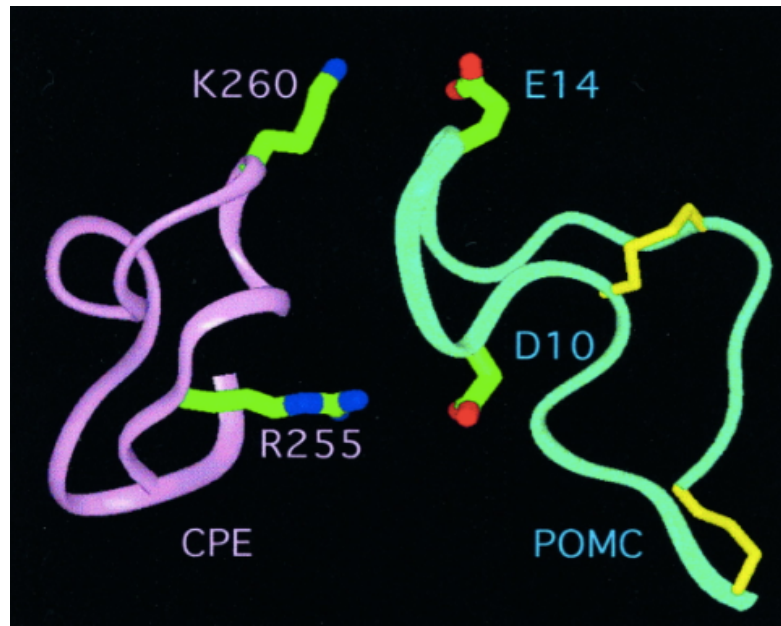


Figure 7. Schematic representation for complementarity of CPE₂₅₄₋₂₇₃ and N-POMC. Arg255 (R255), Lys260 (K260) in CPE bind to the acidic chains of N-POMC; Asp10 (D10) and Glu14 (E14) (Zhang et al., 1999).

Further analysis also suggests that several pathways are involved in the RSP. Granins are the major cargo proteins of large dense-core secretory granules (LDCGs), and secretogranin III and chromogranin A (CgA) are the members of granins. Studies showed that secretogranin III has a synergistic function with CPE in the sorting of POMC and its peptides (Cawley et al., 2016a). When it was silenced, protein secretion from the RSP in AtT20 cells reduced (Cawley et al., 2016b). Secretogranin III binds to CgA which is an important component in the process of regulated secretion by regulating the LDCG biogenesis. The movement of CgA to the granules of the RSP enables secretogranin III to bind CgA, which may cause the sorting of other cargo proteins via interaction with CgA (Montero-Hadjadje et al., 2009). In the formation of the vesicle, CgA functions as an assembly factor and has a role in determining the number of secretory granules formed within the cell (Loh et al., 2004).

1.1.3.3. Regulated Secretion of POMC

The RSP was defined by Gumbiner and Kelly as the place where the release of the contents of the secretory vesicles are controlled by external stimuli, called secretagogues (Gumbiner and Kelly, 1982). Without secretagogues, exocytosis of secretory granule contents decreases to a minimum level. These vesicles are electron dense-core and their turn over is slow since they store bioactive peptides (Harno et al., 2018). LDCGs are the major vehicles for proteolytic enzymes and active peptides in the regulated secretion. The formation of these LDCGs are required for the secretion of neuropeptides. Under low pH and high calcium levels, neuropeptides are aggregated to be destined for regulated secretion, after which they bind to a sorting receptor. Since LDCGs contain a high amount of cholesterol (Dhanvantari and Loh, 2000), ISGs are thought to bud off from the TGN containing lipid raft-rich microdomains. To become LDCGs, ISGs should undergo maturation, and constitutive-like vesicles should be pinched off in order to remove mis-sorted proteins. Then, neuropeptides continue to be processed into mature peptides (Loh et al., 2004). Also, ISGs remove the vesicle-associated membrane protein-4 (VAMP-4) which is associated with LDCGs, although there is still some VAMP-2 associated with mature LDCGs. It was shown that removal of VAMP-4 has an important role for the LDCGs to be releasable. Upon stimulation by secretagogues, LDCGs secrete their peptide contents (Eaton et al., 2000).

In some early analysis, radiolabeling of the sulfates on carbohydrate chains linked to POMC in the TGN showed how POMC is processed and trafficked into the constitutive and secretory granules (Dumermuth and Moore, 1998). Processing of POMC to ACTH starts in ISGs, but some incompletely processed POMC is released from ISGs through the constitutive-like pathway. Using sensitive immunoassays, POMC and ACTH release from AtT20 cells was measured. Thus, it was shown that AtT20 cells cultured under basal conditions released a much higher concentration of POMC than ACTH. But after stimulation for 2 hours, there was 2-fold increase in the concentration of released ACTH, with no changes in ACTH precursors. This

suggested that POMC is secreted through the constitutive-like pathway, whereas ACTH is secreted through the RSP (Seger and Bennett, 1986).

1.1.4. Processing of POMC

During movement of POMC from the ER to vesicles in cells, prohormone POMC is proteolytically cleaved and undergoes chemical transformations in a time- and compartment- specific way to yield biologically active neuropeptide hormones, such as ACTH, α -MSH, β -MSH, γ -MSH, β -END and β -LPH specific for that cell type (Cawley et al., 2016). Post-translation modifications of prohormone POMC have been shown to occur by individual processing in a tissue-specific manner (Bekdash et al., 2014). The peptides produced in the mature granules are stored here by forming an electron-dense core until stimulated by a secretagogue for release from the cell (Cawley et al., 2016).

Peptides produced by extensive posttranslational processing of POMC are cleaved by prohormone convertases (PC) at the well-defined single or dibasic amino acid residues (Pritchard et al., 2002; De Jonghe et al., 2011). PCs are a member of the family of Ca^{2+} -dependent serine endoproteases of the subtilisin/ kexin type (Böhm and Grässel, 2012). There are many types of PCs; PC1/3, PC2, furin (PACE), PACE4, PC4, PC6 and PC7. However, only PC1/3 and PC2 are considered the major endoproteolytic enzymes for POMC processing, since only these PCs are found in the dense secretory granules as shown by analysis of tissue expression and cellular localization of the convertases (Pritchard et al., 2002; Catania et al., 2004). Like prohormone POMC, these enzymes are also synthesized as a pro-form, and moved to and proteolytically cleaved into dense secretory granules where the majority of POMC processing occurs (Cawley et al., 2016).

PC1/3 has a signal peptide and a 80-90 amino acids pro-segment at the N-terminal (Stijnen et al., 2016). In the ER, this pro-segment is cleaved and the remaining 84 kDa

PC1/3 moves to TGN, and then to ISGs where its COOH-terminal peptide is removed. This yields a 66 kDa PC1/3 which is considered to be more active than the 84 kDa form of PC1/3. This 66 kDa form is the mature and fully active form and cleaves the POMC prohormone (Zhou and Lindberg, 1994).

Besides processing of POMC within the immature secretory granules, the presence of an active form of PC1/3 in the Golgi indicates a role in the first cleavage of POMC. In the anterior pituitary, the proprotein POMC is initially cleaved by PC1/3 between the C-terminal of ACTH and NH₂-terminal of β -LPH in the dibasic region Lys-Arg to generate an ACTH-biosynthetic intermediate (ABI)/pro-ACTH (1-136) and β -LPH (Eipper and Mains, 1980). Then the next step of cleavage occurs between the COOH-terminal of the joining peptide and the NH₂-terminal of ACTH (1-39). This cleavage results in the generation of ACTH, a 16-kDa NH₂-terminal peptide containing N-POMC (pro- γ MSH) and a joining peptide (Figure 8). In the human gene, the joining peptide was known as 'missing fragment' (Seidah et al., 1981). This is because it is amidated and released predominantly as a homodimer formed by a cysteine bridge between the two molecules (Bertagna et al., 1988).

Further processing of POMC occurs in the hypothalamus, pars intermediate of pituitary and skin. The degree of processing depends on which enzymes are active in the different tissues. The production of peptides is determined by the tissue-specific processing of POMC. PC1/3 mRNA is abundant in the anterior pituitary corticotropes, but there are also low levels of PC2 mRNA (Day et al., 1993). However, in the intermediate pituitary, the PC1/3 mRNA level is lower than PC2. Since PC2 is not present in the anterior pituitary, processing cannot go further in that region (Pritchard et al., 2002). There is more extensive processing in other areas such as hypothalamus and skin where PC2 is expressed. Since the activation of PC2 is late within the ISG, it has predominantly important roles in the later steps of processing by being responsible for the cleavage of ACTH to produce ACTH (1-17) and CLIP; β -LPH to yield β -endorphin and γ -LPH (further cleaved to β -MSH); and pro- γ -MSH to yield γ -MSH (Figure 8) (Day et al., 1993; Rousseau et al., 2007). Cleavage of ACTH yields a 4.5

kDa ACTH (1-17) and CLIP (ACTH 18-39). ACTH (1-17) is then further cleaved by CPE removing COOH-terminal amino acids to generate ACTH (1-13). Then it is amidated to give ACTH (1-13)-NH₂ at the COOH-terminal by peptidyl-glycine α -amidating monooxygenase (PAM). PAM exists as a bifunctional enzyme containing two catalytic domains. The first domain is the peptidylglycine α -hydroxylating monooxygenase (PHM) and catalyzed the first stage in the process. The second domain is the peptidyl- α -hydroxyglycine α -amidating lyase (PAL) and catalyzed the second stage. Completion of the α -amidation reaction depends on these two domains (Milgram and Mains, 1994). Since PAM is found in the large dense-core granules, the amidation process occurs when POMC peptides move to the secretory granules (Eipper et al., 1993). ACTH (1-13)-NH₂ is also called DA- α -MSH, and acetylated at the N-terminal by *N*-acetyltransferase (*N*-AT) to give a final 13 amino acid product, α -MSH (Glauder et al., 1990). This step is thought to protect peptides from aminopeptidases by increasing their stability (Wilkinson 2006), however there is still no satisfactory explanation for the differences in potencies and biological functions of des-acetyl α -MSH and mature α -MSH.

Other processes occur by cleavage of β -LPH in between the C-terminal of γ -LPH and NH₂-terminal β -END with PC2 (Figure 8). Next γ -LPH is cleaved at a Lys-Lys site from its C-terminal to yield β -MSH. Only humans have this Lys-Lys site and it is not seen in rats or mice, so β -MSH is not found as a separate peptide in rodents (Eberle, 2000). Another product of cleavage of β -LPH is β -END which consists of 31 amino acids at the C-terminal of POMC. Some studies have been reported that further processing of β -END(1-31) may occur to yield β -END(1-27) and β -END(1-26) via CPE found in the pituitary and brain (Young et al., 1986).

Another process is the cleavage of N-POMC to the γ -MSH by PC2. Pro- γ -MSH is also called N-POMC or N-POC (1-76). In humans, N-POMC is cleaved at a pair of dibasic amino acid residues at 49/50 to generate N-POMC (1-49) and γ_3 -MSH (27 amino acids). Then, further processing produces γ -MSH consisting of 11 amino acids (Harno et al., 2018).

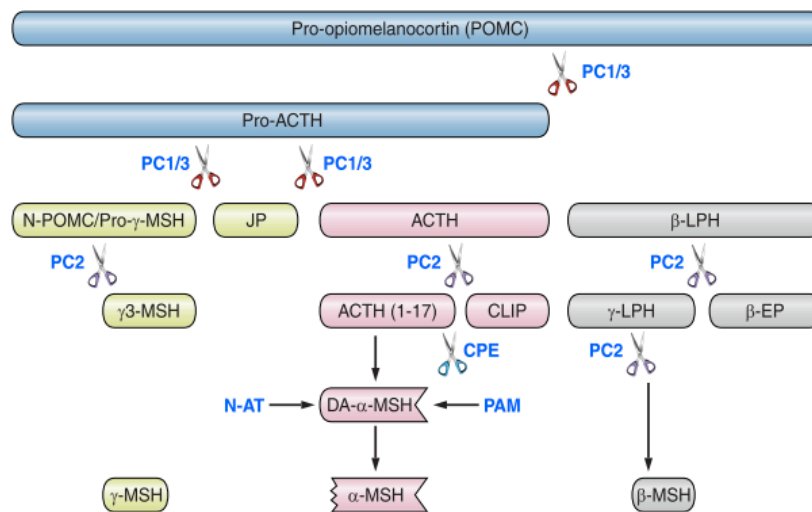


Figure 8. Overview of human POMC processing in a tissue specific manner. PC1/3 firstly cleaves the POMC to yield proACTH, then cleaves proACTH into N-POMC, JP, ACTH and β -LPH in anterior lobe and pars intermediate of pituitary, hypothalamus and skin. Further cleavages of peptides cannot occur in the anterior lobe of pituitary, due to absence of PC2. In the pars intermediate, hypothalamus and skin, ACTH is subsequently cleaved by PC2 to generate ACTH (1–17) and CLIP. Then, basic amino acid residues are removed from the C-terminal end of ACTH (1–17) by CPE. The remaining peptide is amidated by peptidyl-amidating mono-oxygenase (PAM) to yield des-acetyl α -MSH (DA- α -MSH). Acetylation of DA- α -MSH by N-acetyltransferase (N-AT) generates α -MSH. PC2 also cleaves the β -LPH to yield β -endorphin (β -EP) and γ -LPH, which results in β -MSH; and the N-terminal peptide N-POMC to generate γ -MSH (Harno et al., 2018).

Processed peptide hormones of POMC have been analyzed using Western blotting. In AtT20 cells, a secretion assay was performed to detect the levels of POMC peptides in both basal and stimulated secretion (Cawley et al., 2008). In Figure 9, pro-hormone POMC, its intermediate and ACTH were detected by Western blot using anti-ACTH. In the stimulated secretion (S), the level of ACTH increased compared to basal medium (B). Also, in the lysate of cells (L), only small amount of pro-hormone POMC could be detected, whereas processed peptides could not be found. Other smaller peptides such as α -MSH, β -END and β -LPH can be also detected in other study (Creemers et al., 2008).

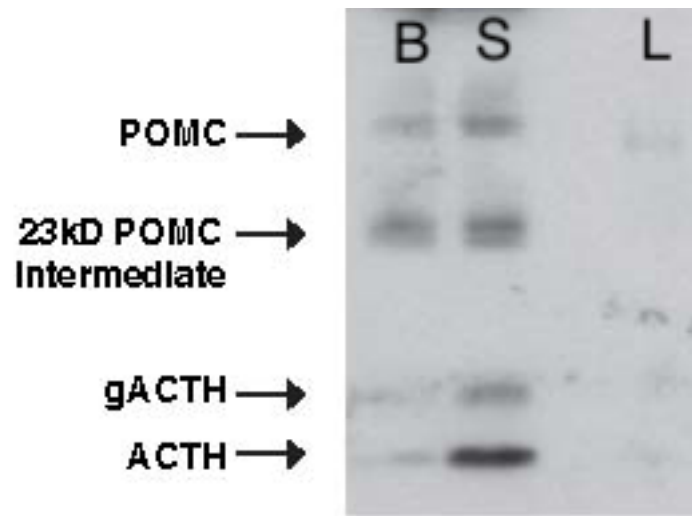


Figure 9. Western Blot analysis of POMC related peptides. A secretion assay was performed on AtT20 cells, and basal secretion (B), stimulated secretion (S) and lysates of cells (L) were analyzed using anti-ACTH antibody. POMC related peptides were detected. Pro-hormone POMC, its intermediate and ACTH were detected in both secretions, with an increased amount of ACTH in S. Only low levels of pro-hormone POMC was detected in L (Cawley et al., 2008).

1.2. THE FUNCTIONS OF THE POMC SYSTEM

The brain controls the appetite and energy homeostasis regulated by a complex neuroendocrine system. The hypothalamus in the brain is the main center for the regulation of food intake and energy homeostasis. Over the last decade, studies have focused on the role of neurons expressing POMC in the hypothalamus, and revealed their critical role in the regulation of appetite and energy expenditure (Xiao et al., 2016). However, peptides derived from POMC have distinct biological roles, including regulation of the stress response, food intake, and melanogenesis. The active peptides bind to their respective receptors on the specific cell type, so that they can function differently depending on where they exist and which receptor they bind to (Day et al., 1993).

1.2.1. The Role of POMC Peptides

The term melanocortins consists of a family of structurally related peptides including ACTH, α -MSH, β -MSH and γ -MSH. The name 'melanocortin' comes from their ability to stimulate melanogenesis in the melanocyte and steroidogenesis in adrenal cells (Coll, 2007).

The processing of POMC is mediated in a tissue-specific manner to generate four main melanocortin peptides which are α -MSH, β -MSH, γ -MSH and ACTH. These peptides share structural homology (Figure 10). All of them have the tetrapeptide sequence His-Phe-Arg-Trp at their core that enables receptor binding (MacNeil et al., 2002).

ACTH	NH ₂ -Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH
α -MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH ₂
β -MSH	NH ₂ -Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
γ 1-MSH	NH ₂ -Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH ₂
γ 3-MSH	NH ₂ -Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Ser-Ser-Ser-Ser-Gly-Ser-Ser-Gly-Ala-Gly-Gln-OH

Figure 10. Common sequence among human melanocortin peptides. Peptides, α -MSH, β -MSH, γ 1-MSH, γ 3-MSH and ACTH, share the invariant tetrapeptide sequence of four amino acids (His-Phe-Arg-Trp) on the 6-9 amino acids of ACTH and α -MSH, in order for melanocortin receptors to bind (Kastin, 2013).

Each POMC-derived peptide has a different role and functions in different tissues by the action of five melanocortin receptors. One of the first products of cleaved POMC is ACTH which contains 39-amino-acids and has the ability to activate all melanocortin receptors. Its major function is to mediate the adrenocortical response to stressful situations. When the hypothalamus-pituitary-adrenal (HPA) axis response is

stimulated by the stress, the anterior pituitary releases ACTH that moves to the adrenal gland, and stimulates glucocorticoid production in the adrenal gland as well as melanogenesis in the skin. (Harno et al., 2018; Wintzen and Gilchrest, 1996). Evidence showed that ACTH can also stimulate melanogenesis, as seen in patients having excessive concentrations of ACTH in the blood (Ray et al., 1995) Also, it has a role in adrenal cortical development (Karpac et al., 2007).

The other product, α -MSH is produced in the skin where it affects pigmentation via binding to one of the melanocortin receptors, MC1R, and shows anti-inflammatory and immunomodulatory properties (Coll 2007). In human melanocytes, it stimulates melanogenesis. Also, α -MSH produced in the hypothalamus has a role in reducing appetite and increasing energy expenditure through the other melanocortin receptors MC3 and MC4 (Pritchard and White, 2007).

Earlier studies have reported that β -LPH has a role in mobilizing lipid and it is thought to be an aldosterone stimulating factor (Matsuoka et al., 1980). However, recent studies showed its primary functions as a precursor of β -MSH and β -endorphin. In humans, there is no further processing of β -LPH due to lack of cleavage sites required for PC2. β -MSH which consists of 22 amino acid residues binds to MC4Rs with high affinity in vitro. In the hypothalamus, β -MSH acts on receptors to control body weight in humans, but with a lesser extent than α -MSH (Cone 2005). β -endorphin does not have the tetrapeptide sequence which is shared by other the melanocortins and interacts with μ -opioid receptors. Its role is seen in pain perception and analgesic response (Raffin-Sanson et al., 2003). The role of γ -MSH is less than clear compared to others, but thought to be involved in the regulation of the cardiovascular system (Mendiratta et al., 2011).

1.2.2. Melanocortin Receptors

The distinct physiological functions regulated by POMC-derived peptides of the melanocortin signaling system are controlled through the action of five melanocortin receptors. Different roles of these peptides, such as controlling of food intake and energy homeostasis, autonomic functions and exocrine secretions, depend on the receptor-subtype expression in different tissues (Anderson et al., 2016).

Melanocortin receptors were cloned firstly as the melanocyte-stimulating hormone receptor and adrenocorticotrophic hormone receptor in 1992 (Mountjoy et al., 1992). The five MCRs were cloned and named melanocortin 1 receptor (MC1R), MC2R, MC3R, MC4R and MC5R (Navarro, 2017). The actions of melanocortin peptides are exerted through these five melanocortin receptors (Böhm and Grässel, 2012). All these receptors have high sequence homology, and belong to the rhodopsin-like family of seven transmembrane G-protein-coupled receptors (Anderson et al., 2016; Coll, 2007). Upon binding to the heterotrimeric G protein, it dissociates. Ligand-bound MCRs activate the $G\alpha$ subunit types, which are $G_{\alpha s}$, $G_{\alpha q}$, and $G_{\alpha 11}$. (Anderson et al., 2016). These coupling and dissociation stimulate adenylyl cyclase activity in the signaling cascade, and induce cyclic 3',5'-adenosine monophosphate, which results in the accumulation of cAMP with an increase in protein kinase C (PKC) and diacylglycerol (DAG) downstream (Cone, 2006; Millington, 2007; Navarro, 2017).

Each receptor has a different expression pattern and binding affinity for melanocortin peptides (Table 1). MC1R, a 317-amino acid protein, was the first cloned melanocortin receptor (Gantz et al., 1994). MC1 receptors are expressed within a range of cell types in the skin, hair follicles, and immune cells including monocytes, neutrophils and lymphocytes, is also found in endothelial cells, melanoma cells and glial cells (Catania et al., 2004; Ständer et al., 2002). Pigmentation is regulated by the signaling through MC1R (Lin and Scott, 2012). However, it has also different biological roles, such as regulation of anti-inflammatory and immunomodulatory responses (Navarro,

2017). MC1R binds both ACTH and α -MSH with almost the same affinity (Böhm and Grässel, 2012). Binding of α -MSH to MC1R leads to the synthesis of eumelanin (brown-black pigment). However, if Agouti (agouti signaling protein) produced in the hair follicle binds to MC1R, it competitively inhibits the binding of α -MSH and cAMP activation due to its high affinity to MC1R and stimulates the synthesis of the red/yellow pigment pheomelanin (Anderson et al., 2016).

MC2R (297-amino acid protein) is the classical adrenocortical ACTH receptor, which was cloned firstly from the adrenal gland (Chhajlani and Wikberg, 1992; Coll, 2007). It only binds to ACTH. Activation of MC2R in the adrenal cortex, where it is exclusively expressed, regulates cell proliferation and promotes expression of steroidogenic enzymes, and results in the secretion of glucocorticoids such as cortisol and corticosterone which have roles in stress mechanisms and the immune system (Anderson et al., 2016; Catania et al., 2004).

MC3R (363-amino acid peptide) binds to all melanocortins with similar affinities except γ -MSH. MC3Rs are expressed in the brain, chiefly in the ARC of hypothalamus and limbic system with high expression (Lee 2012). It may have a minor role in energy homeostasis and feeding compared to MC4R, but its primary role is being the inhibitory 'auto-receptor' on POMC neurons in the ARC (Harno et al., 2018).

MC4R is a 333 amino acid protein and more highly expressed than MC3R. It is found in many areas of brain, such as in the ARC of hypothalamus, thalamus, hippocampus, limbic system, brainstem and spinal cord within the CNS with a striking presence (Coll 2007) as well as in the peripheral nervous system and in intestinal L cells (Anderson et al., 2016). Due to its wide expression areas, it mediates many different neurobiological functions such as pain, neuroperception, sexual behaviors, inflammation and fever (Argiolas et al., 2000; Catania and Lipton, 1993). However, the main function of MC4R is to regulate food intake and energy expenditure and most research recently focused on this function (Anderson et al., 2016). It shows a similar

potency to α -MSH, β -MSH and ACTH, and a lower potency to γ -MSH (Navarro, 2017).

MC5Rs are expressed primarily in exocrine glands (Anderson et al., 2016) and at low levels in numerous peripheral tissues, but not in the CNS (Coll, 2007). All melanocortin peptides can bind to it but the most potent ligand for this receptor is α -MSH. Its function is less understood compared to others, but recent studies have reported that MC5R may be involved in the regulation of exocrine gland function and certain immune responses (Harno et al., 2018).

Table 1. Binding affinity and locations of the five melanocortin receptors. MC2 receptor is also named as ACTHR (Fridmanis et al., 2017).

Receptor	Potency of ligands	Site of expression
MC1R	α -MSH = ACTH > β -MSH > γ -MSH	Melanocytes
ACTHR	ACTH	Adrenal cortex, adipocytes
MC3R	α -MSH = β -MSH = γ -MSH = ACTH	Hypothalamus, limbic system, placenta, digestive tract
MC4R	α -MSH = ACTH > β -MSH > γ -MSH	Hypothalamus, limbic system, cerebrum, brain stem
MC5R	α -MSH > ACTH > β -MSH > γ -MSH	Muscles, liver, spleen, lungs, brain, adipocytes

1.2.3. Central Melanocortin System in the ARC

The hypothalamus located in the forebrain is implicated as the main region for controlling diverse body functions, such as energy metabolism, sexual behavior and the stress response. It consists of many nuclei having different roles in the body. The ARC next to the third ventricle is one of the several nuclei of hypothalamus, and has the most extensively studied neuronal populations which are critical for the control of appetite and energy metabolism (Nazarians-Armavil et al., 2014). It is believed that the ARC is the primary central regulator of energy homeostasis, consists of two distinct and essential populations of neurons that counterbalance each other in controlling body weight (Figure 11). These neurons sense both short-term and long-term nutrient and hormonal signals from the periphery and send projections to secondary nuclei targets, where the signals are integrated to regulate food intake and energy expenditure (Sohn, 2015). One of these neuron populations are the orexigenic neurons, which synthesize agouti related peptide and neuropeptide Y (AgRP/NPY), and the other population contains anorexigenic neurons which synthesize POMC/cocaine- and amphetamine-regulated transcript (CART), which are also defined as first-order neurons (De Jonghe et al., 2011). These neurons are well positioned to integrate intensive peripheral and central signals and to respond to them by controlling of feeding behaviour. Both are located in the ARC (Xu et al., 2011), but in a different subset of neurons (Adan et al., 2006).

Anabolic and catabolic effects on energy balance are mediated opposingly by these NPY/AgRP and POMC/CART neurons. The opposing actions of appetite-promoting NPY/AgRP which produce AgRP (an endogenous MCR antagonist) and appetite-inhibiting POMC/CART which produce endogenous ligands for the MC4R such as α -MSH and ACTH, modulate the pathway of feeding regulation. Intensive projections from these two populations of neurons within the hypothalamus are projected to the downstream second-order neurons, mainly located particularly in the paraventricular nucleus (PVN) and the lateral hypothalamus, which express MC3R and MC4R (Belgardt et al., 2009; Coll 2007). Ligands produced from anorexogenic POMC

neurons bind to the receptors of secondary neurons. In PVN, these receptors are highly expressed and their activation decreases food intake and increases energy utilization (Ramamoorthy et al., 2015). However, another anorexogenic neuropeptide, CART, is less studied than POMC because there is not much known about its receptors. CART is highly expressed than POMC throughout the brain and peripheral tissues, and has a similar role to reduce food intake and increase energy utilization (Lau and Herzog, 2014).

On the other hand, orexigenic neurons NPY/AgRP produce neuropeptide Y (NPY) (agonist of the Y receptors), AgRP (inverse agonist at the MC4Rs), and γ -aminobutyric acid (GABA) (inhibitory neurotransmitter) (Sohn, 2015). Their functions have been demonstrated in the studies showing that NPY, AgRP, and GABA have a synergistic role in the control of food intake, and they increase food intake (Krashes et al., 2013). NPY in the hypothalamus binds to Y1 and Y5 receptors and shows an effect on food intake and energy homeostasis. The same role of NPY is also exerted by GABA, which affects the initial phase of feeding by acting as a substitute for NPY (Ramamoorthy et al., 2015).

However, AgRP has a more well-established role in the regulation of food intake and energy metabolism in the melanocortin system. Its role is considered the underlying mechanism of orexigenic effects, but recent studies showed that orexigenic effects of NPY/AgRP are not dependent on AgRP, which was demonstrated by the deletion of MC4Rs. Thus, it is now considered that the roles of NPY/AgRP neurons in food intake are independent of the central melanocortin system. At this point, it is believed that most of the orexigenic effects of NPY/AgRP neurons are mediated by the release of GABA. Findings from studies showed that GABA release is important for the maintenance of normal energy homeostasis (Xu et al., 2011). In the ARC, the GABAergic signals from NPY/AgRP are sensed by POMC neurons, and this produces a local circuit to mediate appetite and energy metabolism within the ARC by inhibiting anorexogenic effects (Sohn, 2015).

In POMC neurons of the ARC, neuropeptide precursor POMC is cleaved to produce α -MSH and released from axon terminals via the membrane depolarization of neurons. α -MSH acts as an agonist of MC3 and MC4 receptors. Activation of these receptors by binding of α -MSH leads to a decrease in food intake and an increase of energy utilization (Fischer et al., 2016; Ramamoorthy et al., 2015). However, the activity of the melanocortin system is not only regulated by POMC products, but also by AgRP released from NPY/AgRP neurons (Adan et al., 2006). AgRP is an antagonist of MC4R and directly inhibits the action of α -MSH, via blocking the activation of MC4R mediated by α -MSH (Belgardt et al., 2009). This antagonizing action of AgRP decreases energy expenditure by competitively blocking α -MSH binding and inhibiting receptor activation (De Jonghe et al., 2011; Wardlaw, 2011).

Since the first order melanocortin neurons in the ARC act as sensors, the melanocortin signaling system is under direct regulation of various central and peripheral stimuli (Xu et al., 2011). The hormonal and nutrient-related signals can act upon these first-order NPY/AgRP and POMC/CART neurons to regulate food intake and energy homeostasis. The hypothalamus by responding to such signals coordinates a neuronal network that controls distinct aspects of the whole body metabolism such as caloric intake, thermogenesis and spontaneous physical activity (Souza et al., 2016).

Circulating hormones, such as leptin and insulin act on orexigenic and anorexigenic neurons, since they can cross the blood brain barrier. Both sets of neurons express leptin receptors (LepR) and insulin receptors (IR) (Figure 11). Leptin is directly released by adipocytes and has a role in the regulation of body composition. The binding of leptin to LepR on POMC/CART and NPY/AgRP neurons reduces food intake and increases energy expenditure by excitation of POMC neurons and suppression of NPY/AgRP neurons (Ramamoorthy et al., 2015). This is consistent with the findings of studies which showed that deletion of LepR from POMC or AgRP neurons results in obesity and hyperleptinemia (Balthasar et al., 2004).

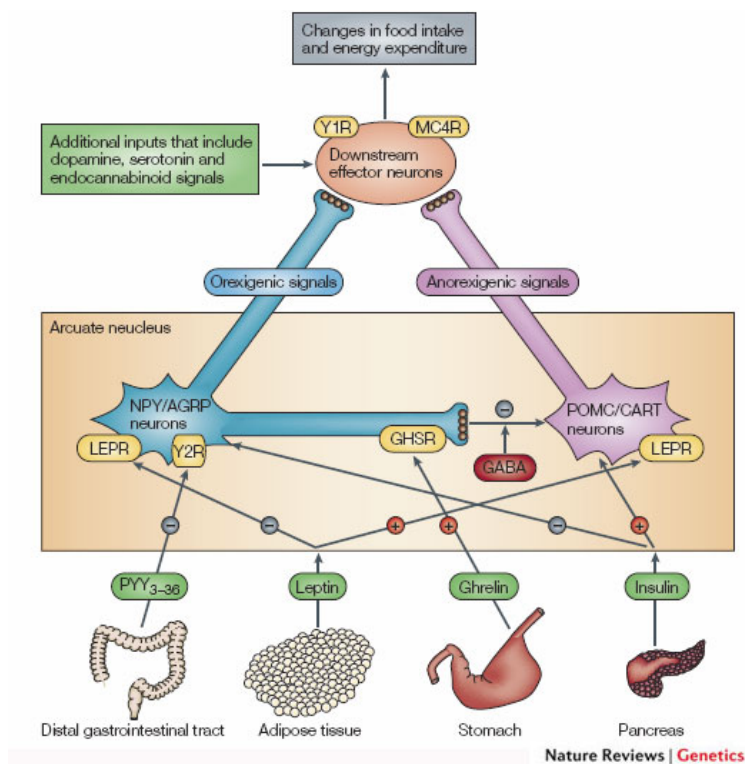


Figure 11. Physiological regulation of energy expenditure and food intake in the Arcuate nucleus of hypothalamus (ARC). Two sets of neurons in the arcuate nucleus synthesize the pro-opiomelanocortin (POMC)/cocaine and amphetamine related transcript (CART) peptides and neuropeptide Y (NPY)/agouti-related protein (AGRP) in order to function oppositely to regulate energy balance. Several endocrine hormones send signals to these two set of neurons. Leptin and insulin, secreted from adipose tissue and pancreas respectively, stimulate the POMC/CART neurons and inhibit the NPY/AGRP neurons, via the binding to their receptors located on both neurons, and lead to anorexigenic effects which are reduced food intake and increased energy expenditure. Ghrelin released from the stomach shows an orexigenic effect by stimulating the NPY/AGRP neurons via binding to growth hormone secretagogue receptors (GHSRs). The other peripheral signal is peptide YY₃₋₃₆ (PYY₃₋₃₆) synthesized in the distal gastrointestinal tract. It binds to Y2 receptors and produces an inhibitory effect on NPY/AGRP neurons. Moreover, γ -aminobutyric acid (GABA) is released from NPY/AGRP neurons and causes the inhibition of the POMC/CART neurons. These first-order neurons, POMC/CART and NPY/AGRP, project their signals through the Y1 and melanocortin 4 (MC4R) receptors to downstream second-order effector neurons to change the food intake and energy expenditure. Also, additional inputs from dopamine, serotonin and endocannabinoid signals are received by these second-order neurons (Bell et al., 2005).

Similar to leptin receptors, insulin receptors are found in both POMC and AgRP neurons since they are widely expressed throughout the central nervous system (CNS). The peptide hormone insulin is secreted by pancreatic β cells and functions in the ARC by producing an anorexigenic effect by reducing food intake and body weight (Woods et al., 1979). Thus, the anorexigenic effect results from binding of insulin to its receptor which stimulates POMC/CART neurons and inhibits AGRP/NPY neurons (Bell et al., 2005).

Other peripheral signals which have roles in the regulation of appetite are ghrelin and peptide YY₃₋₃₆ (PYY₃₋₃₆). These are involved in the short-term regulation of adiposity, whereas leptin and insulin affect the long-term regulation of adiposity. The gut-derived orexigenic peptide hormone, ghrelin, released from the stomach and duodenum, stimulates NPY/AgRP neurons via the growth hormone secretagogue receptor (GHS-R) which is found only in NPY/AgRP neurons. The ghrelin level in serum rises before eating and decreases after eating (Kohno et al., 2003). It is also thought that the activation of GHSR by ghrelin might stimulate GABA release from NPY/AgRP neurons (Sohn, 2015). Another signal is generated by PYY₃₋₃₆ which is secreted from the gastrointestinal tract on ingestion of food. It binds to Y2 receptors found only in NPY neurons, and leads to a decrease in food intake (Bell et al., 2005).

1.2.4. The Role of POMC in Obesity

The melanocortin signaling system mediates different physiological functions of the body including energy homeostasis, pigmentation, the HPA axis and exocrine gland function, since there are many POMC-derived peptides and receptors which show different tissue-specific roles. Therefore, disruption of this signaling system caused by the deficiency in one of the peptides or receptors may lead to diverse diseases. In the pituitary, processing of POMC peptides is critical for the maintenance of the HPA axis, thus, the abnormalities in POMC processing in the pituitary results in the disruption of HPA axis (Harno et al., 2018). Due to expression of POMC in many

peripheral tissues, POMC deficiency is also characterized by adrenal failure and altered pigmentation (Millington, 2007). However, since the discovery of POMC in 1979, the role of POMC in the regulation of energy homeostasis and feeding behavior has long been studied intensively (Singhal and Hill, 2018).

The maintenance of stable body weight depends on the balance between intake and expenditure of energy. A decrease in energy expenditure with an increase in food intake can cause increased fat storage due to disruption of the energy balance (Zegers et al., 2010). Obesity is characterized as a state of excessive fat accumulation. Many different factors can cause the obesity, which may be genetic, environmental, or gene-environment interactions (Yang and Tao, 2017).

Hypothalamic circuits especially in the ARC are involved in the regulation of energy homeostasis and are critical for obesity. Many rare monogenic human obesity syndromes are associated with gene deficiencies, which result in hyperphagia. One of genes in the hypothalamus that regulate the weight balance is *POMC* and its respective peptides and receptors. POMC is defined as a key element of energy homeostasis mediated by melanocortin signaling (Millington, 2007).

Indeed, there are many factors in POMC processing, and defects in any of them may lead the obesity by disrupting the POMC process. Studies showed that obesity may be caused due to the deficiencies in prohormone convertases, CPE, POMC gene and MC3/MC4 receptors. Since single amino acid changes in any of them may lead to defective POMC processing in the CNS, the melanocortin signaling system can be disrupted and ultimately result in obesity (Pritchard et al., 2002).

1.2.4.1. MC3R/ MC4R and Obesity

It is important to understand the mechanism of obesity caused by the melanocortins system to design novel therapeutic strategies. Receptors highly expressed in the hypothalamus are one of these therapeutic targets. Deletions or mutations of melanocortin receptors lead to diseases such as obesity. MC3R and MC4R are more critical than MC1R, MC2R and MC5R for the regulation of body weight. Even though the disruption of all receptors causes negative consequences on the body and homeostasis, MC3 and MC4 receptors are of more importance and have been extensively studied for the role in the pathogenesis of obesity (Yang and Tao, 2017).

Genetic studies have demonstrated the function of MC3R and MC4R in the regulation of energy homeostasis. They both have a role in the regulation of body weight, but they function differently. MC3R has a dominant role in inhibition of energy storage and regulates feeding efficiency and feeding rhythm (Chen et al., 2000; Lee, 2012), whereas MC4R is involved in the regulation of food intake and energy expenditure (Balthasar et al., 2005).

Homozygous deletion of MC3R in knockout mice results in increased adiposity, which is associated with reduced lean body mass, reduced bone density and increased feeding efficiency (Anderson et al., 2016). These mice models also exhibit hypophagia, higher energy efficiency, hyperleptinemia and reduced linear growth without increased body weight compared to WT mice (Aris et al., 2015; Lee 2012). Some studies showed a significant association between polymorphisms of MC3R with early childhood adiposity (Aris et al., 2015). However, human MC3R mutations have not been extensively described, whereas human MC4R mutations are well known, since mutations of the MC4R are a relatively common cause of severe childhood obesity, and heterozygous mutations in MC4R are the most common cause of monogenic obesity (Mendiratta et al., 2011). POMC neurons release α -MSH, which is an agonist of the anorectic MC4R, to suppress appetite, thus deficiencies in MC4R result in hyperphagia and obesity in mice and humans (Sohn, 2015). Mice having

homozygous MC4R deficiency cannot respond to the anorectic effects of α -MSH (Catania et al., 2004). In the *Mc4r* knockout mouse model, it was shown that a lack of both copies of the gene resulted in early onset obesity with hyperphagia associated with pathological lack of satiety, hyperinsulinemia with hyperglycemia, and hyperleptinemia with an increase in adiposity and linear growth, and normal lean body mass (Anderson et al., 2016; De Jonghe et al., 2011; Lee, 2012). Loss of one single allele of *Mc4r* displays an intermediate phenotype between wild type and homozygous models, suggesting a gene dosage effect with respect to body weight and food intake (Anderson et al., 2016; Xu et al., 2011).

Analysis of MC4R mutations in human and mice display a more or less similar phenotype. Several mutations in *MC4R* have been described in humans. They range from missense and nonsense to frameshift mutations and are inherited in an autosomal dominant manner (Censani et al., 2014). The mutations of MC4R in humans can result in increased bone mineral density and longitudinal growth rate, hyperphagia, hyperinsulinemia with increased fat mass and lean body mass, without the changes in gonadotropin, cortisol levels, thyroid and sex steroid levels (Adan et al., 2006). Therefore, MC4R may be a promising target in the obesity treatment (Brumm et al., 2012).

1.2.4.2. POMC Deficiency and Obesity

Intracellular processing of POMC and its peptides have a role in the regulation of energy metabolism. In response to alterations of energy requirements, levels of bioactive peptides secreted from POMC neurons are controlled by the regulation of post-translational processing of POMC. Therefore, any disruption in this regulation can lead to obesity. In the obesity research significant contributions have been made based on studies using genetically modified mice. These studies showed that POMC products are involved in the anorectic response. It was shown that mice lacking either the whole coding region of POMC or the whole *Pomc* displayed significant obesity

and an imbalance in energy homeostasis (De Jonghe et al., 2011; Millington, 2007). Deficiencies in POMC cause lack of melanocortin functions and ultimately result in the altered feeding behavior. After unveiling role of POMC peptides it was found that many inherited deletions in *POMC* are associated with obesity (Jameson and De Groot, 2010).

Remarkable similarity between the murine and human POMC gene implicates the conserved role of *POMC* in the controlling of energy homeostasis (Yaswen et al., 1999). *Pomc* knockout mice which do not have endogenous melanocortins led to the obese phenotype, with other diseases such as adrenal insufficiency with very low levels of corticosterone, causing hypersensitivity to metabolic effects of glucocorticoids (Adan et al., 2006). It was also shown that *Pomc* ^{-/-} mice displayed a higher fat amount in the body than wildtype mice and a reduced basal metabolic rate (Mendiratta et al., 2011). The results seen in POMC-null mutant mice were also displayed in humans. The first human POMC mutation is the POMC-null mutation, which was described in 1998 by Krude et al. it was found in two children, who congenitally lacked *POMC* products. Red hair, secondary hypercortisolism and early-onset and extreme obesity were observed in these patients with complete loss of *POMC* gene function (Krude et al., 1998).

POMC deficiency in patients causes the interruption of the hypothalamic signaling cascade of weight regulation. Since the CNS responds to an increase of leptin and insulin by the activation of MC3 and MC4 receptors, in the case of POMC deficiency, absence of POMC-derived peptides results in lacking of receptor activation resulting in interruption of the hypothalamic signaling cascade (Krude et al., 2003).

The POMC deficiency syndrome is a recessive trait, because symptoms shown above are not observed in all heterozygous mutation carriers. However, the studies showed that heterozygosis in POMC mutations increases the risk for obesity (Krude et al., 2003; Mencarelli et al., 2012). With these findings, several mutation-screening studies have been reported. Various single point mutations of *POMC* have been studied to

assess its association with obesity or increased the risk for obesity (Mendiratta et al., 2011). Some mutations found in these obese patients showed that alterations in POMC folding and turnover in the ER. For example, at position 28, POMC cysteine-to-phenylalanine mutation has been found in patients having early onset obesity (Creemers et al., 2008). Due to this mutation, the mutated POMC cannot be targeted for proteasomal degradation and aggregates in the ER by destabilizing the protein (Kim et al., 2018).

In another study, a missense (A15G) mutation found in *POMC*, particularly in signal peptide, of patients with early onset diabetes, central obesity and hyperphagia was analyzed. The A15G POMC mutation was found to completely abolishing the production and overall secretion of POMC protein (Mencarelli et al., 2012). Other signal peptide mutations, Ser7Thr and Ser9Leu, were also identified in the heterozygous state in obese children (Del Giudice et al., 2001).

When POMC cannot not be secreted from cells, its anorexigenic function (appetite-suppressing) is suppressed, hence leading to an increase in body weight. Therefore, it is important to know how these mutations result in the obesity. Adequate knowledge of POMC physiology by detailed investigation of its mutations helps researchers to understand the pathophysiology of obesity and new targets for its treatment.

1.3. SER9LEU MUTATION

Molecular screening of the coding region of POMC led to the discovery of several mutations in patients with obesity. In one of these kind of studies, the Ser9Leu POMC mutation was found (Del Giudice et al., 2001). Del Giudice and his colleagues have screened the coding region of POMC by analysis of single-strand conformation polymorphism (SSCP) in eighty-seven unrelated Italian obese children with very early onset obesity aged 4.7 ± 2.5 years. Three new heterozygous mutations were reported

in three patients as a result of mutational screening. One is the missense mutation G3824C, which leads to the change of a serine to threonine at codon 7 of POMC. Another missense mutation is C3840T, resulting in the change of a serine to leucine at codon 9 on POMC. A third missense mutation C7406 results in the change of arginine to glycine at codon 236 within β -endorphin peptide of POMC. the first two mutations are the first amino acid substitutions which are found within the signal peptide of POMC. They lie in the N-tail of signal peptide. Particularly, serine at codon 9 (Ser9) locates at the end of the amino-terminal region. The long hydrophobic stretch of 11 amino acids which belongs to h-region begins after Ser. Since Ser9 is located in this border region, a change of the hydrophilic Ser9 to hydrophobic leucine (Leu) may affect the translocation of the overall protein into the ER.

Because of the importance of the location of the Ser9Leu POMC mutation and the lack of knowledge of its cellular mechanism in the literature, in this thesis, the cellular mechanisms of Ser9Leu POMC mutation was investigated.

1.4. AIM OF THE STUDY

The Ser9Leu POMC mutation is located within the signal peptide which is critical for the translocation of POMC. Other studies have found that mutations in the signal peptide of the secreted proteins lead to the accumulation of mutant prohormones in the ER and these mutations resulted in the inhibition of the further processing and secretion of the proteins (Shen and Loh, 1997). Based on the literature the cellular mechanism of Ser9Leu POMC has not been studied, though it was implicated in the genetic predisposition to obesity (Del Giudice et al., 2001). Therefore, the aim of this study was to investigate the changes of the cellular mechanisms of Ser9Leu POMC in a neuronal cell line. It was hypothesized that Ser9Leu POMC may reduce the translocation of POMC into the ER, hence, escort out the inactive mutant POMC by decreasing the level of further processing.

CHAPTER 2

MATERIALS AND METHODS

2.1. PREPERATION OF PLASMIDS

The POMC plasmid DNA was a gift from Dr. Peng Y. Loh (NICHD, National Institutes of Health (NIH), Bethesda, USA), and kept at 4° C until used.

2.1.1. Plasmid Extracting from Whatman Paper

POMC plasmid was on the Whatman filter paper with a circle drawn on it and kept at 4° C. To extract the plasmid, circle containing the DNA was cut out and put into an eppendorf tube. One hundred µl of 1X TE buffer containing 10mM Tris (Sigma Aldrich, Germany) and 1mM EDTA (Sigma Aldrich, Germany) was added to the tube with DNA and incubated for 15 minutes at room temperature. Then the eppendorf was centrifuged at high speed for 5 minutes with a bench top mini centrifuge. The supernatant of centrifuged sample was taken into another tube. The concentration of DNA sample was measured in NanoDrop2000 (BioDrop, UK).

2.1.2. Generation of WT and Ser9Leu POMC Mutation

The POMC plasmid DNA belongs to *Bos taurus* (bPOMC). For this study which investigated a mutation found in human, bPOMC was compared with human POMC (hPOMC), and different nucleotides between hPOMC and hPOMC were identified, which some lead to changes in amino acid (aa) sequences between *Bos Taurus* and human, whereas some do not because of the degeneration of the genetic code.

Firstly, aa sequences of both species were compared using BLAST (Basic Local Alignment Search Tool) and different amino acids were identified. Then, bPOMC was sent to a company, Sentegen (Ankara), for the de novo analysis. De novo analysis of bPOMC plasmids was performed by comparing it with SNP region of hPOMC (rs139750421², 501 bases). The circular map of bPOMC plasmid (6704 bp) and the result of de novo analysis were provided in Figure A.1 and Figure A.2, respectively in Appendix A. All regions of the plasmid and the restriction sites which were used for site-directed mutagenesis (Bachman, 2013) were shown in the circular map of plasmid. In the de novo analysis, the differences between bPOMC and the human SNP region were highlighted as red (Figure A.2).

Based on the de novo analysis, the differences in nucleotides of the signal peptides cause two different amino acids in the signal peptides of bPOMC and hPOMC. hPOMC contains Ser and Cysteine (Cys) on codons 4 and 6, respectively, and bPOMC contains Leu and Ser on codons 4 and 6, respectively (Figure 12). Ser and Cys are polar and have non-charged side chains, whereas Leu is non-polar and has aliphatic side chain. Since the signal peptide is the important region for the protein translocation within the cell, these differences in the signal peptide of bPOMC would cause fallacy in the results. For this reason, only two aa's in the signal peptide of bPOMC was changed to hPOMC signal peptide with two aa's modification.

• Human = MPRSCCSRSGALLLALLLQASMEVRG
 • Bos taurus= MPRLCSSRSGALLLALLLQASMEVRG

Figure 12. Comparison of signal peptides (1-26 aa) of WT human and *Bos taurus* POMC. There are 2 different amino acids, highlighted as red at the codons of 4 and 6. Serine (Ser) at codon 9, highlighted as yellow, was mutated to Leu (L).

² More information about this SNP can be retrieved from the NCBI dbSNP website (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=rs139750421)

For the generation of Ser9Leu POMC mutation and other two modifications (Leu4Ser and Ser6Cys) in the signal peptide of bPOMC, plasmid DNA was sent to Sentegen (Ankara). These two different amino acids (4th and 6th aa's) of the signal peptide of bPOMC was changed to aa's in the signal peptide of hPOMC with site-directed mutagenesis. At the same time using the same primer sequence, 9th aa of POMC was changed from Ser to Leu to generate the Ser9Leu POMC mutation. The procedures of site-directed mutagenesis were provided in Appendix B.

After the generation of WT and Ser9Leu POMC, Sanger sequencing (Sanger et al., 1977) was performed to see nucleotide sequences of both WT and Ser9Leu POMC, and the result of sequencing was provided in Appendix C.

2.2. BACTERIAL GROWTH

Two prepared plasmids were transformed to competent cells (*E. coli*) in order to amplify the number of plasmids. Manufacturing information about the products that were used in the experiments were given in the appendices, unless stated in this chapter.

2.2.1. Plasmid Transformation

Transformation of bacteria is the process by which exogenous DNA is introduced into the host cell. Transformations in this study were performed by heath shock method (Inoue et al., 1990).Plasmids were transformed to the DH5αTM competent cells (Invitrogen, USA) by using heat shock. Procedures were performed as stated in kit's manual³.

³ <https://assets.thermofisher.com/TFS-Assets/LSG/manuals/18258012.pdf>

Competent cells and SOC (Super Optimal broth with Catabolite repression) medium were taken out of the -80°C freezer and cells were thawed on ice for 5 minutes. One μL of plasmid DNA was mixed with 100 μL of competent cells and incubated on ice for 30 minutes. Then, the competent cell/DNA mixture was given a heat shock by incubation for 45 seconds in the 42° C water bath, which led to some of bacteria taking up the plasmids. After the heat shock, the mixture was again incubated on ice for 2 minutes and 900 μL of SOC medium was added to mixture. This mixture was incubated in the 37°C shaking incubator for 1 hour to allow the growth of bacteria with plasmids. Then, a range of serial dilutions of bacteria from transformation mix was prepared for spreading on agar plates. This procedure was performed for both WT and mutant POMC (MT) plasmids. Also, pUC19 vector provided by the kit was used as a control to determine the transformation efficiency.

2.2.2. Colony Selection and Plasmid Isolation

Luria-Bertani (LB) agar plates and media were prepared for growth of bacterial colonies (Sambrook et al., 1989), and provided in Table D.1 of Appendix D. Serial dilutions; 10 μL , 50 μL and 100 μL of transformed cells containing same plasmid were streaked on LB/ampicillin plates and incubated overnight at 37°C. Next day, several colonies resistant to antibiotic were chosen for the plasmid isolation. Three single colonies from three plates were picked with a tip of pipette and inoculated into six 5 mL of LB/ampicillin media overnight in a shaking incubator at 37°C at 225 rpm. Then, all plasmids were isolated with GeneJET Plasmid Miniprep Kit (Thermo Fisher, USA) according to manufacturer's manual provided in Appendix E, and nucleic acid concentrations were measured by NanoDrop2000. This procedure was performed for WT, MT, pUC19 and empty vector (EV), separately. All plasmids were kept at -20°C until the transfection cells.

2.2.3. Agarose Gel Electrophoresis

Gel electrophoresis is used to separate DNA fragments based on their size and charge. Isolated plasmids were examined with the Agarose Gel Electrophoresis to check their size (Koontz 2013), after digestion with XbaI (Thermo Scientific, USA). Gels of 1% agarose concentrations were used in this study. Briefly, 0.5 g agarose was weighed and dissolved in 50 mL of 1X TAE buffer prepared from 50X stock (Appendix D) in a flask, heated into the microwave until boiling and completely dissolved. After letting the solution cool down, 1 μ L of EtBr which makes DNA visible under UV light was added. The mixture was poured into a gel tray with the well comb in place. After the gel solidified, the gel box was filled with 1X TAE until the gel was covered. Then samples (1:5 ratio with 6X loading dye) and marker were loaded onto gel. The gel was run at 100V for around 90 minutes depending on the properties of the loaded samples and agarose content of gel. Later DNAs were visualized under UV light, and assessed with Quantum ST4 (Montreal Biotech, Canada) for image acquisition.

2.3. CELL CULTURE

All chemical and buffers for cell culture were listed in Appendix D.

2.3.1. Growth of N2A and AtT20 Cells

Mouse neuroblastoma (N2a) cells were obtained from ATCC (American Type Culture Collection) and mouse pituitary corticotrope tumor (AtT20) cells were a gift from Dr. Peng Y. Loh (NICHD, National Institutes of Health (NIH), Bethesda, USA). All cells kept in liquid nitrogen were quickly thawed in 37°C water bath. All thawed contents of cells were added to 10 mL of growth medium and centrifuged for 5 minutes at 800 rpm to remove DMSO (dimethyl sulfoxide) from the freezing medium.

After discarding of supernatant, cells were resuspended in 10 mL growth medium and added to a 75 cm flask (T75). Cells were grown in DMEM with L-glutamine supplemented with 10 % FBS and 1% pen-strep solution (Day et al., 1993). Cells were maintained in an incubator at 37°C temperature, 95% air and 5% CO₂ culture conditions.

2.3.2. Cell Passaging

After the confluence of cells reached almost 80%, they were transferred into another culture flask (T75). Old medium was discarded, and cells were washed two times with PBS (Phosphate-buffered saline). After washing steps, 3mL of Trypsin (Biological Industries, Israel) was added onto the cells and they were placed into the incubator for 2 minutes in order to allow cells detach. Then 7 mL of complete medium was added onto cells to inhibit trypsin. Detached cells were harvested, and each 2 mL of detached cells was transferred to new T75 flask containing 8 mL complete medium. Subculture was done at 1:5 for both cell types.

Cells from one of the flasks were cultured into 6-well plate for transfection. After the N2a cells reached confluency of 80% in the 6-well plate, cells were ready for transfection.

2.3.3. Transfection of N2a Cells with POMC DNAs

N2a cells in 6-well plate were transfected with EV, WT and MT plasmids using Lipofectamine2000 reagent (Thermo Fisher, USA). Each 3 µg of DNA was mixed with 250 µL of OptiMEM (Gibco, USA) and 10 µL of Lipofectamine2000 reagent was mixed with 250 µL of OptiMEM, separately. These two mixtures were incubated separately for 5 minutes at room temperature, then, they were mixed into one vial and

incubated for 20 minutes at room temperature. Old medium of cells on the 6-well plate was discarded and cells were washed with OptiMEM two times. Then, 2 mL of transfection medium (Appendix D) was transferred to the plate by the adding of the DNA/Lipofectamine/OptiMEM mixture. Cells were incubated overnight at 37°C temperature, 95% air and 5% CO₂ culture conditions.

2.4. SECRETION ASSAY

After transfection, the medium was removed, and cells were rinsed twice with 1 mL serum-free DMEM supplemented with 0.01% BSA which is called as basal medium. The cells were then incubated with 1.5 mL of basal medium for 30 minutes in the incubator. The medium was collected and labelled as basal medium 1 (B1). Then, fresh basal medium was added to cells and incubated for another 30 minutes. This medium was also collected and labeled as B2. Similarly, B3 and B4 samples were collected. Then, cells were incubated with 1.5 mL of stimulation medium (DMEM supplemented with 0.01% BSA, containing 50mM KCl and 70mM NaCl) for 30 minutes in the incubator. After 30 minutes stimulation of the cells, the medium was collected and labeled as stimulated secretion (S). All these samples; B1, B2, B3, B4 and S were centrifuged at 3000 rpm for 3 minutes and the supernatants were transferred to new vials to be stored in -20°C. The experimental steps of this procedure were shown in Figure 13.

Finally, the cells were rinsed twice with 1 mL ice-cold PBS and lysed in the mixture of 200 µL M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA) and 33.3 µL EDTA-free protease inhibitor (7X) (Roche, Germany). This lysed content of cells was labelled as L and centrifuged at 13.000 rpm for 20 minutes to pellet the cell debris. Supernatants (soluble cell extracts) were transferred to the new vials, and protein concentrations were measured by BCA Assay (Thermo Scientific, USA).

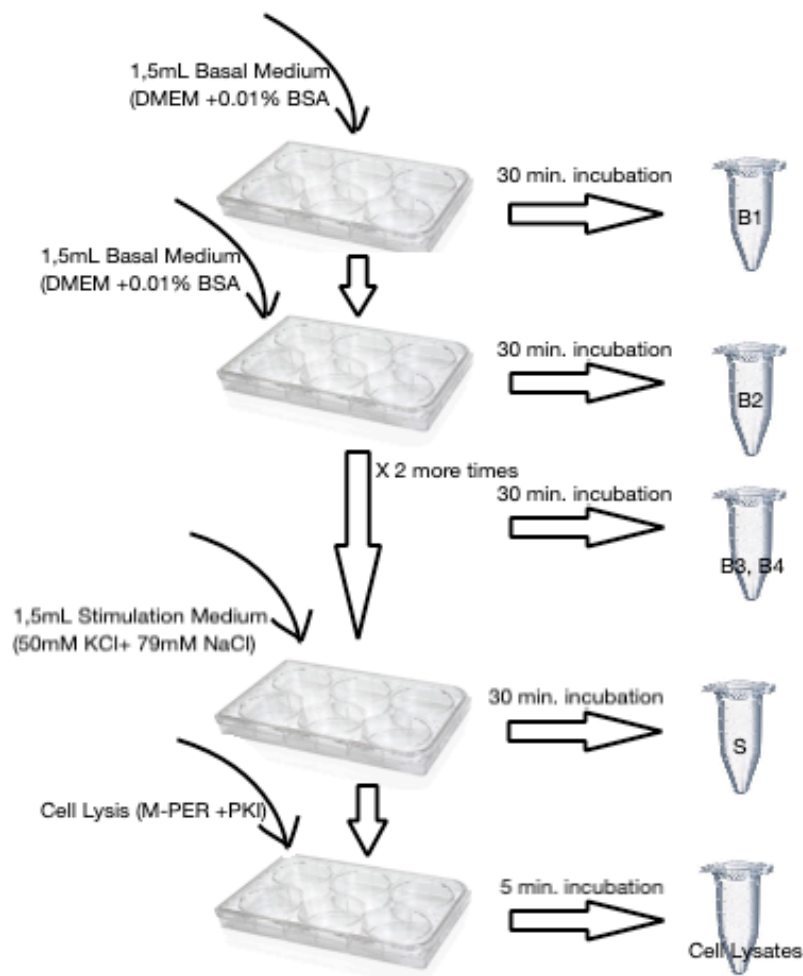


Figure 13. Experimental Steps of Secretion Assay. B1, B2, B3 and B4 samples were collected after each 30 minutes incubation with basal medium. After 30 minutes incubation with stimulation medium, S sample was collected. Cell contents were lysed with M-PER and protease inhibitors.

AtT20 cells are positive control cells for POMC since they endogenously produce POMC. Therefore, they were also cultured in 6-well plate and same procedure of the secretion assay was also performed for AtT20 cells without transfection.

2.4.1. Concentration of Proteins with TCA

To concentrate the proteins in B1, B2, B3, B4 and S medium samples, TCA (Trichloroacetic acid) precipitation was performed (Cawley et al., 2016). Seven hundred twenty μL of B1, B2, B3, B4 and S samples were added to 80 μL of 100% TCA (Appendix D), and the mixtures were precipitated overnight at 4°C. Then, TCA-precipitated proteins were centrifuged at 13.000 rpm for 20 minutes. The supernatant was discarded after the centrifugation. To remove any residual TCA, protein pellets were washed twice with ice-cold acetone. 500 μL of acetone was added onto pellet. This acetone/pellet mix was incubated for 1 hour at -20°C, then centrifuged at 13000 rpm for 15 minutes. After doing this twice, the supernatant was discarded carefully, and pellet was allowed to air-dry for a while. Then, the protein pellet was reconstituted in 72 μL 1X SDS-PAGE sample buffer for gel electrophoresis.

2.4.2. BCA assay

Total concentration of proteins was measured by Pierce Rapid Gold BCA Protein Assay Kit (Thermo Scientific, USA). It was performed according to manufacturer's instructions (Thermo Scientific) (Appendix F). The analysis of protein concentrations was performed with multiplate spectrophotometer (Thermo Scientific, Finland).

2.5. WESTERN BLOT

Standard Western blotting techniques (Mahmood and Yang, 2012) were performed to analyze proteins from cell lysates and secretion media using ACTH antibody. All the buffers used in Western blot procedure and both stacking and separating gels were self-manufactured from various components available in the laboratory. The list of buffers and compositions of gels were provided in the Appendix G.

Based on the optimization of the Western blot procedures, all samples were run at 120 V for approximately 90 minutes. Transfers of proteins from gels to the methanol activated PVDF membranes were conducted at constant 100 mA for 2 hours. Membranes were drenched in blocking solutions (%3 BSA, 0.1% T-TBS) with a gentle agitation for 1 hour at room temperature. Then, membranes were incubated with primary antibody for overnight at 4°C, whereas secondary antibody incubation was 1 hour at room temperature.

Also, gel was stained with Coomassie Blue for 30 minutes, then, washed with destaining solution until dye was removed. PVDF membranes after blotting were stained with Ponceau S to check the amount of samples. They were incubated with Ponceau S solution for 5 minutes. Then, they were rinsed with distilled water to achieved desired staining.

Primary antibodies used were mouse monoclonal anti-ACTH (1:5000, Santa Cruz, USA), rabbit polyclonal anti-Actin (1:2000; Thermo Fisher Scientific, USA), and secondary antibodies were anti mouse M-IgG BP-HRP (1:10000, Santa Cruz, USA) and anti-rabbit IgG-HRP (1:10000, Santa Cruz, USA). The Western blot protocol was provided in the Appendix G. Blots were visualized using ChemiDoc™ MP System of BioRad and analyzed with Image Lab™ Software provided by BioRad.

2.5.1. Statistical Analysis

Cell culture, secretion assay and Western blots were repeated at least four times independently. Statistical analysis was performed by Student's t-test to assess the significance of results for the secretion assay ($*p < 0.05$ considered with significance). Western blotting of secretion assay samples' band intensity was measured using a software Image Lab provided by the BioRad imaging system. Secretion assay bands were normalized within each replicate (n=4) by the division of the average band intensity.

CHAPTER 3

RESULTS

3.1. CONSTRUCTS OF WT AND SER9LEU POMC

The result of BLAST showed that bPOMC and hPOMC are 80% identical (Figure 14). There were two different aa in the signal peptide (first 26 aa). Therefore, the signal peptide of hPOMC with the Ser9Leu mutation was generated by changing the amino acids in 4th and 6th codons of the signal peptide of bPOMC (Figure 15).

Sequence ID: Query_118513 Length: 267 Number of Matches: 1

Range 1: 1 to 267 [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
402 bits(1032)	9e-148	Compositional matrix adjust.	220/274(80%)	236/274(86%)	16/274(5%)
Query 1	MPRLCSSRSGALLLLALLLQASMEVRGWCLESSQCQDLTTESNLLACIRACKPDLSAETPV				60
Sbjct 1	MPR C SRSGALLLLALLLQASMEVRGWCLESSQCQDLTTESNLL CIRACKPDLSAETP+				60
Query 61	FPGNGDEQPLTENPRKYVMGHFRWDRFGRNRGSSSSGVGAAQKREEEVAVGE-----				113
Sbjct 61	FPGNGDEQPLTENPRKYVMGHFRWDRFGRNRN SSSSG GA QKR E+V+ GE				118
Query 114	-GPGPRGDDAETGPREDKRSYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAQAFPLEFKR				172
Sbjct 119	GP PR D A+ GP RE KRSYSMEHFRWGKPVGKKRRPVKVYPNGAEDESA+AFPLEFKR				178
Query 173	ELTGERLEQARGPEAQAES-AAARAELEYGLVAEAEAEAAEKKDSGPYKMEHFRWGSPPK				231
Sbjct 179	ELTG+RL + GP+ A+ A A+A+LE+ L+ AAEKKD GPY+MEHFRWGSPPK				233
Query 232	DKRYGGFMTSEKSQTPLVTLFKNAIKNAHKKGQ 265				
Sbjct 234	DKRYGGFMTSEKSQTPLVTLFKNAIKNAHKKGE 267				

Figure 14. BLASTp result of bPOMC and hPOMC amino acids. Query represents *Bos taurus* POMC amino acids, subject represents human POMC amino acids.

Based on the Sanger sequencing, the colorful electropherogram result shows that nucleotides on the 4th and 6th codons were successfully changed to nucleotides present in humans, and the Ser9Leu mutation was successfully generated (Figure 15). Therefore, bPOMC now can be used for trafficking of hPOMC in N2a cells. In Figure 15, there was a mistake in 3rd codon (thymine) of the mutated plasmid which is highlighted as red indicating a change, but it was due to wrong annotation of sequencing algorithm. In 3rd codon of both WT and MT, same color (green, indicating Adenine) were displayed in the electropherogram, but it was wrongly annotated in the result due to little peak of red in the electropherogram. Therefore, it was ignored in this study.

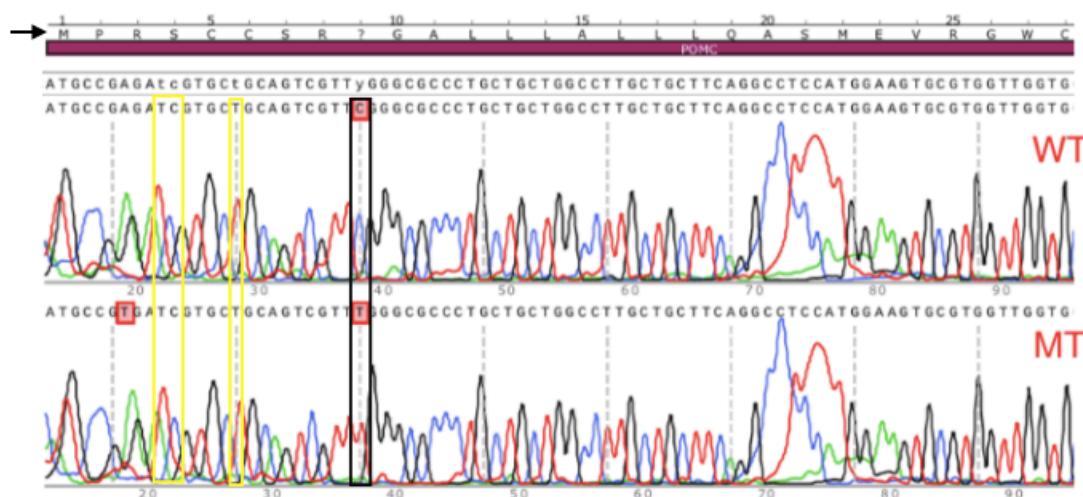


Figure 15. Signal peptide of POMC after Sanger sequencing. The top sequence is WT, below sequence is Ser9Leu POMC (MT). In the 9th codon of WT, there is cysteine, whereas in Ser9Leu POMC, it is thymine, represented in the black box. Other two modifications are shown in yellow boxes to resemble bPOMC to hPOMC. Nucleotides in codon 4 and 6 of bPOMC were changed to nucleotides of hPOMC. ‘tc’ in codon 4 and ‘t’ in codon 6 are the nucleotides of hPOMC, and present in both WT and MT. Black arrow shows the amino acid sequence.

After the plasmid transformation and isolations, nucleic acid concentrations of all plasmids were measured by NanoDrop2000, and DNA with the best qualities was chosen for further experiments. The ratio of absorbances at three levels which are absorbances at 230, 260 and 280 gives the purity of DNA. The ratio of A260/A280 should be around 1.8 for good quality DNA. Lower ratios indicate that there are protein contaminants in DNA sample. Secondary measure of purity is the ratio of A260/A230 which indicates the presence of organic contaminants and should be close to 2.0. lower ratios below 1.8 indicates that there are significant number of contaminants (BioDrop manual, 2018). Concentrations of plasmids were provided in Table H.1 and Table H.2 in Appendix H.

3.1.1. DNA Gel Electrophoresis

POMC DNAs (digested with XbaI) were analyzed with the gel electrophoresis to check their presence. Since the shorter molecules move faster through the gel, EV which does not have POMC insert was migrated farther than WT and Ser9Leu POMC plasmids. This finding confirms that POMC regions were inserted into the plasmids, making then heavier (Figure 16).

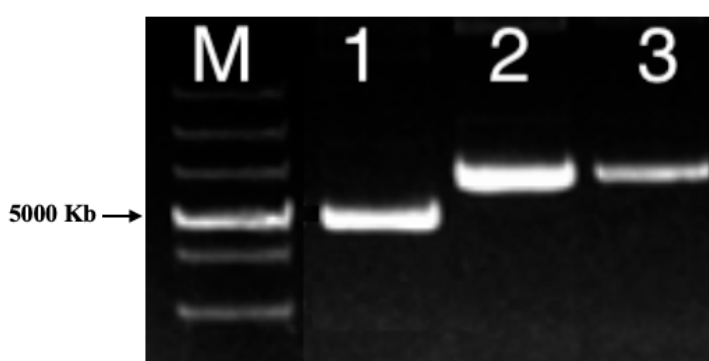


Figure 16. Agarose gel imaging of POMC plasmids. M: 1 Kb Plus DNA Ladder; Lane 1: empty vector (EV); Lane 2: WT POMC plasmid and Lane 3: MT POMC plasmid, digested with XbaI.

3.2. PROTEIN CONCENTRATION ANALYSIS

After the transfection and secretion experiments of N2a cells and AtT20 cells, their cells were lysed, and proteins were collected to measure the concentrations with BCA protein assay. A protein standard curve was created by plotting the absorbance at 480 nm for each BSA dilution versus its concentration in $\mu\text{g/mL}$ in order to determine the total protein concentration of unknown samples (Figure 17). R^2 value of the curve should be between the values of 0.95 and 1.05, so that the standard curve can be valid.

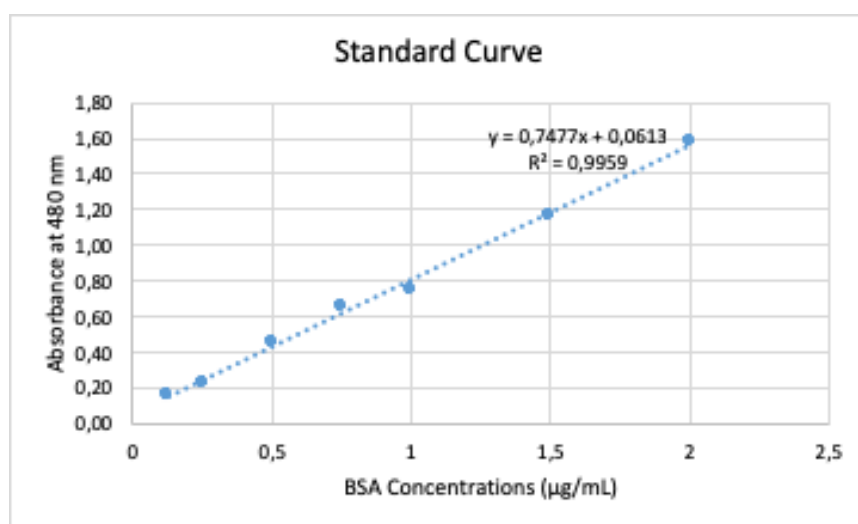


Figure 17. Standard curve for the determination of protein concentration with BCA assay. Curve was plotted by using BSA concentrations (x-axis) versus their absorbance value at 480nm (y-axis).

After the plotting of standard curve (Figure 17), the equation ' $y=0,7477x+0,0615$ ' was used to determine the concentrations of protein samples. In this equation, y represents the absorbance value of unknown protein samples, and x represents the concentration of that proteins. Using this equation, concentrations of proteins used in this assay were determined. Standard curves were also plotted for other replicates.

3.3. WESTERN BLOT ANALYSIS OF PROTEINS

The functional role of the mutation in the processing and/ or sorting of POMC protein was evaluated by blotting of the samples from N2a cells transfected with WT and Ser9Leu POMC. B3 and B4 samples from basal mediums of cell culture and S sample from stimulation medium reveal the secretion pathway of the protein. Intracellular levels of POMC were detected on both WT and MT samples with almost equal amounts. In Figure 18, samples from control (untransfected N2a cells) and EV (cells transfected with empty vector which does not contain POMC insert) were used for negative control. As expected, there is no POMC secretion in these samples, which also confirms that N2a cells cannot produce POMC endogenously. It also shows that antibody did not recognize components of the medium, such as BSA, used for secretion assay.

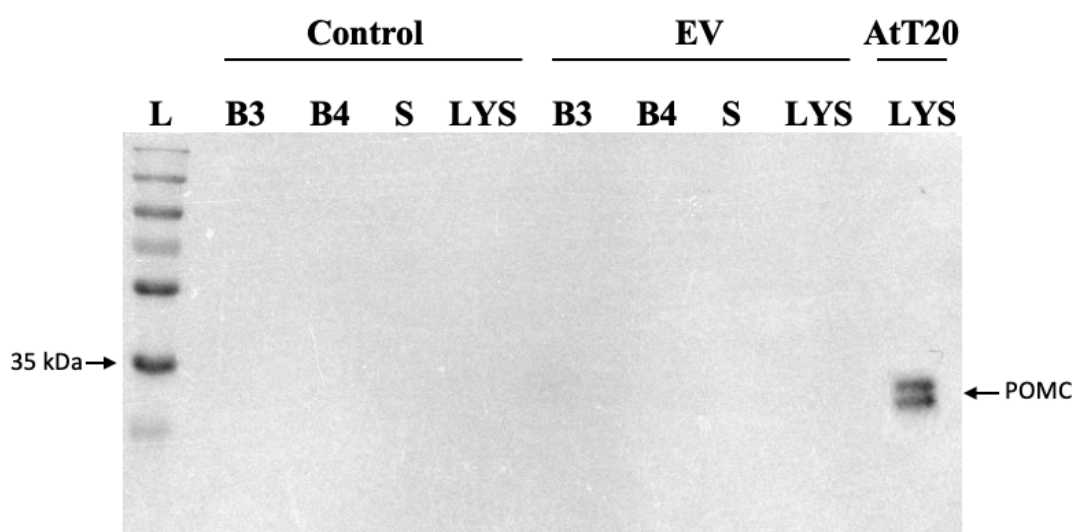


Figure 18. Western blot analysis of untransfected N2a cells and empty vector (EV) for the secretion assay. Lysate of AtT20 cells were used to check if antibody works. B: basal secretion, S: stimulated secretion and LYS: cell lysate, L; protein ladder.

In the all images of Western blot (n=4; Figure 19, Figure 20, Figure 21 and Figure 22), all bands appear around 35 kDa, which indicates the presence of inactive pro-hormone POMC. While basal mediums (B3 and B4) represent the constitutive secretion of protein without being processed, after stimulation with KCl and NaCl, POMC was expected to be secreted with RSP while being processed to small peptides.

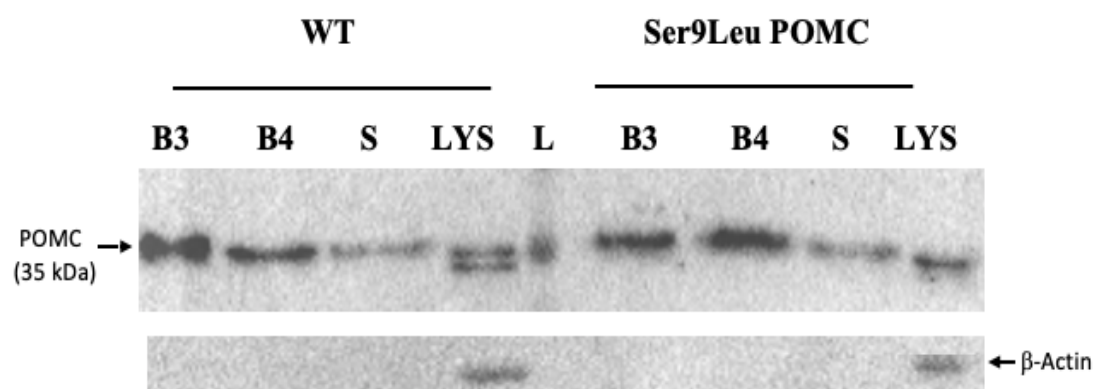


Figure 19. First experiment of the secretion assay (n=4). Image of Western blot for the secretion assay in WT and MT POMC transfected N2a cells is shown. Western blot was carried out in which proteins in basal medium (B3 and B4), stimulated medium (S) and lysates (LYS) of N2a cells transfected with WT and Ser9Leu POMC using anti-ACTH. Bands around 35 kDa belongs to inactive POMC secreted constitutively. Anti-β-actin was used as loading control for cell lysates. L: protein ladder.

For the N2a cells transfected with WT POMC, in the absence of stimulus, pro-hormone POMC was secreted in high amount in B3 and B4 samples (Figure 19). However, after the addition of stimulus to cells, large decrease in the secretion of inactive pro-hormone POMC was observed in S of cells transfected with WT POMC. The same result was also observed in the cells transfected with Ser9Leu POMC. Lysates of both cells transfected with WT and Ser9Leu POMC still contain high amount of POMC, although intracellular POMC was not much in Figure 9. Moreover, the same results were also observed in other three replicates of same experiments (Figure 20, Figure 21, Figure 22).

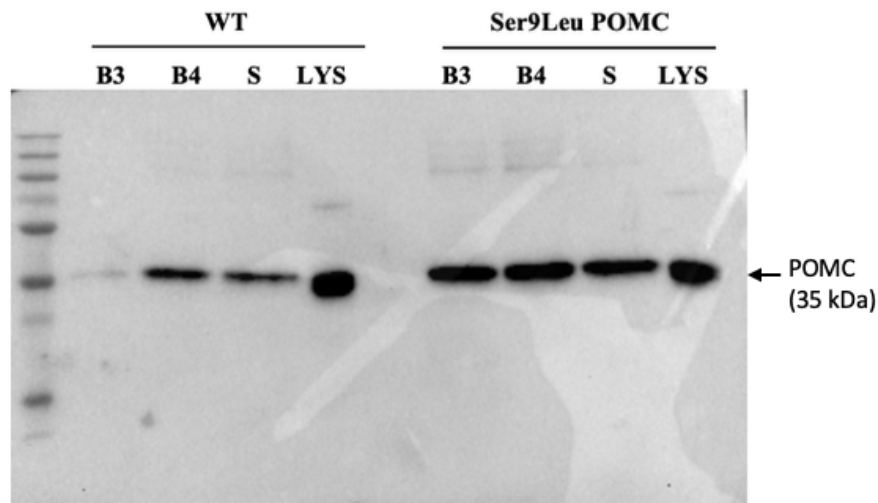


Figure 20. Second experiment of the secretion assay (n=4). Image of Western blot for secretion assay in N2a cells is shown. Bands in Ser9Leu POMC were overexposed. Lysates were loaded as 5 μ g. B: basal secretion, S: stimulated secretion and LYS: cell lysate.

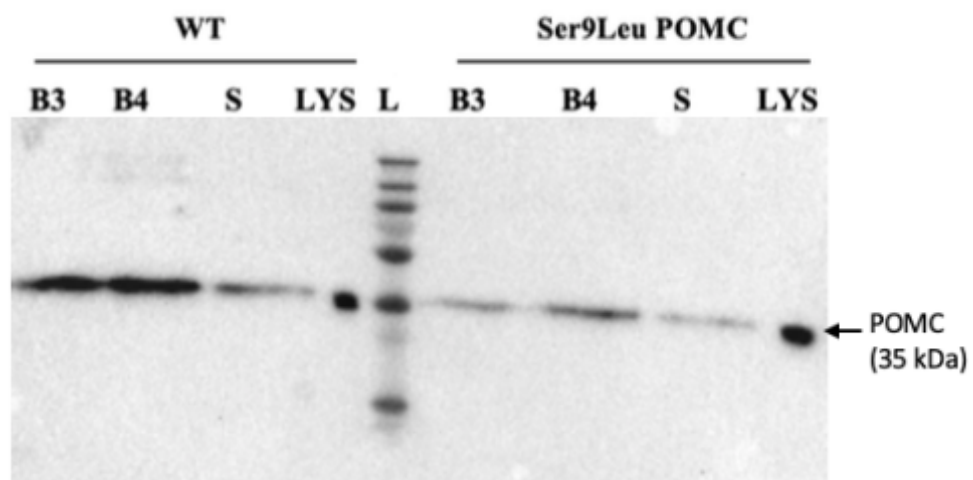


Figure 21. Third experiment of the secretion assay (n=4). Secretions in S were reduced in both WT and Ser9Leu POMC. Lysates were loaded as 5 μ g. B: basal secretion, S: stimulated secretion and LYS: cell lysate, L: protein ladder.

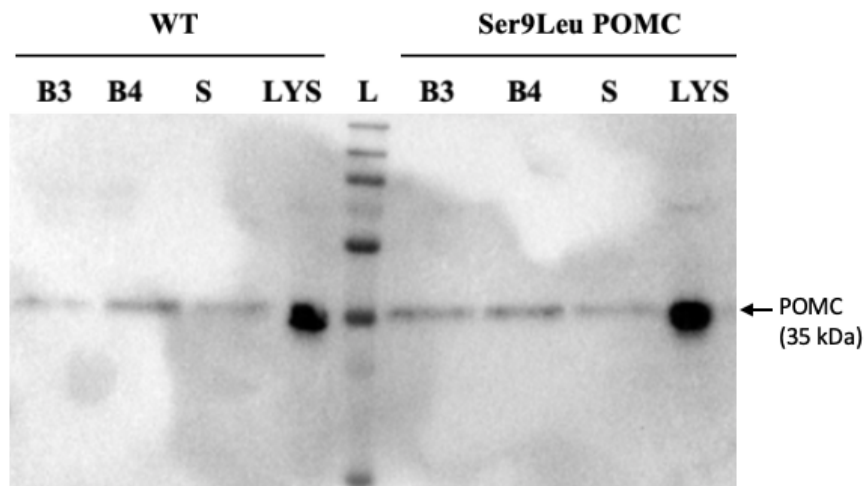


Figure 22. Fourth experiment of the secretion assay (n=4). Secretions in S were reduced in both WT and Ser9Leu POMC. Lysates were loaded as 5 μ g. B: basal secretion, S: stimulated secretion and LYS: cell lysate, L: protein ladder.

To ensure the relative loading amount of samples, the gel was stained with Coomassie Blue (Figure 23A) and PVDF membrane was stained with Ponceau S after blotting (Figure 23B). In all lanes of the Coomassie Blue stained gel and the Ponceau S stained membrane, the loading looked similar (Figure 23 A and B).

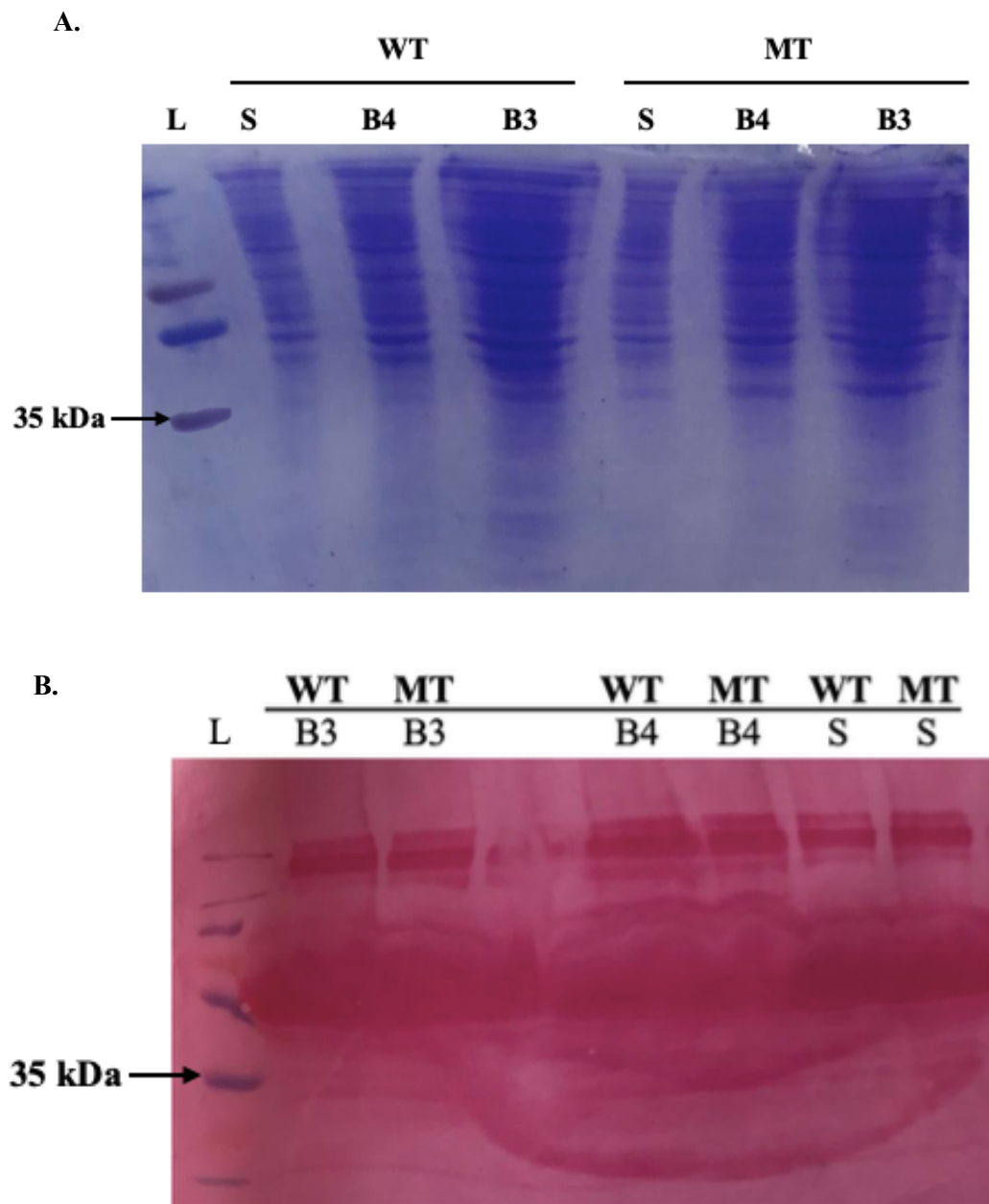


Figure 23. Secretion assay samples staining. A. Gel was stained with Coomassie Blue. B. PVDF membrane after blotting was stained with Ponceau S. WT POMC B3, B4, and S secretion samples and MT POMC B3, B4 and S samples. L: Protein ladder

Figure 24 shows the average of band intensity for the secretion assays. For the statistical analysis, bands were normalized within each replicate to analyze band intensity (n=4) between two 30 min periods of basal secretions (B3 and B4), some amount of inactive POMC (35 kDa) was detected in the medium of cells expressing WT and MT POMC. For B3 and B4, a 1.08 ± 0.4 SEM-fold increase (n=4) in WT POMC B3 samples, a 1.36 ± 0.22 SEM-fold increase in WT POMC B4 samples, and also a 0.99 ± 0.21 SEM-fold increase in MT POMC B3 samples, a 1.18 ± 0.13 SEM-fold increase in MT POMC B4 samples were detected. Upon depolarization of these cells for 30 minutes, a 0.66 ± 0.04 SEM-fold increase (n=4) WT POMC S samples and a 0.82 ± 0.21 SEM-fold increase in MT POMC S samples in stimulated secretion of inactive POMC were observed. Student t-test was used to determine the level of secretion relative to basal and stimulated secretion samples. The statistical differences of secretion levels between B3 of WT and MT POMC, B4 of WT and MT POMC and S of WT and MT POMC were not significant. The statistical secretion levels of B4 to S for both WT and MT POMC were significant ($*p<0.05$) (Figure 24).

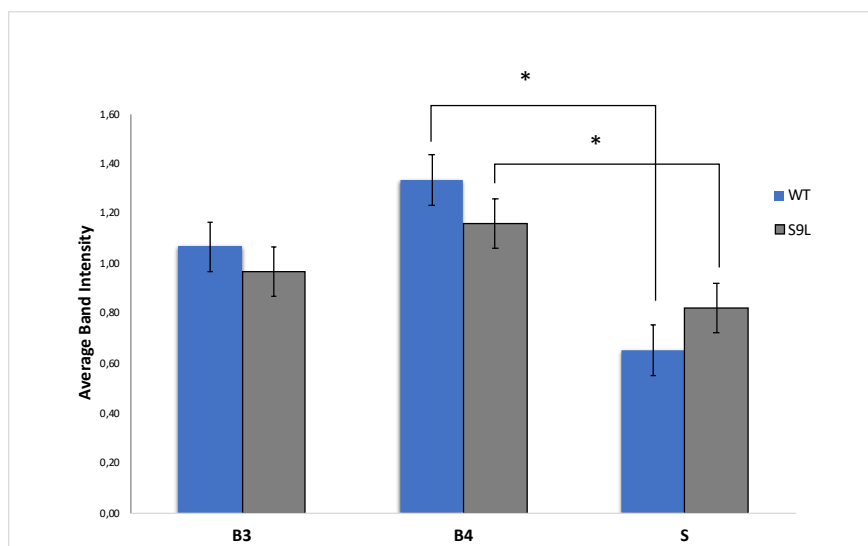


Figure 24. Average band intensities of WT and Ser9Leu POMC for the secretion assay. The secretion of inactive pro-hormone POMC between B4 to S are significantly reduced in both WT and S9L POMC (n=4) ($*p<0.05$).

AtT20 cells were used as the positive control for the synthesis and the secretion assay. In all AtT20 cells, POMC was secreted as aggregated around 35 kDa, resulting double bands in the blot (Figure 25). Two replicates of AtT20 cells secretion were depicted in Figure 25. Due to ability of AtT20 cells, each time different amount of POMC was produced and secreted. However, the large decrease in the secretion of inactive POMC was observed after the stimulation of cells (S) in both replicates.

Moreover, lysates of N2a cells transfected with WT and MT POMC and AtT20 cells were loaded into one gel (Figure I.1 in Appendix I). Lysates of AtT20 cells (two replicates) displayed as double bands of POMC, whereas lysates of N2a cells showed only one band of POMC. Also, other samples such as B1 and B2 of N2a cells and AtT20 cells were shown in Figure I.2 of Appendix I.

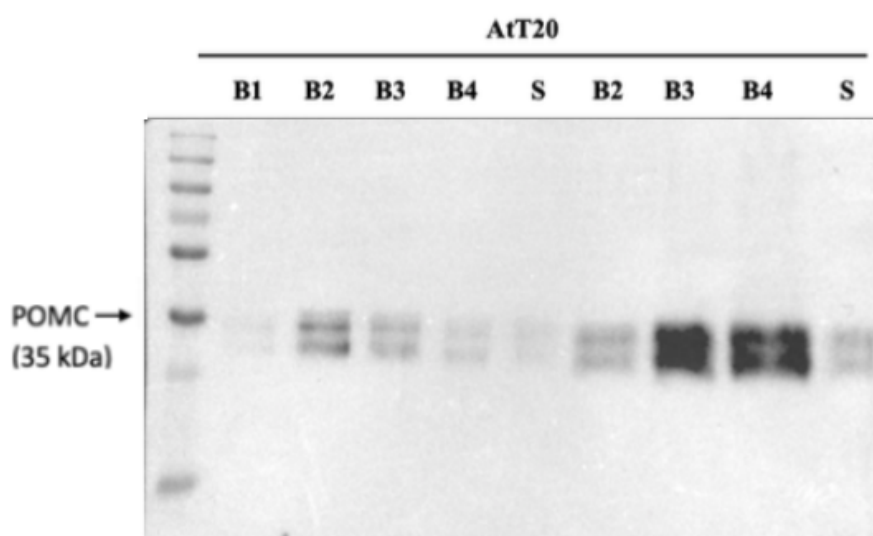


Figure 25. Western blot analysis of AtT20 cells for the secretion assay. AtT20 cells were used as a positive control for POMC secretion and analyzed by Western blot probing with anti-ACTH. B: basal secretion, S: stimulated secretion and LYS: cell lysate, L: protein ladder. POMC secretion was reduced in AtT20 cells during S.

CHAPTER 4

DISCUSSION

The mammalian physiology is regulated by peptide hormones. Many human diseases are caused by the disruption in synthesis or secretion of hormones (Kim et al., 2018). POMC is one of the peptide hormones and a key regulator of mammalian physiology which is found in many tissues. Peptides derived from POMC are involved in diverse body functions from cortical development to skin pigmentation (Bertagna 1994). POMC peptides produced in the neurons of ARC in the hypothalamus appear to be most important weight regulator (Schwartz et al., 2000), because it was shown in the studies that humans and mice that lack POMC display hyperphagia and obesity (Coll et al., 2004). Since the description of whole human POMC deficiency, there have been many reports and studies that associate the mutations in *POMC* with an increased chance of weight problems which cause obesity (Hinney et al., 1998; Krude et al., 1998). Mutations within this gene are known to be a cause of severe monogenic early-onset obesity in humans. Most of those mutations have an effect on the structure or features of the peptides, or on the cleavage from their precursor. Mutations in the N-terminal region of *POMC* likely affect the processing and sorting of POMC protein to the RSP.

POMC mutations are also associated with early-onset obesity (Creemers et al., 2008). Ser9Leu POMC mutation in the N-terminal region of the *POMC* was found in an Italian child having early-onset obesity (Del Giudice et al., 2001). It differs from other mutations in that it does not lie within, or close to, the melanocortin peptides, but lies in the signal peptide of POMC which is removed in the ER. Since the signal peptide of proteins is required for the translocation of proteins within the ER, it was hypothesized that the Ser9Leu POMC mutation may impair the translocation of POMC and leads to decreased secretion of the protein in the RSP.

In the present study, cellular mechanisms of Ser9Leu mutation located on the signal peptide of *POMC* was investigated. Our results showed that cells transfected with WT *POMC* secreted the proteins in B1, B2, B3 and B4 samples around 35 kDa from the constitutive secretory pathway, since unprocessed and inactive *POMC* proteins are mostly secreted from constitutive secretory pathway. After the stimulation of cells with KCl, *POMC* was anticipated to be processed into its peptides and secreted from RSP. Thus, the reason of reduction of inactive *POMC* level in S may be because of that some portion of *POMC* underwent the proteolytic cleavages to be secreted from RSP. However, samples of cells transfected with Ser9Leu *POMC* showed similar results as cells transfected with WT *POMC*. Statistical analysis of all samples showed that the reduction from B4 to S was found statistically significant for both cells transfected with WT and Ser9Leu *POMC*.

Moreover, our antibody could recognize only 35 kDa *POMC*. Due to lack of appropriate antibody which distinguishes the smaller products of *POMC*, these smaller peptides could not be displayed in this study as they were detected in the other studies (Figure 9) (Creemers et al., 2008; Kim et al., 2018; Y. Peng Loh et al., 2004). Thus, we could not analyze the changes in amount of smaller peptides released from RSP between the cells transfected with WT and Ser9Leu *POMC*, thereby; the effect of mutation on sorting and processing of *POMC* remains unsolved. Nevertheless, Ser9Leu mutation may not be resulted in a major problem on the translocation of *POMC* as well as the protein synthesis.

We aimed to investigate the cellular mechanisms of Ser9Leu *POMC* mutation, so that may be a reason for the obesity state of the child with this mutation. Our results were not sufficient to reveal the cellular mechanisms of Ser9Leu *POMC* which leads to obesity because of lack of the appropriate antibody against to *POMC*. Therefore, further studies are needed to analyze Ser9Leu *POMC* secretion pathways using appropriate antibodies against *POMC* that show all cleaved forms of *POMC* products.

CHAPTER 5

CONCLUSION

It is well known that hypothalamic POMC neurons have critical roles in maintaining energy balance. Many mutations in *POMC* of obese patients were associated with obesity. Ser9Leu POMC mutation which was identified in an Italian child having early-onset obesity was considered to be a reason of his obesity since other reasons such as leptin levels could not found different in this patient. In this project, the cellular mechanisms of Ser9Leu POMC mutation was investigated in the neuronal cells (N2a) to understand the changes of POMC secretion which may explain the obese state in patients with this mutation.

It was hypothesized that the impaired translocation of Ser9Leu POMC which could lead to decrease in the secretion of active peptides, and ultimately the association with early-onset obesity. However, our results showed that the mutation may not cause the problem on the translocation of POMC, as well as the synthesis of the protein. Nevertheless, the lack of appropriate POMC antibody which displays all POMC products limits analysis of mutant POMC peptides secretion from N2a cells. Therefore, same experiments could be carried out with another antibody that recognize all peptides of POMC in order to analyze the secretory pathway. Also, the visualization of WT and MT POMC within the cells using immunocytochemistry (ICC) could show the localization of POMC and support the secretion assay for POMC. Thus, the effect of mutation could be analyzed better.

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APPENDICES

A. SEQUENCES OF bPOMC AND hPOMC PLASMIDS

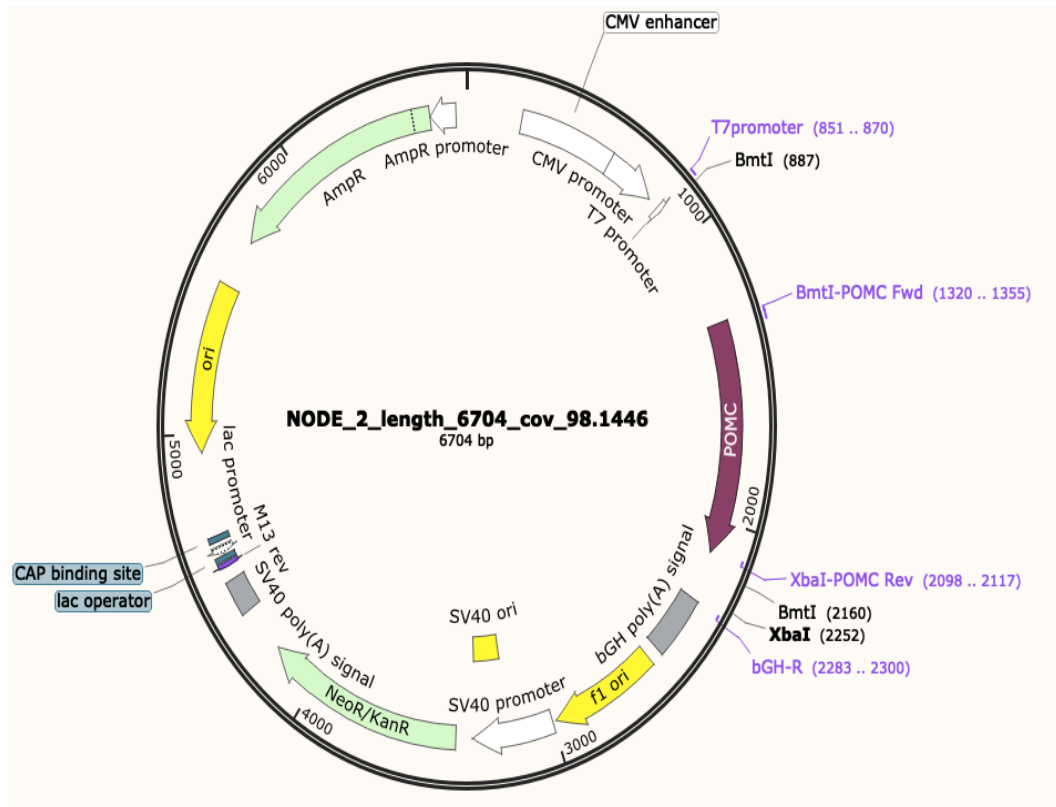
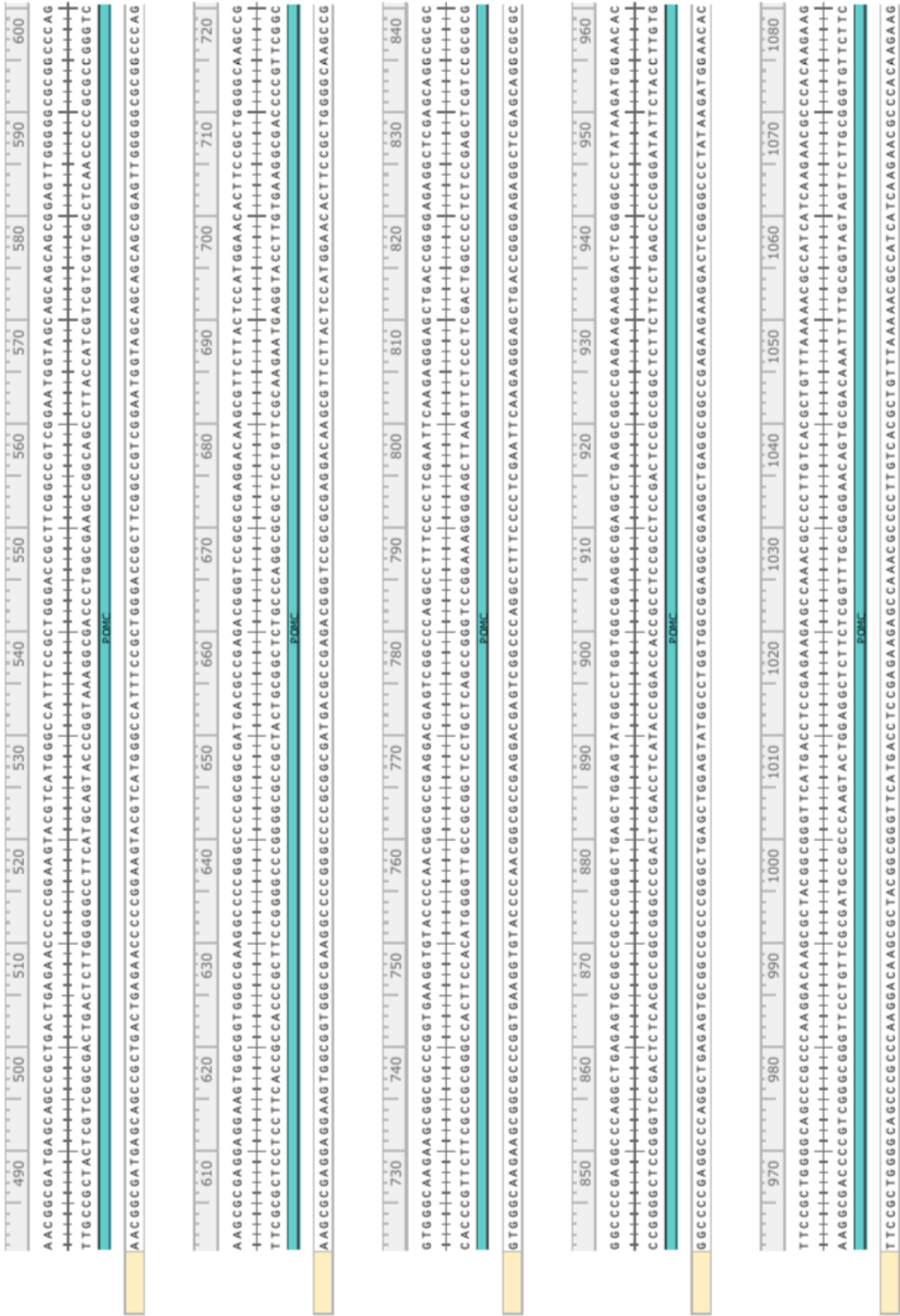


Figure A.1. Circular plasmid map of pcDNA3.1 containing bPOMC. After the de novo analysis, all sequence of plasmid was defined and regions of plasmid with the restriction sites were seen in the plasmid map.

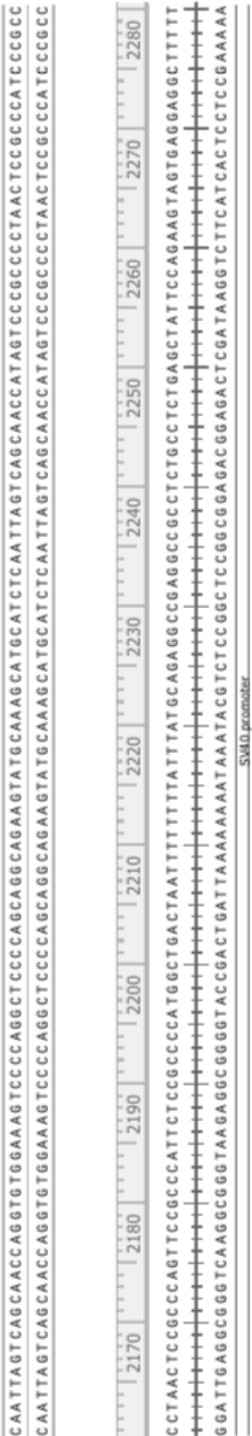
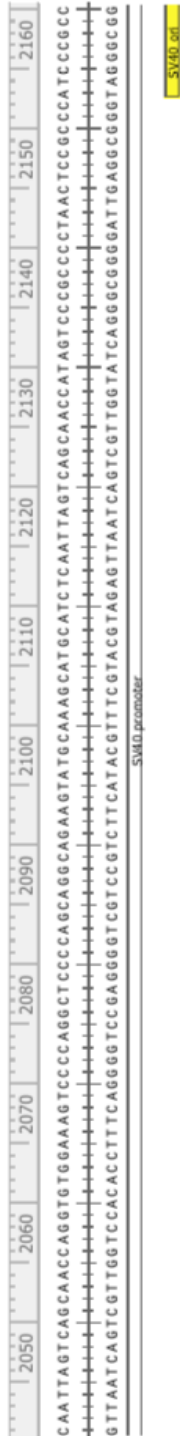




Genomic tracks showing the POME and NGH poly(A) signal across a region from 1090 to 1450. The POME track (red) shows a strong signal at approximately 1120-1130. The NGH poly(A) signal track (green) shows a strong signal at approximately 1360-1370. The tracks are labeled with their respective names and the NGH poly(A) signal is labeled with its name.

Position	POME	NGH poly(A) signal
1090	+	+
1100	+	+
1110	+	+
1120	+	+
1130	+	+
1140	+	+
1150	+	+
1160	+	+
1170	+	+
1180	+	+
1190	+	+
1200	+	+
1210	+	+
1220	+	+
1230	+	+
1240	+	+
1250	+	+
1260	+	+
1270	+	+
1280	+	+
1290	+	+
1300	+	+
1310	+	+
1320	+	+
1330	+	+
1340	+	+
1350	+	+
1360	+	+
1370	+	+
1380	+	+
1390	+	+
1400	+	+
1410	+	+
1420	+	+
1430	+	+
1440	+	+
1450	+	+

[illegible]



2410 2420 2430 2440 2450 2460 2470 2480 2490 2500 2510 2520
TCCGCCCGCTTGGGTGGAGAGCTATTGCGCTATGACTGGGCACAACAGACAAATCGGCTGCTGTGATGCCGCCCGTGTCCGGCTGTCAAGCGAGGGGCGCCCGGTTCTTTTGTCAAGAC
+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
AGGCCGGCGAACCCACTCTCGATAAGCCGATACTGACCCGTGTTGTCGTTAGCCGACGAGACTACGGCGGCACAAAGCCGACAGTCCGCTCCCGCGGGCCCAAGAAACAGTTCTG
Pro Ala Trp Val Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Thr Ile Gly Cys Ser Asp Ala Val Phe Arg Leu Ser Val Leu Phe Val Lys Thr
Nest/Kent

TCCGCCCGCTTGGGTGGAGAGCTATTGCGCTATGACTGGGCACAACAGACAAATCGGCTGCTGTGATGCCGCCCGTGTCCGGCTGTCAAGCGAGGGGCGCCCGGTTCTTTTGTCAAGAC
TCCGCCCGCTTGGGTGGAGAGCTATTGCGCTATGACTGGGCACAACAGACAAATCGGCTGCTGTGATGCCGCCCGTGTCCGGCTGTCAAGCGAGGGGCGCCCGGTTCTTTTGTCAAGAC

2530 2540 2550 2560 2570 2580 2590 2600 2610 2620 2630 2640
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+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
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Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Val Leu Asp Val Val Thr Glu Ala Gly Asp Trp
Nest/Kent

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CGACTGTCCGGTGCCTGAATGAAGTGCAGGACGAGCGCGGCTATCGTGGCTGACCAGCGGGGCTTCTTGGCGAGCTGTGCTCGACGTGTGCTCACTGAAGCGGGAAGGACTG

2650 2660 2670 2680 2690 2700 2710 2720 2730 2740 2750 2760
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+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
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Leu Leu Leu Gly Glu Val Pro Gly Gln Asp Leu Leu Ser His Leu Ala Pro Ala Glu Lys Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro Ala Thr Cys
Nest/Kent

GCTGCTATTGGCGAAGTCCCGGGCAGGATCTCCTGTATCTCACCTTGCTCTGCTGCTCCGAGAAAGTATCCATCATGCTGATGCAATCGCGGGCTGECATACGCTTGATCGGGTACCTG
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2770 2780 2790 2800 2810 2820 2830 2840 2850 2860 2870 2880
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+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
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Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Leu Asp Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe
Nest/Kent

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2890 2900 2910 2920 2930 2940 2950 2960 2970 2980 2990 3000
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Ala Arg Leu Lys Ala Arg Met Pro Asp Gly Glu Asp Leu Val Thr His Gly Asp Ala Cys Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ile Asp Cys Gly Arg
Neor/KanR

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3010 3020 3030 3040 3050 3060 3070 3080 3090 3100 3110 3120
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CGACCCACACCGCTGGCGATAGTCTGTATCGCAACCGATGGGCACTATAACGACTTCGGAACGCGCTTACCGACTGGCGAAGAGCAAGAAATGCCATAGCGCGGAGGCTAAG
Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala Thr Arg Asp Ile Ala Glu Leu Gly Gly Trp Ala Asp Arg Phe Leu Val Tyr Gly Ile Ala Ala Pro Asp Ser
Neor/KanR

GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTTGATTTGCTGAAGAGCTTGGCGGGAATGGGCTGACCGCTTCCTCGTGTTCACGGTATCGCGCTCCCGATTTC
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3130 3140 3150 3160 3170 3180 3190 3200 3210 3220 3230 3240
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Gln Asp Ile Ala Phe Tyr Arg Leu Leu Asp Glu Phe Phe
Neor/KanR

GCAGCGCATCGCCTTCATCGCCTTCCTGACGAGTTCTTCTGAGCGGGACTCTGGGTTGCGAAATGACCGACCAAGCGAGCGCCCAACCTGCCATCAGGAGTTTCGATTCCACGCGCGCC
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SV40 poly(A) signal

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TTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCGGGACGCGCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCGCACCCCAACTTGTTTATTGCAGCTTATAAT

3370	3380	3390	3400	3410	3420	3430	3440	3450	3460	3470	3480
------	------	------	------	------	------	------	------	------	------	------	------

SV40 poly(A) signal

3

55

— 100 —

lac operator

413 rev

▲

CAP binding site

3

[illegible]

॥

[illegible]

4

[illegible]

2

[illegible]

4

NODE_2_length_6704_cov_98.1446.dna (Circular / 6704 bp)

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Asp Ile Arg Ser Leu Val Ala Gly Cys Leu Leu Val Lys Phe Thr Ser Met Met Pro Phe Arg Glu Pro Arg Phe Ser Glu Leu Ile Lys Gly Ser Asp Leu Asp Glu Ile Tyr
AmpR

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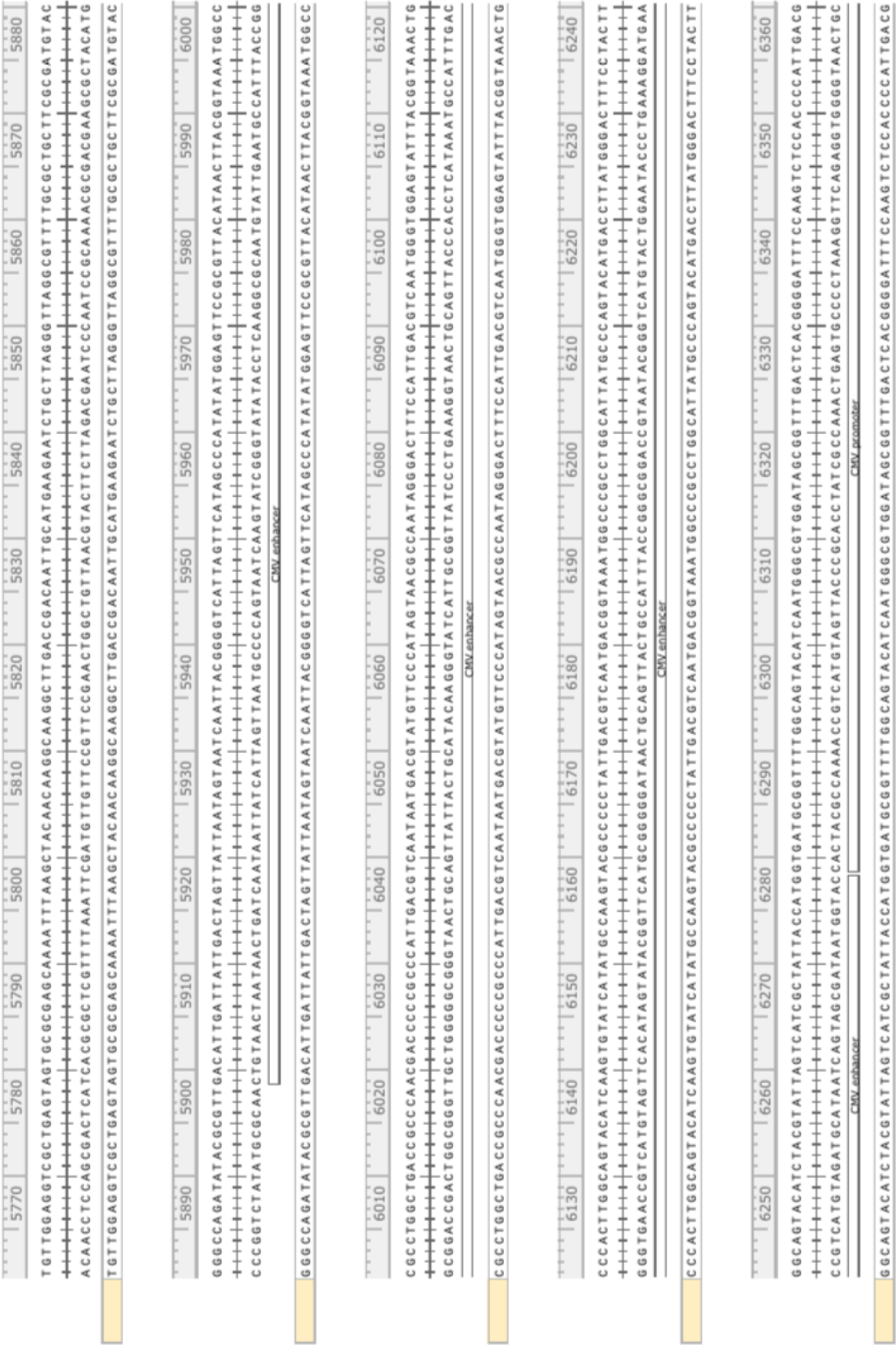
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Gly Val Arg Ala Leu Glu Asp Glu Ala Asp Lys Val Lys Val Leu Thr Glu Pro His Ala Phe Val Pro Leu Cys Phe Ala Phe Pro Ile Leu Ala Val Arg Phe His Gln
AmpR

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5530 5540 5550 5560 5570 5580 5590 5600 5610 5620 5630 5640
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U Ser Met
Signal sequence
AmpR

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5650 5660 5670 5680 5690 5700 5710 5720 5730 5740 5750 5760
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NODE_2_length_6704_cov_98.1446.dna (Circular / 6704 bp)

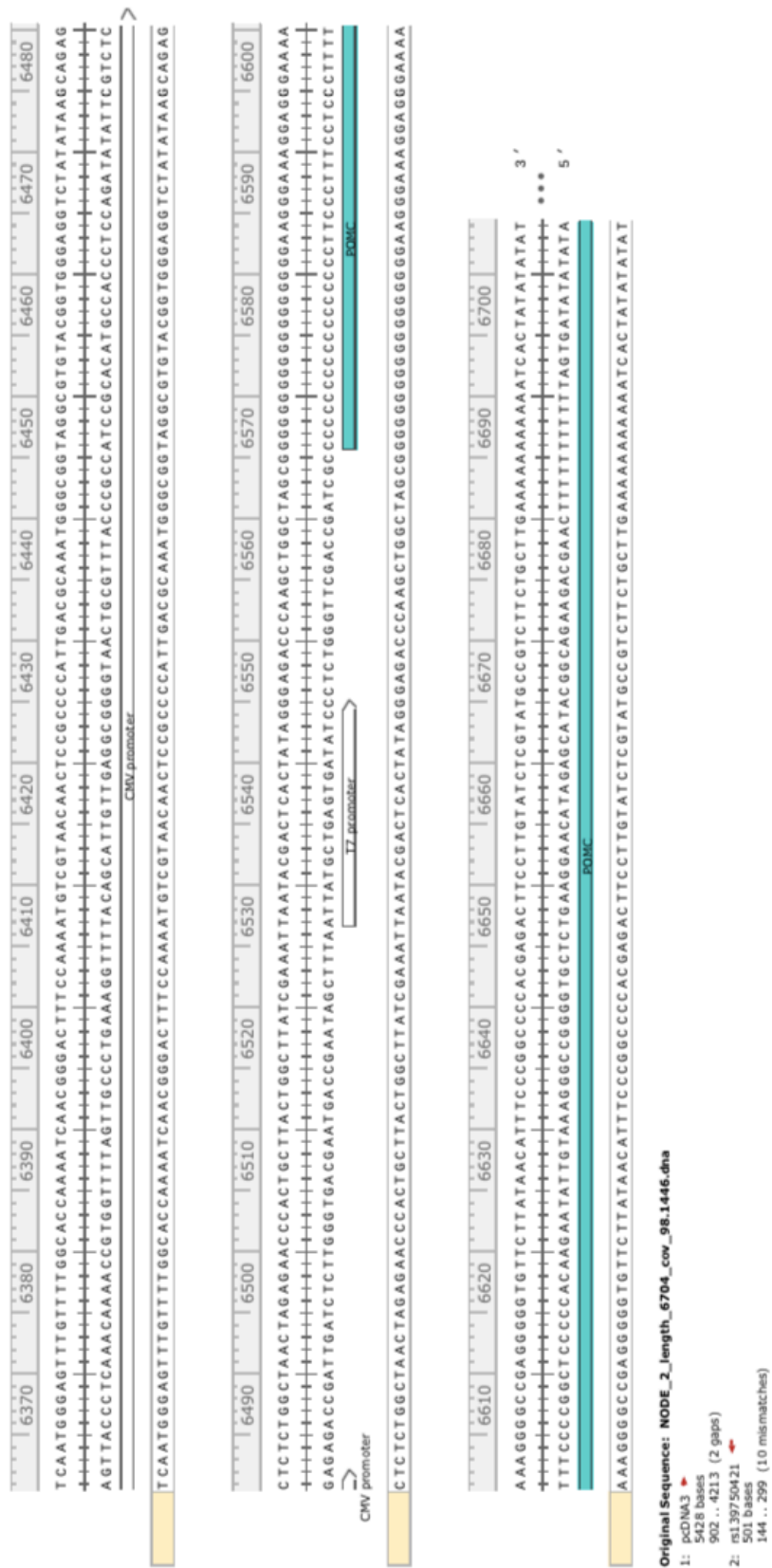


Figure A.2. De novo analysis of bPOMIC. All regions of gene and pcDNA3.1 + plasmid are shown.

B. PROCEDURES FOR SITE-DIRECTED MUTAGENESIS

The procedures for the generation of constructs which have a mutation with other two modification are given below.

1. First, the plasmid was cut with BmtI restriction enzyme in two BmtI sites (Figure A.2) in order to get empty plasmid (5431 bp). After removing of the insert which was the POMC region (1273 bp), linear empty pcDNA3.1 was ligated at the ends of BmtI restriction sites to get the circular plasmid. The reagents used in the restriction and ligation procedures were listed in Table B.1 with the amounts used in procedures.
2. After the ligation of empty plasmid, it was transformed to the bacteria to be amplified. Then, PCR (Polymerase Chain Reaction) was performed for several colonies in order to check whether the colony has any additional insert in the site of POMC or not. The 177 bp region between T7 promoter and bGH poly(A) signal of plasmids isolated from colonies were amplified with T7 promoter and bGH-R primers (Table B.3). The amplified region was analyzed with agarose gel electrophoresis to check if there is more than 177 bp in the plasmid. Colonies which did not have more than 177 bp were chosen for next processes. The procedure and reagents of colony PCR were provided in Table B.2.
3. To generate the mutation and modifications, POMC gene was amplified on the plasmid with PCR. Two primers were used, BmtI- POMC Forward and XbaI- POMC Reverse. These primers contain the changed nucleotides which were indicated as lowercase letters (Table B.3). The PCR reagents and procedure were listed in Table B.4. After the amplification of POMC insert, it was run on the agarose gel to check the presence of 822 bp POMC.

4. Then, PCR products of POMC and the empty vector were cleaned up to generate sticky ends for the ligation. The restricted POMC fragment was ligated into the corresponding restriction sites of the pcDNA3.1 vector using T4 DNA ligase. The reagents for clean-up and ligation of POMC and empty vector were provided in Table B.5 and Table B.6, respectively.
5. After the ligation, colony PCR was performed again for POMC insert with the same procedure, except that extension at 68°C was 1 minutes rather than 1.5 minutes. The products of this PCR were run on agarose gel to check if POMC could be inserted into the plasmid.

Table B.1. Reagents for restriction enzyme cut and ligation procedures

Restriction Enzyme Cut of Plasmid	Amount
200 ng/μL pcDNA3.1-POMC	5 μL
10 X Cut Smart Buffer	5 μL
20 μg/μL BmtI- HF	0.5 μL
DEPC water	39.5 μL
Total	50 μL
Proceed at 37°C for 3 hours	
Ligation of Empty Plasmid	Amount
pcDNA3.1 cut with BmtI	4.5 μL
5 X Ligation Buffer	4 μL
T4 DNA Ligase	0.5 μL
DEPC water	11 μL
Total	20 μL
Proceed at 22°C for 1 hour	

Table B.2. Reagents and procedure for colony PCR. Cycles=35

Colony PCR	Amount	X 8
10 X Standard Taq Buffer	1 μ L	8 μ L
10 mM dNTPs	0.2 μ L	1.6 μ L
10 μM T7 promoter primer	0.2 μ L	1.6 μ L
10 μM bGH-R primer	0.2 μ L	1.6 μ L
Taq DNA polymerase	0.05 μ L	0.4 μ L
DEPC water	8.35 μ L	66.8 μ L
Total	10 μ L	80 μ L
PCR Steps	Temperature	Duration
Initial Denaturation	95°C	30 seconds
Denaturation	95°C	30 seconds
Annealing	53°C	30 seconds
Extension	68°C	90 seconds
Final Extension	68°C	5 minutes
Incubation	10°C	Indefinitely

Table B.3. Primers used for site-directed mutagenesis

Primers	Sequence of primers
BmtI-POMC Fwd	5`-GCGGGCTAGCCACCATGCCGAGAtcGTGcTGCA GTCGTTyGGGCGCCCTG-3`
XbaI-POMC Rev	3`-GGGTGTTCTTCCCGGTCACTAGATCTGGCG-5`
T7 promoter	5`-TAATACGACTCACTATAGGG-3`
bGH-R	3`-GGAGCTGACACGGAAGAT-5`

Table B.4. PCR reagents and procedure. POMC region was amplified with the primers for generation of mutation and modifications

Reagents for PCR		Amount
5 X Q5 Buffer		10 μ L
10 mM dNTPs		1 μ L
10 μ M BmtI-POMC Fwd primer		2.5 μ L
10 μ M XbaI-POMC Rev primer		2.5 μ L
50 ng/ μ L pcDNA3.1-POMC		0.5 μ L
Q5 HF DNA polymerase		0.5 μ L
DEPC water		33 μ L
Total		50 μ L
PCR Steps	Temperature	Duration
Initial Denaturation	98°C	30 seconds
Denaturation	98°C	10 seconds
Extension	72°C	30 seconds
Final Extension	72°C	2 minutes
Incubation	10°C	Indefinitely
Cycle	35	

Table B.5. Reagents for PCR product clean-up. Each proceed at 37°C for 2 hours

Restriction Enzyme Cut for POMC	Amount
104.4 ng/μL POMC-Q5	9.6 μL
10 X Cut Smart Buffer	5 μL
20 μg/μL BmtI- HF	0.5 μL
20 μg/μL XbaI	0.5 μL
DEPC water	34.4 μL
Total	50 μL
Restriction Enzyme Cut for Empty Plasmid	Amount
125.6 ng/μL pcDNA3.1	8 μL
10 X Cut Smart Buffer	5 μL
20 μg/μL BmtI- HF	0.5 μL
20 μg/μL XbaI	0.5 μL
DEPC water	36 μL
Total	50 μL

Table B.6. Reagents for ligation of POMC and empty plasmid. Proceed at 22°C for 3hours

Reagents	Amount for a vial	Amount for a vial
25.2 ng/ μL pcDNA3.1 BmtI+ XbaI	2 μL	-
38.7 ng/ μL POMC-Q5 BmtI+ XbaI	2 μL	2 μL
5 X Ligation Buffer	4 μL	4 μL
T4 DNA Ligase	0.5 μL	0.5 μL
DEPC water	11.5 μL	13.5 μL
Total	20 μL	20 μL

C. SANGER SEQUENCING OF PLASMIDS

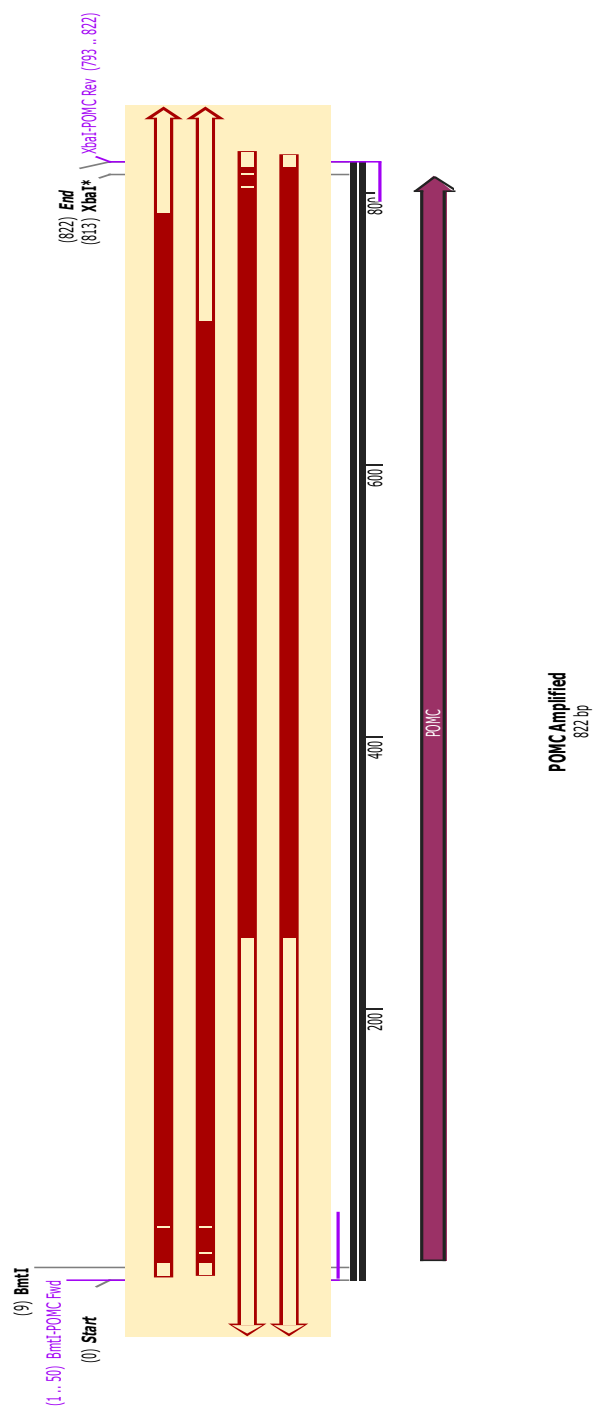
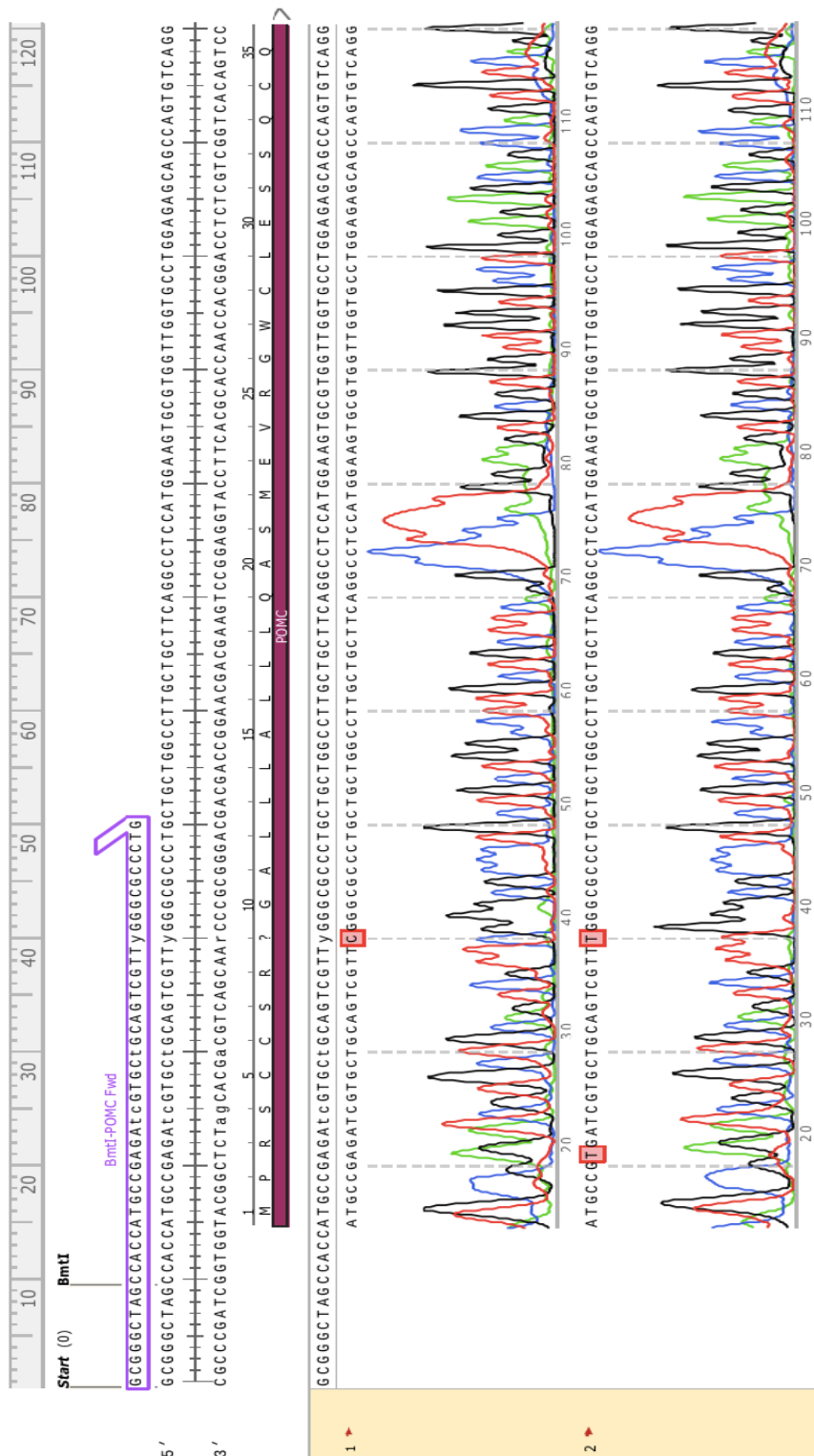
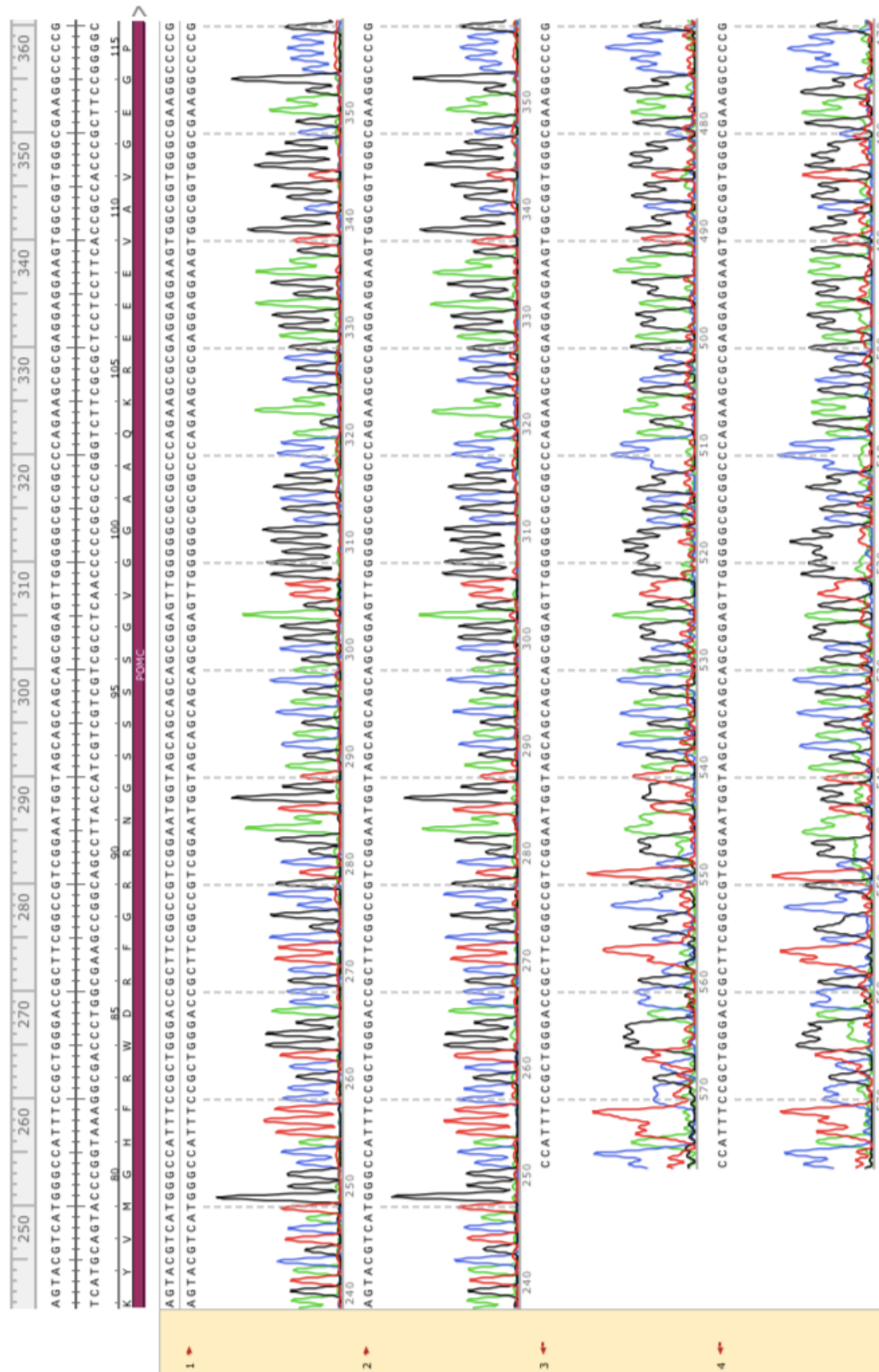
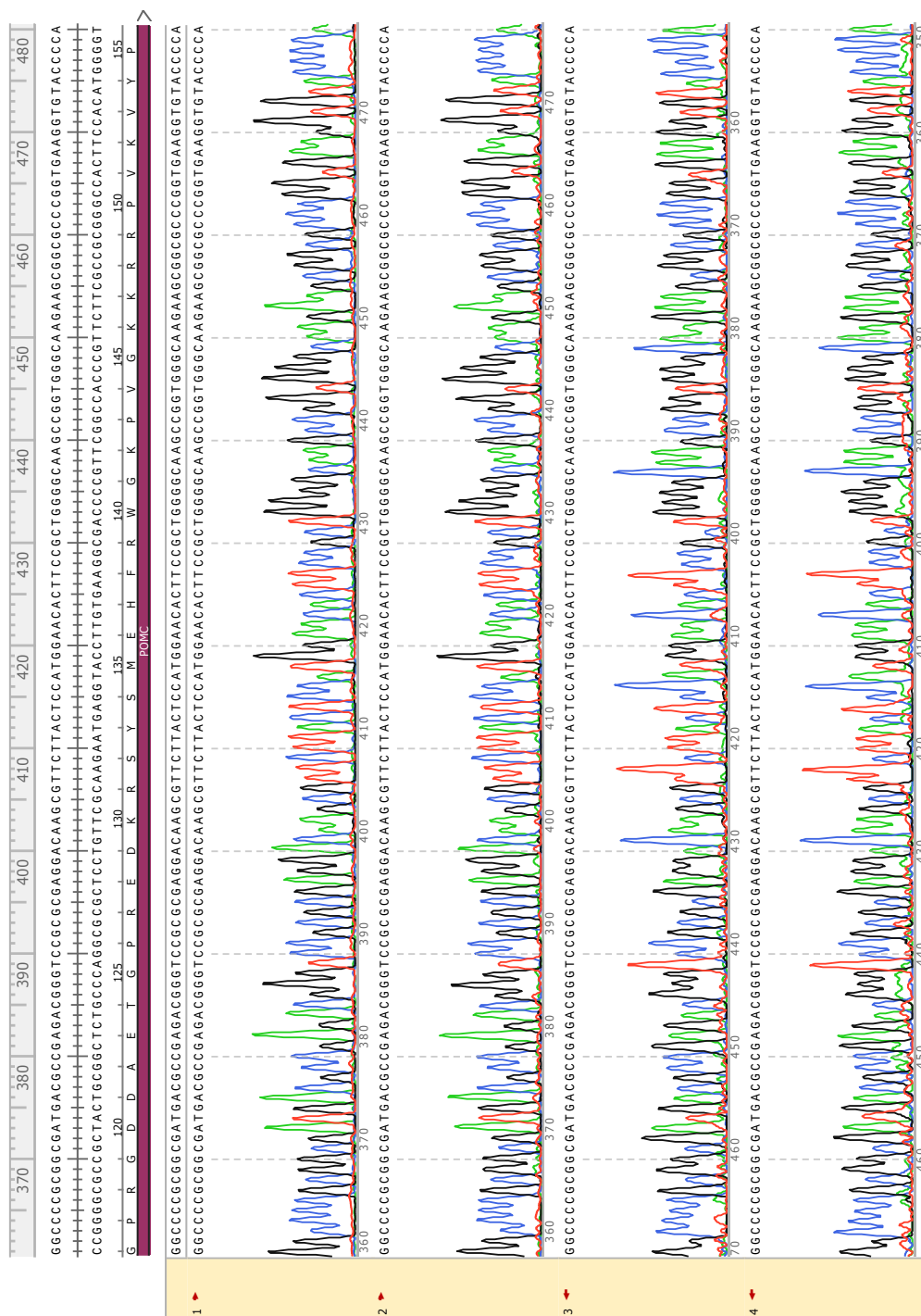
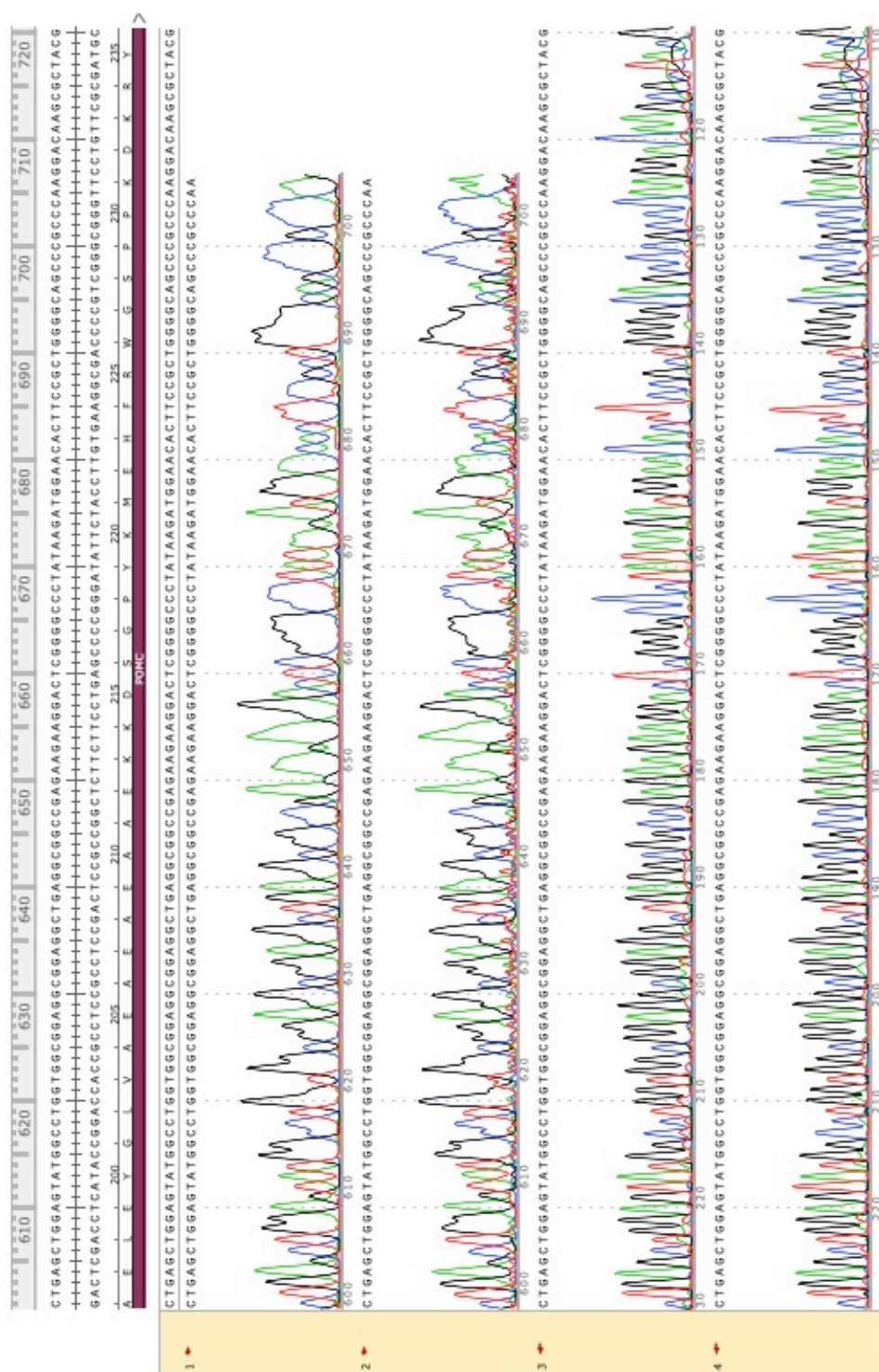


Figure C.1. The result of Sanger sequencing. Two primers from opposite strands were used to determine the sequence. These primers are T7 promoter and bGH.









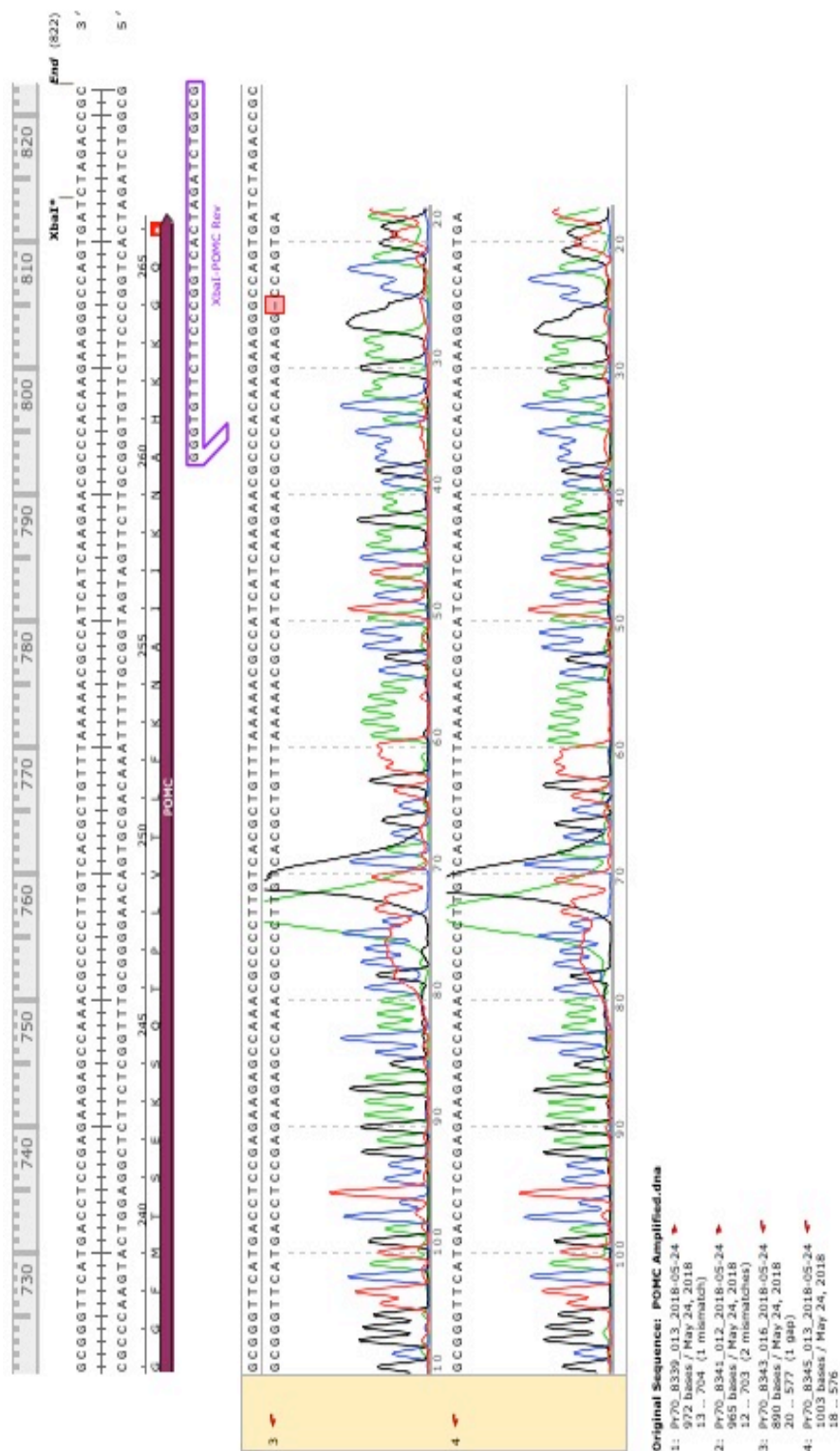


Figure C.2. Sanger Sequencing result of amplified POMC plasmids. The mutated nucleotide is highlighted as red; C in wild type, T in mutated plasmid.

D. SOLUTIONS AND BUFFER

Table D.1: The solutions and buffers used for plasmids and cells. Sterility was achieved by autoclaving solutions at 121°C for 20 minutes.

Luria-Bertani (LB) agar plates	3.5 g LB broth (Merck, Darmstadt, Germany) 5 g agar (Sigma Aldrich, Germany) 100 mL distilled water Autoclave Cool down and add 100 µg/mL Ampicillin
Luria-Bertani (LB) media	4 g LB broth 100 ml distilled water Autoclave Add 100 µg/mL Ampicillin prior to use
Ampicillin stock (AppliChem, Darmstadt, Germany)	50 mg/mL stocking concentration was prepared 100 µg/mL working concentration was used
50X stock TAE buffer	242 g Tris free base 18.61 g Disodium EDTA 57.1 mL Glacial Acetic Acid Deionized water to 1 liter
1X TAE buffer	20 ml 50 x TAE Final volume 1000 mL with distilled water

Table D.1. (Cont`d)

Complete growth medium	Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) with 10% Fetal bovine serum (FBS) (Biological Industries, USA), 1%Penicillin/streptomycin (Biological Industries, USA), 2mM L-Glutamine (Gibco, USA)
PBS (Phosphate buffered saline) (Sigma Aldrich, Saint Louis, USA)	Each PBS tablet was dissolved with 100 mL of distilled water.
Transfection medium	DMEM with 2mM L-glutamine and 10% FBS, without antibiotic
Basal medium	DMEM with 2mM L-Glutamine and 0.01% BSA, without antibiotic and FBS
Stimulation medium	DMEM with 2mM L-Glutamine, 0.01% BSA, 50mM KCl (Merck, Darmstadt, Germany) and 79mM NaCl (Merck, Darmstadt, Germany), without antibiotic and FBS
7X EDTA-free protease inhibitor (Roche, Germany)	One tablet of protease inhibitor was dissolved with 1.5 mL distilled water
100% TCA (Serva, Heidelberg, Germany)	100 g of TCA was dissolved with 100 mL of distilled water

E. PLASMID ISOLATION PROTOCOL

1. Buffers were prepared as it is stated in the kit's manual.
 - RNase A solution was added to the Resuspension Solution and mixed.
 - Ethanol (96-100%) was added to the Wash Solution prior to first use as provided in Table E.1.

Table E.1. Required ethanol to add to wash solution

Solution	Amount
Wash Solution (concentrated)	20 mL
Ethanol 96%	35 mL
Total volume	55 mL

2. After single colony was inoculated in 5 mL LB medium overnight, bacterial culture was harvested by centrifugation at 8000 rpm for 2 minutes at RT.
3. Then all supernatant was discarded, and remaining pellet was resuspended in 250 μ L of the Resuspension Solution by pipetting up and down. The cell suspension was transferred to a microcentrifuge tube.
4. 250 μ L of the Lysis Solution was added to cell suspension, and tube was mixed by inverting the tube 4-6 times until the solution became viscous and clear.

5. 350 μ L of the Neutralization Solution was added to it and mixed immediately and thoroughly by inverting the tube 4-6 times.
6. Then tube was centrifuged for 5 minutes to pellet cell debris and chromosomal DNA.
7. The supernatant was transferred to the supplied GeneJET spin column by pipetting.
8. Transferred supernatant was centrifuged for 1 min. The flow-through was discarded and placed the column back into the same collection tube.
9. 500 μ L of the Wash Solution was added to the GeneJET spin column. Column was centrifuged for 60 seconds and the flow-through was discarded. The column was placed back into the same collection tube
10. Wash procedure (step 9) was repeated using 500 μ L of the Wash Solution.
11. Then the flow-through was discarded. Column was centrifuged again for an additional 1 minutes to remove residual wash solution.
12. The GeneJET spin column was transferred into a fresh 1.5 mL microcentrifuge tube. 50 μ L of the Elution Buffer was added to the center of the column membrane to elute the plasmid DNA. It was incubated for 2 min at room temperature and centrifuged for 2 min.
13. Column was discarded, and purified plasmid DNA was stored at -20°C.

F. MICROPLATE BCA ASSAY PROTOCOL

1. Diluted albumin (BSA) standards were prepared with some modifications according to kit's protocol given in Table F.1.

Table F.1. Dilution scheme of protein standards

Vial	Volume of Diluent (μL)	Volume and Source of BSA (μL)	Final BSA Concentration (mg/mL)
A	0	300 (Stock)	2.0
B	62.5	187.5 (Stock)	1.5
C	100	100 (Stock)	1.0
D	100	100 (vial B dilution)	0.75
E	100	100 (vial C dilution)	0.50
F	100	100 (vial E dilution)	0.25
G	100	100 (vial F dilution)	0.125
I	200	0	0

2. 200 μL of WR is required for each sample in the microplate procedure. BCA working reagent (WR) was prepared by using following formula;

$$(\# \text{ standards} + \# \text{ unknowns}) \times (\# \text{ replicates}) \times (\text{volume of WR per sample}) = \text{total volume WR required}$$

For example, one 6-well plate with 6 unknowns and 3 replicates of each sample: $(8 \text{ standards} + 6 \text{ unknowns}) \times (3 \text{ replicates}) \times (0.2 \text{ mL}) = 8.4 \text{ mL WR required}$.

So, 10 mL of WR was prepared to ensure enough WR is available for the assay.

3. Prepared WR was mixed with 50 parts Rapid Gold BCA Reagent A with 1-part Rapid Gold BCA Reagent B (50:1, Reagent A: B).
4. Microplate procedure of manual was performed as sample to WR ratio 1:10.

5. 20 μL of each standard and unknown sample replicate was added into each microplate well.
6. 200 μL of WR was added to each well and plate was mixed thoroughly on a shaker for 30 seconds.
7. Then plate was incubated at room temperature for 5 minutes.
8. Absorbance at 480 nm was measured on a plate reader.
9. A standard curve was prepared by plotting the average blank-corrected 480 nm measurement for each BSA standard versus its concentration in $\mu\text{g/mL}$. The standard curve was used to determine the protein concentration of each unknown sample.

G. WESTERN BLOT PROTOCOL

1. 12% separating and 4% stacking gels with 1 mm thickness were prepared as following recipe provided in Table G.1.

Table G.1. Recipes of SDS PAGE 12% separating and 4% stacking gels⁴

12% Separating Gel	Amount for 2 Gels	4% Stacking Gel	Amount for 2 Gels
Distilled Water	5.3 mL	Distilled Water	3 mL
30% Acrylamide: Bisacrylamide	6.4 mL	30% Acrylamide: Bisacrylamide	670 µL
1.5 M Tris Buffer, pH 8.8	4 mL	0.5 M Tris Buffer, pH 6.8	1.25 mL
10% SDS	160 µL	10% SDS	50 µL
10% APS	160 µL	10% APS	50 µL
TEMED (Amesco, Ohio, USA)	16 µL	TEMED	5 µL

2. Sample preparation for loading into the gel;
 - 5 µg of cell lysates mixed with 2X Sample Buffer.
 - Protein pellets from TCA precipitation were resuspended in 72 µL of 1X Sample Buffer. 20 µL of sample was loaded into the gel.
3. After the preparation of blot apparatus with the gels, 1X running buffer was poured into the electrophorator.
4. Pre-stained protein marker (3µL) and protein samples were loaded into the gels, and samples were run at 120V for around 90 minutes until the dye front runs off the bottom of the gel.

⁴ Calculated in <https://www.cytographica.com/lab/acryl2.html>

5. Before the transfer process, 2 polyvinylidene fluoride (PDVF) membranes and 4 filter sheets were cut at the dimensions of 8x6 cm.
6. PVDF membranes (Thermo Scientific, Rockford, USA) were immersed in methanol for 60 seconds, then in distilled water for 2 minutes and in 1X transfer buffer for 15 minutes. Meanwhile, sponge and filter papers were immersed in 1X transfer buffer until used.
7. After running, gels were put into 1X transfer buffer for 15 minutes.
8. Transfer sandwich was prepared as follows;
 - The black sponge was put on the black side of container.
 - One filter paper was put on the black sponge.
 - Gel was put on the filter paper.
 - PVDF membrane was put on the gel and all air bubbles were removed by roller.
 - Another filter paper was out on the membrane.
 - Lastly, other black sponge was put on the filter paper and sandwich was locked.
9. This sandwich was put into the transfer apparatus with an ice pocket and transfer was performed at the constant 100 mA for 2 hours.
10. After transferring, membranes were blocked with 3% BSA-0.1% TBS-T solution for 1 hour at room temperature.
11. Membrane were incubated in a solution of primary antibody prepared in 3% BSA - 0.1% TBS-T with appropriate concentrations for overnight at 4°C.
12. The next day, membranes were washed 3 times with TBS-T each for 15 minutes, then incubated with secondary antibody for 1 hour at room temperature.
13. Lastly, membranes were washed 2 times with TBS-T and once with TBS each for 15 minutes.
14. Then, membranes were covered with ECL substrate solutions prepared as 1:1 ratio in the dark room for 1 minute and visualized using UV transilluminator for 200 seconds for the picture.

Table G.2. Buffers and solutions used in Western blot.

1.5 M Tris Buffer	181.7 g Tris is added, ~800 mL distilled water. pH is adjusted to 8.8 with HCl. Then, add distilled water up to 1L.
0.5 M Tris Buffer	60.57 g Tris is added, ~800 mL distilled water. pH is adjusted to 6.8 with HCl. Then, add distilled water up to 1L.
30% / 8% (W/V) Acrylamide / Bisacrylamide (100mL)	30 g of Acrylamide (Invitrogen, Carlsbad, USA) 0.8 g of Bisacrylamide (Amesco, Ohio, USA) Add distilled water up to 100 mL
10% SDS (100mL)	10 g SDS (Sigma Aldrich, Japan) Add distilled water up to 100 mL
10% APS solution (10mL)	1 g APS (Bio-Rad, Japan) Add distilled water up to 10 mL
10 X Transfer Buffer (1L)	30 g Tris 144 g Glycine (Sigma Aldrich, China) Add distilled water up to 1 L <ul style="list-style-type: none"> • Diluted 1X before use and add 200 mL methanol

Table G.2. (Cont'd)

5 X Running Buffer (1L)	15 g Tris 72 g Glycine 5 g SDS Add distilled water up to 1 L Adjust pH 8.3 with HCl <ul style="list-style-type: none"> • Diluted 1X before use
10 X TBS (1L)	80 g NaCl 24.2 g Tris Add distilled water up to 1 L Adjust pH 7.6 with HCl <ul style="list-style-type: none"> • Diluted 1X before use
0.1% TBS-T (Tween 20) (1L)	100 mL of 10X TBS 900 mL distilled water 1 mL Tween 20 (BioShop, Burlington, Canada)
SDS Sample Buffer (2X)	Tris base 0.379 g (Sigma Aldrich, St. Louis, USA) Glycerol 5g (BioShop, Burlington, Canada) β -ME 2.5 mL (Bio-Rad, Japan) SDS 1.15 g pH to 6.8 with HCl to 50 mL with H ₂ O Add 0.05g bromophenol blue
Primary Antibody Solution	1:2000 ACTH antibody 3%BSA in 0.1% TBS-T
Secondary Antibody Solution	1:10000 Anti-Mouse 3%BSA in 0.1% TBS-T

H. CONCENTRATIONS OF PLASMIDS

Concentrations of plasmids were measured by NanoDrop 2000 and provided in Table H.1 and H.2.

Table H.1. Concentrations of generated plasmids

Sample	Concentration (ng/ μ L)
WT POMC SNP: C	131.1 ng/ μ L
MT POMC SNP: Y	93.3 ng/ μ L

Table H.2. POMC DNA's concentration measured by NanoDrop2000

Sample	Concentration (μ g/mL)	A260/280	A260/230
bPOMC DNA	380.0	1.89	2.03
WT POMC DNA	240.0	1.93	2.17
MT POMC DNA	310.0	1.97	2.10
EMPTY VECTOR (EV)	260.0	1.96	2.24
pUC19	60.0	1.79	2.12

I. WESTERN BLOT RESULTS

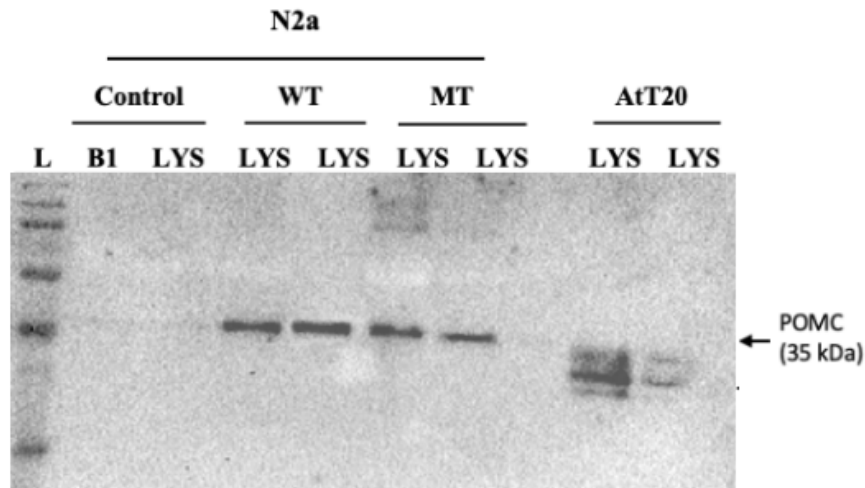


Figure I.1. Image of Western blot for N2a and AtT20 cells lysates. There are two different samples for each WT, MT and AtT20 samples. Each protein lysate sample was loaded as 5 μ g. LYS; cell lysates, L: protein ladder.

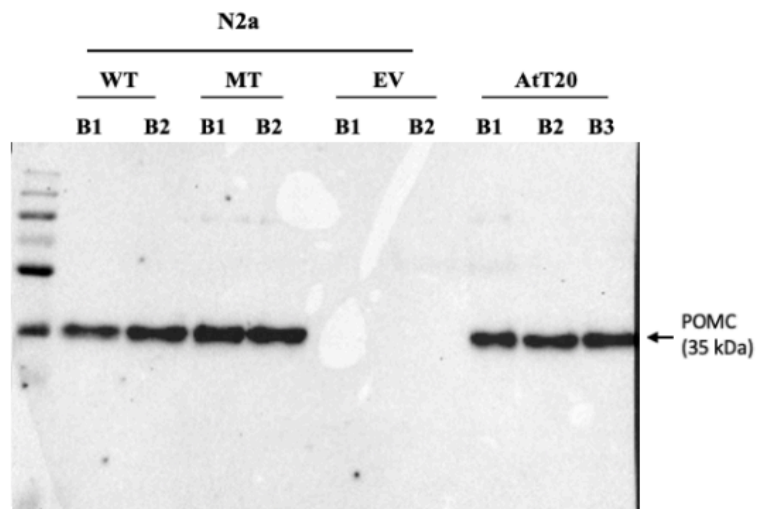


Figure I.2. Western blot analysis for the secretion assay in N2a and AtT20 cells. POMC secretion was showed in B1 and B2 samples of N2a cells transfected with WT and MT plasmids, and B1, B2 and B3 samples of AtT20 cells. B: basal secretion, EV: empty vector.