BIOCHEMICAL CHARACTERIZATION OF PROOPIOMELANOCORTIN VARIANTS IN HUMAN AND OWLS

Travail de Maîtrise universitaire en médecine

Master thesis in medicine

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I. ABBREVIATIONS

α-MSH, β-MSH, γ-MSH: α-, β- and γ-melanocyte-stimulating hormones

ACTH: Adrenocorticotrophin

AGRP: Agouti-related protein

BSA: Bovine Serum Albumin

CLIP: Corticotrophin-like intermediate peptide

CRH: corticotropin-releasing hormone

DMSO: Dimethyl sulfoxide

HRP: Horseradish peroxidase

Hu: Human

IgG: Immunoglobulin G

LHA: Lateral Hypothalamic Area

LPH: β-lipotropin

MC1,2,3,4,5R : Melanocortin receptor 1,2,3,4,5

NPY: Neuropeptide Y

OPD: O-phenylenediamine dichloride

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate Buffered Saline

PBST: Phosphate Buffered Saline with Tween

PC: Proprotein Convertases

POMC: Proopiomelanocortin

PVN: Paraventricular nucleus

PYY : Peptide YY

SA: Strix *aluco*

SDS: Sodium Dodecyl Sulfate

TA: Tyto *alba*

WF: Wessel and Fluegge

II. INTRODUCTION

A. POMC STRUCTURE AND SYNTHESIS

In humans the proopiomelanocortin (POMC) gene is located on the short arm of the chromosome 2 at the 23.3 position and encodes a 39kDa peptide with 241 amino acids. This peptide is the precursor of the proopiomelanocortin prohormone and it is produced in both the anterior and posterior lobes of the pituitary, as well as the arcuate nucleus of the hypothalamus.

Maturation of POMC involves glycosylation, acetylation, and selective, sequential and tissue specific processing events mediated by proprotein convertases PC-1/3 and PC-2. Proteolytic cleavage of POMC at 9 different locations produces a variety of hormones, including adrenocorticotrophin (ACTH), α-, β- and γ-melanocyte-stimulating hormones (α-MSH, β-MSH, γ-MSH), corticotrophin-like intermediate peptide (CLIP), β-lipotropin (β-LPH), and β-endorphin (Fig. 1.).

B. PROPROTEIN CONVERTASES AND POMC

The basic proprotein convertases are a family of seven secretory serine proteases - PC-1/3, PC-2, furin, PC-4, PACE4, PC-5/6, PC-7 - that cleave precursor proteins at highly conserved basic motifs. Unlike the other members of basic PCs, PC-1/3 and PC-2 are active at very acidic pH, typically reached in the secretory granules [1].

PC-1/3 and PC-2 are crucial for POMC maturation that gets processed by these enzymes at 9 different sites (Fig.1). The cleavage at a given site is preferentially carried out by either PC-1/3 or PC-2. In turn, the differential processing of POMC depends on the convertase tissue distribution: PC-1/3 is mostly expressed in the anterior pituitary in order to produce mostly corticotropic hormones (ACTH, β-LPH) while PC-2 is mainly expressed in the intermediate pituitary involved in melanotropic hormones (MSHs) secretion.

Apart from the anterior lobe and the intermediate lobe of the pituitary, PC-1/3 and PC-2 are distributed in several other tissues, including endocrine organs involved in food intake and energy homeostasis: PC-2 is present in pancreatic α-cells and is involved in the conversion of proglucagon to glucagon. PC-1/3 is present in the intestinal L-cells and converts proglucagon to glucagon-like peptides [2,3].

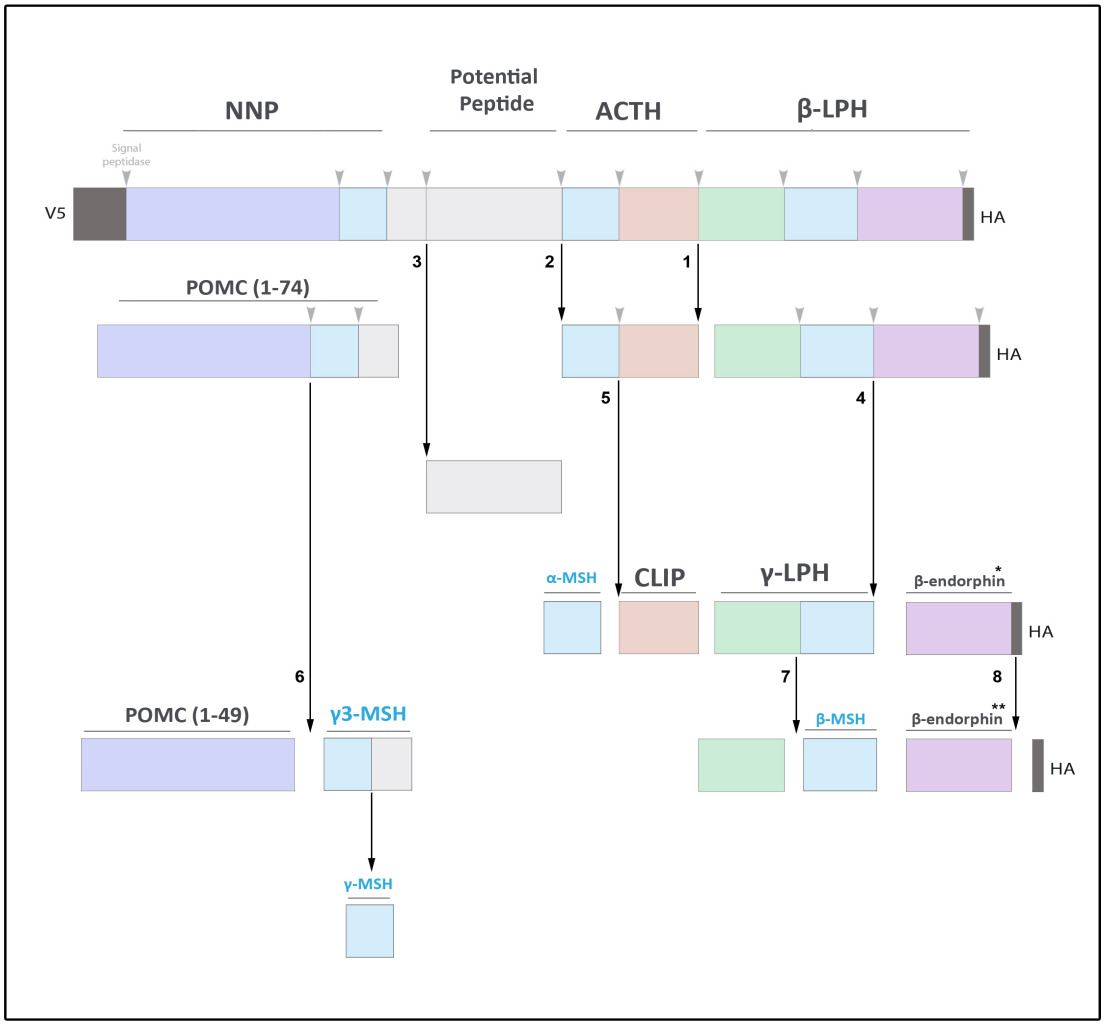

Figure 1. Sequential proteolytic cleavage of the POMC precursor. *The first step involves the cleavage of the signal peptide on the N-terminal end of the precursor. Steps 1,2 and 3 are mediated by PC-1/3 only, and happen early in the proteolytic cleavage. Steps 4 and 5 are mediated by PC-1/3 and strongly enhanced by PC-2. Steps 6 and 7 are mediated by PC-2 and happen later in the processing of POMC.* [4]

C. Implications in human diseases

The melanocortin system, and therefore POMC and all its cleavage products, is involved in a great number of body functions such as pigmentation, steroïdogenesis, energy homeostasis, food intake, analgesia, skin pigmentation, sexual function, inflammation, immunomodulation, temperature control, cardiovascular regulation and neuromuscular regeneration [5].

The role of POMC in food intake is well described: POMC neurons present in the arcuate nucleus of the hypothalamus are stimulated directly by leptin, serotonin and insulin and indirectly by PYY which inhibits

the Agouti-related protein/Neuropeptide Y (AGRP/NPY) neurons which have the opposite effect from POMC on food intake. The activation of these POMC neurons creates an anorexigenic stimulus that reduces food intake through the action of α-MSH on melanocortin receptor 4 (MC4R) of corticotropin-releasing and melanin-concentrating hormones of the PVN [6, 7]. In parallel, corticotropin-releasing hormone (CRH) produced in the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA) in response to stress can also increase the production of POMC through direct synaptic contact within the hypothalamus.

Most -if not all- of POMC functions are the consequence of the binding of these hormones and pro-hormones to the melanocortin receptors, and therefore the function depends on hormones relative affinities to them (Tab. 1).

Receptor	Effect on	Agonists	Primary function
MC1R	Immune system, skin, and neuronal cells	α-MSH >= ACTH > β-MSH > γ-MSH	Pigmentation
MC2R	Adrenal glands, skin, adipose tissue and bone cells	ACTH	Glucocorticoid biosynthesis
MC3R	Immune system, GI tract, heart, kidneys, placenta and CNS	γ-MSH > β-MSH >ACTH> α-MSH	Energy homeostasis
MC4R	CNS, heart, lung, kidneys, testis	β-MSH > α-MSH >ACTH> γ-MSH	Energy homeostasis
MC5R	Skin, adipose tissue, GI tract, spleen, sexual organs, bone marrow	α-MSH > β-MSH >ACTH> γ-MSH	Exocrine gland regulation

Table 1. Summary of melanocortin receptors functions and their affinity to the different possible agonists [8,9,10]

Correct expression and proteolytic processing of POMC by PC-1/3 and PC-2 are crucial for normal metabolic function and pigmentation. In human, recessive mutations of POMC and PC-1/3, and dominant mutation of Melanocortin-4 Receptor have been documented and associated with obesity [7]. POMC deficiency is characterized by severe obesity from the first month of life, associated with congenital adrenal insufficiency, pale skin as well as red hair, hypocortisolism and low α-MSH.

Lack of functional PC-1/3 also due to a recessive genetic disorder leads to obesity from the first month of life associated with gonadotropic and corticotropic insufficiency as well as hyperproinsulinaemia. In humans, the receptor MC1R is highly polymorphic with specific genetic variants associated with hair and skin pigmentation phenotypes [10].

III. AIM OF THE PROJECT

The aim of the project is to biochemically characterize POMC variants of barn owl and humans. Genotyping analysis of wild barn owls revealed the expression of a POMC variant characterized by the insertion of poly-serine (Ser) sequences of different length. Notably, the poly-Ser inserts found in barn owls resemble the Ser-Ser-Gly insertions found in human POMC associated with an elevated body mass index [11]. How exactly the polymorphism is related to the disease in human is not known.
In order to assess owl POMC cleavage into the melanocortin hormones, the PC-1/3 and PC-2 orthologues have been used. This study will allow first insights into the molecular mechanisms of differential processing and glycosylation of owl/human POMC variants. Considering the structural and functional conservation of POMC among vertebrates, we expect that findings obtained from these studies can be translated into a better understanding of the role of these factors in human physiology and disease.

Part 1: Production and purification of the different POMCs and PCs

In the first part of the project, we produced and purified POMC of Tyto *alba* (TA-POMC) containing 18Ser, Strix *aluco* (SA-POMC) containing 3Ser, and human (Hu-POMC), as well Tyto *alba* PC-1/3 and PC-2.

Protein production: Proteins were produced in mammalian HEK293 cells at the EPFL platform directed by Dr. Hacker. Plasmids of Hu-POMC, SA-POMC, and TA-POMC were tagged with HA and V5 epitope whereas PC-1/3 and PC-2 were tagged with V5 epitope. Plasmids encoding each protein were expanded to final 1.5mg which is the quantity required for 0.5L production of conditioned media by cell suspension. Identity of each plasmid has been confirmed by sequencing prior to providing them to the EPFL platform.

Protein purification: 18 Serine TA-POMC, SA-POMC, and Hu-POMC were HA tagged at the N-terminus and V5 tagged at the C-terminus. Protein purification was attempted using anti-HA and anti-V5 matrix

columns. Briefly, equilibrated matrix was loaded with 0.5L of conditioned supernatant. HA/V5-tagged POMC protein retained in the column was eluted using HA/V5 peptide solution. Aliquots of eluates and flowthrough were analyzed by coomassie gel to check for the efficiency of purification.

Part 2: PC-1/3 and PC-2 *in vitro* analyses and competition assay

In vitro analyses of non purified Tyto alba PC-1/3 and PC-2: Optimal *in vitro* activity of TA-PC-1/3 and TA-PC-2 was assessed using as substrate the synthetic fluorogenic peptide Pyr-RTKR-MCA in buffer solutions of different pHs. The proteases cleave the peptide releasing the MCA moiety, which becomes fluorescent, allowing the monitoring of the reaction of cleavage. Mouse PC-1/3 and PC-2 provided by our collaborators were used as controls.

Competition assay: To determine the affinity of each POMC protein for the cognate enzyme, TA-POMC, SA-POMC and Hu-POMC were pre-incubated with each proprotein convertase PC-1/3 and PC-2, prior to addition of the fluorogenic substrate. POMC-fluorogenic substrate competition was monitored *in vitro* as release of fluorescence/time.

Part 3: Testing of the custom anti-POMC antibodies

Non-purified POMC was used to assess the specificity of custom antibodies generated against TA-POMC and SA-POMC using the enzyme-linked immunosorbent assay (ELISA) and western blotting (WB). Briefly, supernatants from POMC over-expressing cells were immobilized on a 96 well plate. Primary antibodies (custom antibodies) were used in serial dilutions, followed by Horseradish peroxidase-linked (HRP) secondary antibodies. Upon addition of O-phenylenediamine dichloride (OPD), reagent detection was performed by measuring absorbance at 450nm. Western blot analysis was performed as described below.

Part 4: Purification of POMC using custom antibodies

Custom antibodies were selected according to their specificity against the different POMCs to purify SA-POMC with better efficiency than with the anti-HA and anti-V5 matrix columns. This second attempt of purification used magnetic Dynabeads coated with the custom antibody and compared to input samples through western blot analysis.

IV. Material and Methods

A. Library of antibodies against POMC

The library contains 27 different antibodies targeting ACTH, γ-MSH or β-MSH. Antibodies are of diverse origin (custom made or commercially available) and stored at -20C for > 2years. All antibodies have been thawed and aliquoted in 500µl aliquots (ca. 5ml stock; except commercially available antibodies) prior to testing.

Target Hormone	Target species	Antibody name	Antibody type	Species raised in	Peptide raised against
ACTH	Human	Santa Cruz SC-57018 (clone O2A3)	purified IgG1 from hybridoma	mouse	SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEF (hu ACTH 1-39)
		A-1A12 (Anne White)	purified IgG1 from hybridoma	mouse	SYSMEHFRWGKPVGKKRRPVKVYP (hu ACTH 1-24)
		A-2A3 (Anne White)	purified IgG1 from hybridoma	mouse	SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEF (hu ACTH 1-39)
	Tyto *alba* And Strix *aluco*	1-ACTH-1E3-4-1	hybridoma supernatant	mouse	GRKRRPIKVYPNGVC (PJ 126)
		1-ACTH-2G9-4-1	hybridoma supernatant	mouse	GRKRRPIKVYPNGVC (PJ 126)
β-MSH	Tyto *alba*	1-MSH-2B1-1-1	hybridoma supernatant	mouse	SHRVRPFRWHAPLKDC (PJ 127)
		1-MSH-3A1-1-1	hybridoma supernatant	mouse	SHRVRPFRWHAPLKDC (PJ 127)
		1-MSH-3B1-1-1	hybridoma supernatant	mouse	SHRVRPFRWHAPLKDC (PJ 127)
		1-MSH-3C12-1-1	hybridoma supernatant	mouse	SHRVRPFRWHAPLKDC (PJ 127)

		1-MSH-3F2-1-4	hybridoma supernatant	mouse	SHRVRPFRWHAPLKDC (PJ 127)
		1-MSH-3H10-6-4	hybridoma supernatant	mouse	SHRVRPFRWHAPLKDC (PJ 127)
		1-MSH-4B3-1-3	hybridoma supernatant	mouse	SHRVRPFRWHAPLKDC (PJ 127)
		1-MSH-4F12-4-6	hybridoma supernatant	mouse	SHRVRPFRWHAPLKDC (PJ 127)
	Strix *aluco*	2-MSH-3A1-1-1	hybridoma supernatant	mouse	SYRMRHFRWHAPLKDC (PJ 128)
		2-MSH-4E5-2-1	hybridoma supernatant	mouse	SYRMRHFRWHAPLKDC (PJ 128)
		2-MSH-4G11-1-1	hybridoma supernatant	mouse	SYRMRHFRWHAPLKDC (PJ 128)
		5-MSH-1G10-1-A7	hybridoma supernatant	mouse	SYRMRHFRWHAPLKDC (PJ 128)
		5-MSH-1G6-1-H1	hybridoma supernatant	mouse	SYRMRHFRWHAPLKDC (PJ 128)
γ-MSH	Tyto *alba* and Strix *aluco*	3-MSH-1B10-11-1	hybridoma supernatant	mouse	YVMSHFRWNKFGC (PJ 125)
		3-MSH-2H1-1-1	hybridoma supernatant	mouse	YVMSHFRWNKFGC (PJ 125)
		3-MSH-3G4-4-3	hybridoma supernatant	mouse	YVMSHFRWNKFGC (PJ 125)
		3-MSH-3B6-4-4	hybridoma supernatant	mouse	YVMSHFRWNKFGC (PJ 125)
		3-MSH-4D1-2-2	hybridoma supernatant	mouse	YVMSHFRWNKFGC (PJ 125)
		6-MSH-1M8-8-F12	hybridoma supernatant	mouse	YVMSHFRWNKFGC (PJ 125)

		6-MSH-1B12-C1-6	hybridoma supernatant	mouse	YVMSHFRWNKFGC (PJ 125)
		6-MSH-1B12-F8-5	hybridoma supernatant	mouse	YVMSHFRWNKFGC (PJ 125)

Table 2. Library of custom antibodies raised against different species and different peptides included in the POMC protein.

B. Wessel and Fluegge precipitation protocol

400µl of supernatant were mixed in eppendorf tubes with 400µl methanol and 100µl chloroform. The eppendorf tubes were vortexed for 1min at 12000rpm. Proteins were then located between two liquid phases and the upper phase is removed. Further 400µl of methanol were added, and the Eppendorf tubes were inverted 5 times and centrifuged 1min at 13000rpm. The upper liquid phase was removed and the pellet was left to dry for 5min. Finally, to resuspend protein content, 50µl of water and 10µl of 6x SDS were added in the tubes and heated at 95°C for 8min. The Eppendorf tubes were vortexed to better dissolve the proteins prior gel loading for WB analysis.

C. Standard western blotting and coomassie protocol

Samples were diluted in 2x SDS-PAGE (sodium dodecyl phosphate – polyacrylamide gel) containing 10% Dithiothreitol (DTT) and boiled 5min at 95° before loading. Proteins were separated in a SDS-PAGE gel composed of a stacking and a running part. In order to allow the proteins to reach the running gel, 80V current was applied. Current was then increased to 120V for 1h in order to allow the migration of the polypeptides and the markers through the electrophoresis gel (12% gels).

In order to do a coomassie blue staining, gels were incubated with coomassie blue made of 0.025% Coomassie brilliant blue R250, 40% methanol and 7% acetic acid for 1h, and washed overnight with destaining solution made of 67% dH_2O, 25% methanol and 7.5% acetic acid in order to remove the dye in excess from the gel. The coomassie blue staining solution was prepared by adding 2.5g of coomassie blue dye, 455ml of ethanol, 455ml of water, and 90ml of glacial acetic acid. The destaining solution was composed of 455ml of ethanol, 455ml water, and 90ml of glacial acetic acid.

For Western blotting analysis, separated proteins on the gels were transferred to a nitrocellulose membrane, and then blocked by 3% milkpowder in PBST (Phosphate Buffer Solution with 0.1% Tween-

20). Membranes were incubated overnight with the appropriate primary antibody, being either anti-HA antibody (Roche Diagnostics), anti-V5 antibody (Life Technologies) or one of the custom antibodies present in our antibody library. The next day, membranes were washed twice with PBST (20min) and an HRP-linked secondary antibody was added for 1h at room temperature. To reveal the proteins, membranes were then washed with PBS 1X 0.2 Tween-20 and revealed with the Advansta Westernbright kit according to the manufacturer's protocol.

D. STANDARD ELISA PROTOCOL

For all ELISA done in this work, unless differently specified, this protocol has been applied:
Proteins of interest are diluted in 1% Bovine Serum Albumin (BSA) in PBS and used to coat a 96 wells plate overnight in the cold room at 4°C. The next day, wells are washed three times with PBS. 200µL/well of 1% BSA in PBS are added and incubated 1h at room temperature in order to prevent unspecific binding. Primary antibodies are diluted in 1% BSA in PBS at appropriate concentrations and added to the wells. After overnight incubation in the cold room, wells are washed three times with PBST 0.1% Tween-20. The appropriate secondary antibodies are added and the plates are incubated 1h at room temperature.

As secondary antibodies, anti-rat and anti-mouse antibodies (depending on the primary antibody used) are diluted 1:3000 for 1h at room temperature. The wells are washed three times with PBST. Finally, the wells are washed three times with PBST, prior detection.
Detection is achieved as follows: Fresh OPD solution is made with 10µL H_2O_2 added to 12,5ml of phosphate citrate buffer and one tablet of OPD (Sigma-Aldrich). The solution is vortexed thoroughly until the reagent is dissolved. 100µl of OPD solution are added in every well and incubated 15min at room temperature. The ELISA plate is then read at 450nm. 80µl of sulfuric acid 2,5M is added in every well to block the reaction. The ELISA plate is read again at 490nm.

E. *IN VITRO* ASSAYS

All conditioned media were provided by EPFL platform. *In vitro* digestions were performed using 10µl of conditioned PC-1/3 and PC-2 media, 86µl of buffer of variable pHs (Phosphate or citrate final concentration 90mM), 2µl of $CaCl_2$ (2mM final concentration) and 2µl of Pyr-RTRK-MCA substrate (final concentration 5µM). Enzyme activity was read as fluorescence increase over time, monitoring the release of the fluorescent AMC group (λex 350nm; λem 450nm) using a Berthold Tristar LB941 reader.

Data were elaborated using GraphPaD.
Regarding the competition assays, conditioned media containing either PC-1/3 or PC-2 were pre-incubated 20min with increasing amount of conditioned media containing POMC protein at room temperature. The solutions contained 90mM of Acetate buffer and 2mM of $CaCl_2$ (pH 5 for PC-2 and pH 5.5 for PC-1/3). Following the pre-incubation, substrate was added and reading over time was achieved as described above.

F. PURIFICATION OF POMC FROM THE THE SUPERNATANT BY AFFINITY CHROMATOGRAPHY OR IMMUNOPRECIPITATION

Purification by affinity (anti-HA and anti-V5) chromatography was done using anti-HA and anti-V5 matrices and a peristaltic pump. The matrices were equilibrated using 10 bed volumes (20ml) of equilibration buffer made of 20 mM Tris, pH 7.5, 0.1 M NaCl, 0.1 mM EDTA. The flow rate was adjusted to 1 ml/min.
The affinity column was loaded with 8 ml of SA-POMC supernatant, the flow rate adjusted to 0.8 ml/min, and the column sealed with parafilm.
The column was washed with 20 bed volumes (40ml) washing buffer made of 20 mM Tris, pH 7.5, 0.1 M NaCl, 0.1 mM EDTA and 0.05% Tween 20. For the anti-V5 column, PBS was used as wash buffer.
For elution, 1 bed volume (2ml) of HA or V5 peptides reconstituted to 1 mg/ml in equilibration buffer were added to the column and incubated 15 min at 37°C. Elution fractions were then collected in an eppendorf tube. Elution was repeated three more times in order to obtain 4 elution fractions. Samples were kept at 2-8°C until final analysis. In order to regenerate the column for further use, HA peptide was removed from the column with, 20 bed volumes (40ml) of regeneration buffer (0.1 mM glycine, pH 2), followed by re-equilibration of the column with 20 bed volumes (40ml) of equilibration buffer.

Supernatant purification was also performed using magnetic Dynabeads coupled to custom antibody 1-ACTH-1E3-4-1. Dynabeads were resuspended and 20µl each transferred to three eppendorf tubes. The tubes were placed on a magnet and supernatant was removed and discarded. In order to coat the beads with the antibodies, 10µl of PBST (negative control), anti-HA antibody (negative control) and custom antibody 1-ACTH-1E3-4-1 were added each into the bead containing Eppendorf tubes, along with 200µl PBST. The tubes were incubated in a rotating wheel for 30 min at room temperature, put on a magnet and the supernatant was removed and discarded. The bead-antibody complexes were resuspended in 200µl PBST. The tubes were placed on a magnet and the supernatant was removed. 250µl SA-POMC

sample was then added and incubated at 4°C overnight in a rotating wheel. The tubes were washed three times with 1ml PBS 1X. 50µl 1% SDS buffer were added to each tube and incubated 10 min at 70°C in order to elute the desired protein.

V. RESULTS

A. ANALYSIS OF EPFL LARGE-SCALE PRODUCTION

Plasmids of Hu-POMC, SA-POMC, and TA-POMC were tagged with HA and V5 epitope whereas PC-1/3 and PC-2 were only tagged with V5 epitope. Plasmids encoding each protein were amplified to final 1.5mg (according to manufacturer's protocol) which is the quantity required for 0.5L production of conditioned media by cell suspension. Proteins were produced in HEK293 cells by the EPFL platform directed by Dr. Hacker. In order to assess the quality of the production, Hu-POMC, SA-POMC, TA-POMC, PC-1/3, and PC-2 samples were separated on a 12% SDS-PAGE gel after a Wessel & Flügge precipitation. The Coomassie gel showed an overall good amount of the different proteins contained in the supernatant provided by the EPFL platform, with the exception of PC-2. Hu-POMC was present at high concentrations (Fig.3). Two western blots were then made. The first was loaded with Hu-POMC, SA-POMC, TA-POMC, PC-1/3 and PC-2 and revealed with anti-V5 antibody (Fig. 4A –low exposure- and B – high exposure). The second was loaded with Hu-POMC, SA-POMC, TA-POMC and revealed with anti-HA antibody (Fig. 3C). Molecular weights of all proteins fit the expectations. Western blot analyses are in line with the Coomassie results. Degradation products can be seen in the Hu-POMC lanes of the HA-revealed western blots.

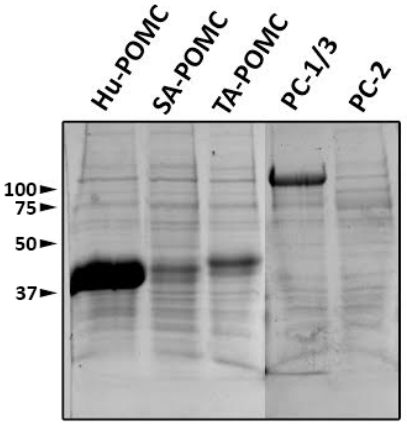

Figure 2. Coomassie gel of the different POMC and PCs acquired through the EPFL platform. *The gel was loaded with Human, Strix aluco and Tyto alba POMCs, as well as with PC-1/3 and PC-2, and revealed with Coomassie blue,*

showing good amount of the different proteins, particularly Hu-POMC and PC-1/3. The amount of proteins loaded corresponds to 400µl of original cell supernatant before Wessel and Fluegge.

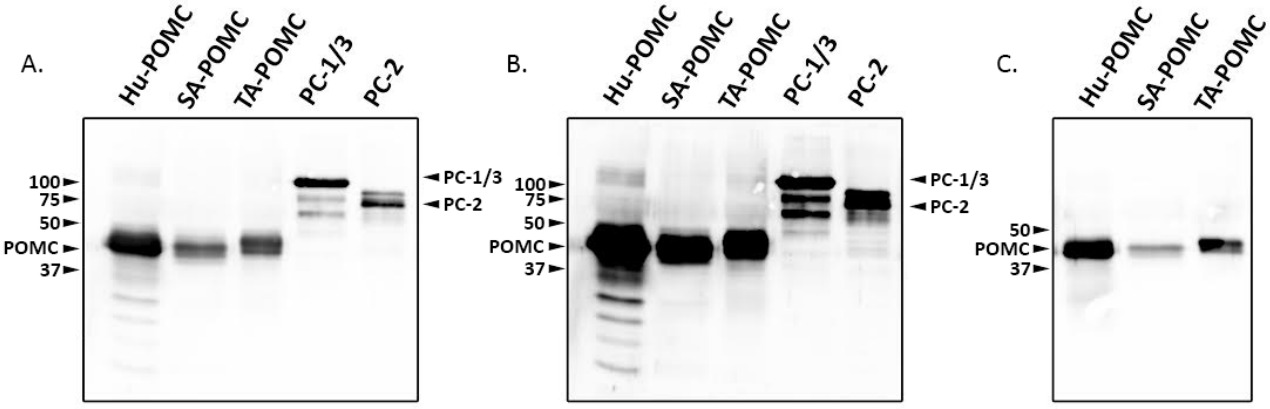

Figure 3. Western blots of the different POMCs and PCs acquired through the EPFL platform. A. and **B.** *The gels were loaded with Human, Strix aluco and Tyto alba POMCs, as well as with PC-1/3 and PC-2, and revealed with anti-V5 antibody. Hu-POMC shows a certain amount of degradation products still containing the V5 tag.* **C.** *The gel was loaded with Human, Strix aluco and Tyto alba POMC and revealed with anti-HA.*

The 87kDa, 81kDa and 63kDa forms of PC-1/3 are visible [12]. The unprocessed protein seems to be present in larger amounts than the mature forms, in line with the expectations (Fig. 3A and 3B).

The overall production was quantitatively acknowledged as adequate for the purpose of the further experiments.

Next, we wanted to verify whether PC-1/3 and PC-2 retained their enzymatic activities. To this purpose, we analyzed *in* vitro the ability of the conditioned media (PC-1/3 and PC-2) to cleave the fluorogenic substrate Ac-RTKR-MCA, carrying the basic PC *consensus* sequence Basic-Xn-Basic. Conditioned media containing SA-POMC was taken as negative control. Briefly, 85µL of supernatant was incubated with 5µM substrate in buffer solutions covering the pH range 4.0-7.5 (see materials and methods for details). Reading of the released AMC fluorescence over time revealed that our preparations contained active enzymes within the expected pH values. In contrast the POMC conditioned medium showed no enzymatic activities (Fig. 4).

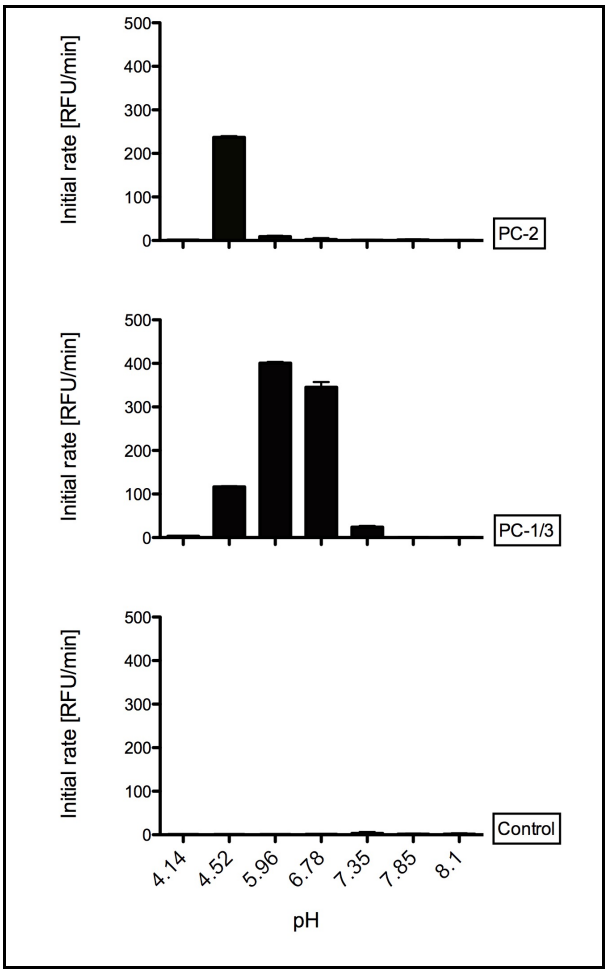

Figure 4. Released fluorescence over time at different pH for PC-1/3 and PC-2

Finally, we investigated the ability of POMC to compete with the synthetic Ac-RTKR-MCA substrate. The competition gives an idea of the affinity of the natural vs synthetic substrate.
Briefly, PC-1/3 and PC-2 media were pre-incubated with increasing volumes of Hu-, TA- SA-POMC conditioned media in acidic conditions. 20min post-incubation 5µM Ac-RTKR-MCA was added and fluorescence monitored as reported above. The presence of POMC did not have any effect on PC-2. In contrast, we observed a trend for up-regulation of PC-1/3 enzymatic activity proportionally to the amount of POMC present in solution (Fig. 5.).
Overall, the *in vitro* analysis of PC-1/3 and PC-2 demonstrated good enzymatic activities against a synthetic fluorogenic substrate. PC-1/3 activity seems to be increased in the presence of high POMC concentrations.

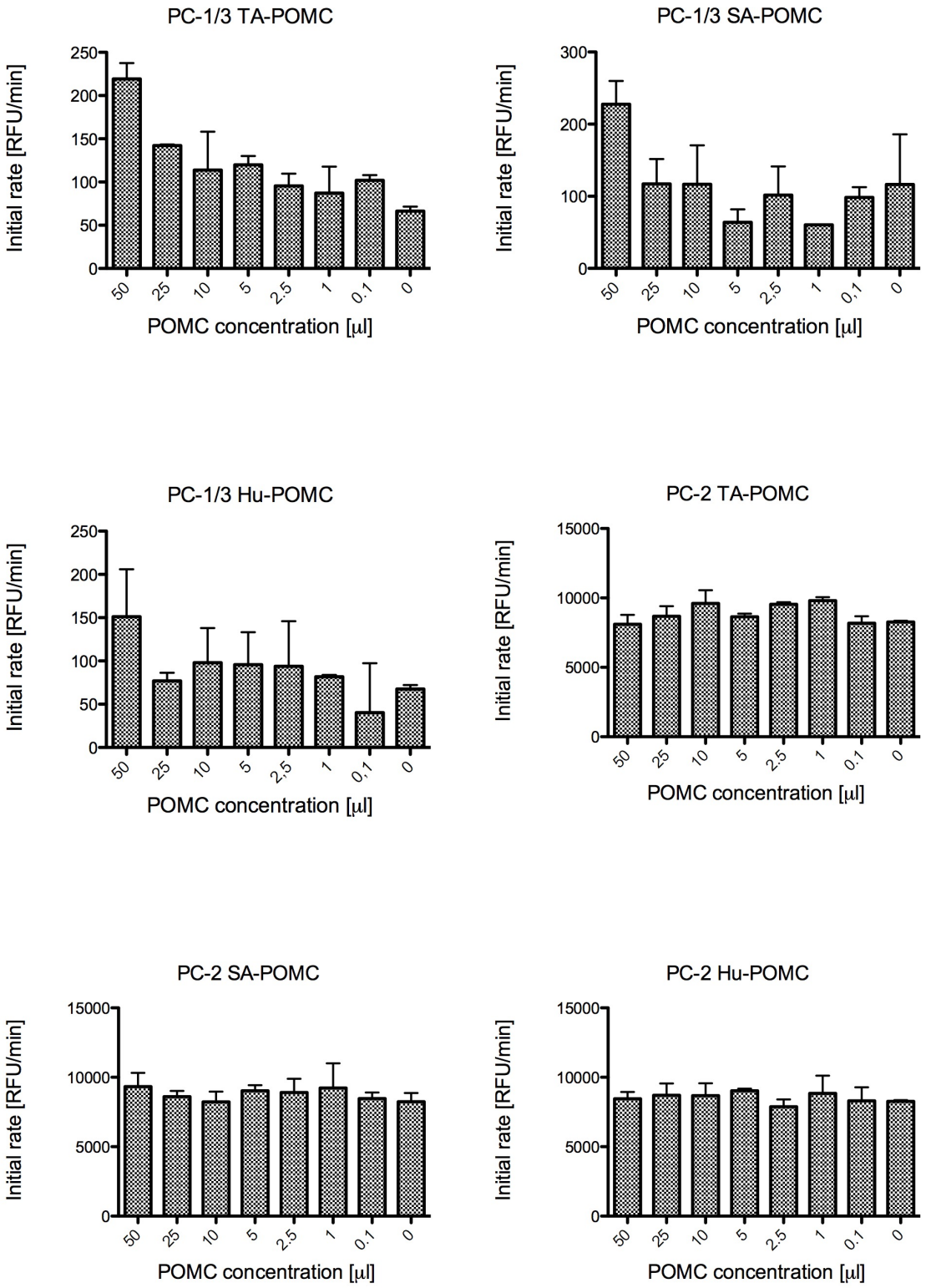

Figure 5. Competitive assay of PC-1/3 and PC-2 protein convertases against Pyr-RTRK-MCA substrate and TA-, SA-, and Hu-POMC.

B. Purification of POMC

POMCs and enzymes were needed for *in vitro* studies, which required high levels of purity. In order to purify the proteins, two affinity purifications of SA-POMC were carried out using either anti-HA or anti-V5 agarose bead affinity columns (Fig. 6.).

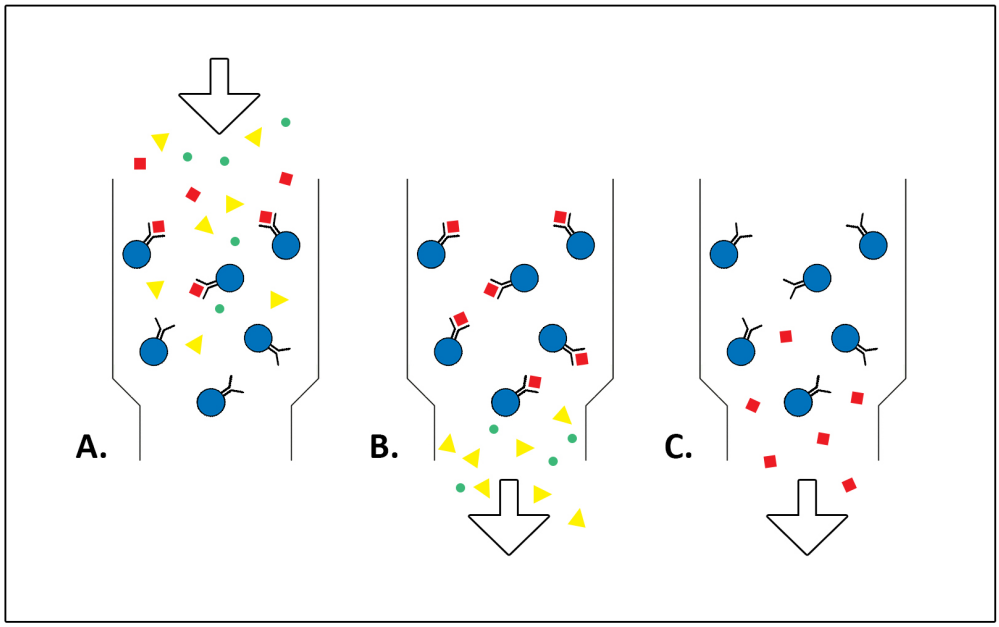

Figure 6. Affinity purification using agarose matrix cross-linked to IgG antibodies. A. *The mixture containing the protein of interest is added to the column.* **B.** *The undesired components are washed away with washing buffer.* **C.** *The protein of interest is eluted into a separate aliquot.*

Firstly, 0.5L of supernatant were loaded overnight on an anti-HA matrix using a peristaltic pump (0.5mL/min). The flow-through and a sample of input were kept in order to perform a coomassie gel to control the efficiency of the purification. POMC was eluted using anti-HA or anti-V5 peptide adjusted to 1mg/ml in equilibration buffer as described in materials and methods. The analysis revealed that the SA-POMC was not purified since it was fully recovered in the flow-through (Fig.7 A). In a second attempt, the same protein was loaded onto an anti-V5 column, following a protocol similar to that described above. Also in this case, the coomassie gel showed huge amount of proteins in the flowthrough (Fig.7 B). No traces of SA-POMC were detected in the eluates, suggesting that none of the tagged POMC bound to neither HA- nor V5-antibodies.

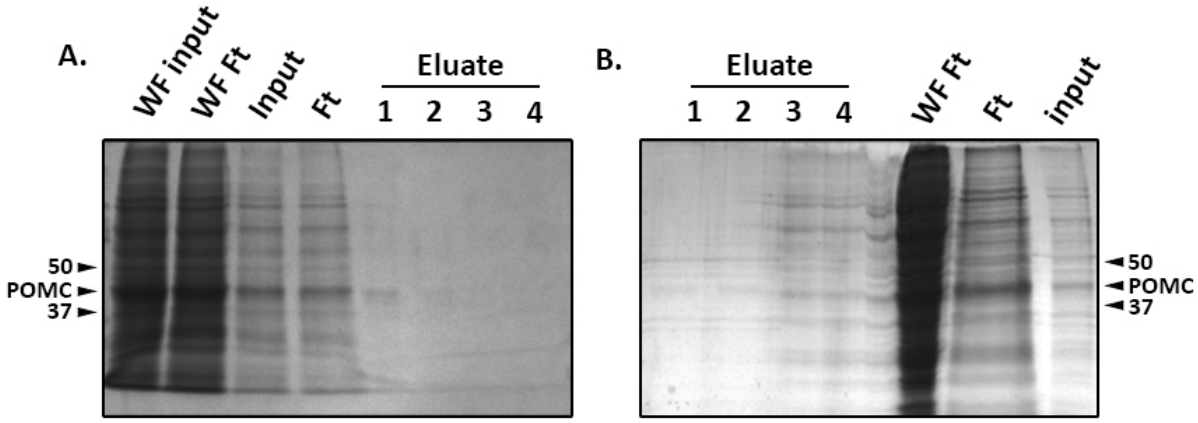

Figure 7. Coomassie gels of SA-POMC after HA- and V5- affinity purification. *Following HA* **(A.)** *or V5* **(B.)** *affinity purifications, flowthrough and input were loaded with and without WF procedure. 4 sequential elutions were performed and samples loaded. (Ft: flowthrough; WF: Wessel & Fluegge)*

C. CHARACTERIZATION OF ANTIBODIES AGAINST POMC:

Our lab has a library of antibodies raised against POMC (see materials & methods for details) to be fully characterized. We initially wanted to use purified protein for this analysis but the inability to purify POMC forced us to use raw material (supernatants from HEK cells overexpressing POMC). The antibodies were tested using two approaches: 1. ELISA, which is based on native protein conformation; 2. Western blotting, which requires protein denaturation.

PART 1: ELISA

ELISA test was performed against all POMCs. Briefly, 96-well plates were coated with POMC containing medium, as well as PC-2 containing medium and BSA (negative controls). As a positive control, C-terminally V5 tagged POMC was revealed with anti-V5 antibodies to verify proper coating. Primary antibodies were used undiluted. ELISA readout followed the procedure described in materials and methods.

Tyto alba β-*MSH antibodies:* None of the anti Tyto *alba* β-MSH antibodies did recognize specifically POMC. Antibodies 1-MSH-2B1-1-1 and 1-MSH-3F2-1-4 that displayed some activity towards β -MSH also yielded a positive signal in wells coated with medium from PC2, but not POMC expressing cells. These antibodies therefore unspecifically detect another component in the supernatant.

Strix aluco β-*MSH antibodies:* In contrast, anti Strix *aluco* antibodies 2-MSH-3A1-1-1 and 5-MSH-1G6-1-H1 gave signals superior to the PC-2 negative control. 5-MSH-1G6-1-H1 antibody bound both, human and Strix *aluco* POMC, but not Tyto *alba* POMC. 2-MSH-3A1-1-1 did not recognize the β-MSH epitope within Strix *aluco* POMC, the species, the b-MSH peptide for immunization was derived from, but recognized human POMC. We acknowledge that the medium from human POMC overexpressing HEK cells contained higher POMC content than the medium from Strix *aluco* expressing HEK cells.

ACTH antibodies: Most of the antibodies raised against ACTH worked well against their epitopes in POMC, usually without significant specificity for the species they were raised against (cross species activity).

Strix aluco/Tyto alba γ-MSH antibodies: antibodies 3-MSH-3B6-4-4 and 6-MSH-1B12-F8-5 reacted with POMC of all species, the latter being much more specific with low PC-2 medium cross reactivity.

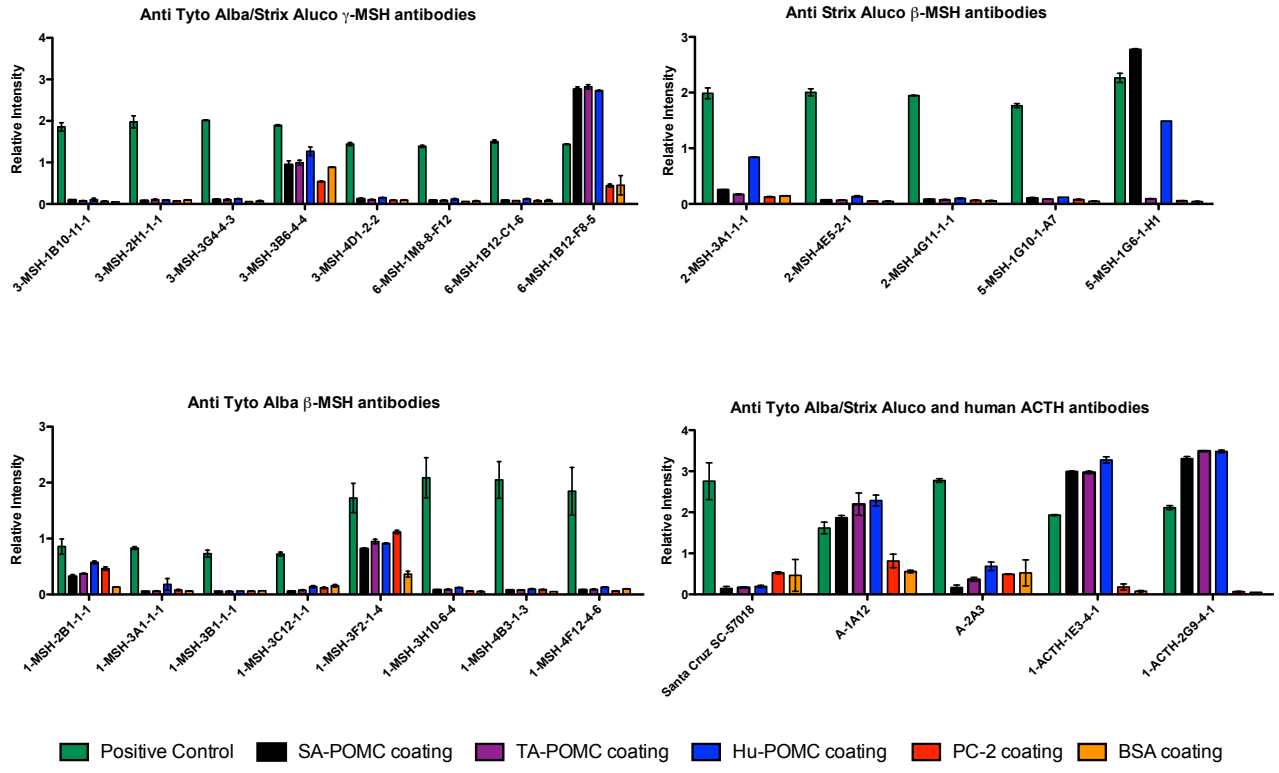

Figure 8. Summary of ELISA of the custom antibodies against TA-, SA-, and Hu-POMC. *Positive control was detected with anti-V5 antibody.*

Overall, POMC recognition by the anti-ACTH antibodies was better than with the other tested antibodies, with antibodies A-1A12, 1-ACTH-1E3-4-1, 1-ACTH-2G9-4-1 showing reactivity superior even to the positive control. Interestingly, the anti-MSHs antibodies specifically raised against owl sequences, antibodies A-1A12 and A-2A3 raised against human ACTH as well as 1-ACTH-1E3-4-1 and 1-ACTH-2G9-4-1 raised against owl ACTH, all recognized their respective epitopes in human as well as owl POMC.

PART 2: WESTERN BLOTTING

The antibodies that showed a significant ELISA response were further tested in western blot analysis. Briefly, 20µL of EPFL conditioned medium containing Hu-, SA-, TA- POMC were loaded on a 12% SDS-PAGE gel. PC-1/3 medium was taken as negative control to eventually identify antibody cross-reactivity with unspecific proteins present in the medium. Each membrane was split into three to be revealed with two custom antibodies as primary antibodies and with anti-HA as primary antibody for positive control to confirm the presence of POMC. The results of the Western blots were in line with those obtained with the ELISA. A discrepancy was found for the antibody SC-57018 which specifically recognized Hu-POMC in WB but not ELISA (Fig. 9 A.) and antibody 2-MSH-3A1-1-1, which recognized not only human but also Tyto *alba* POMC (Fig. 12 B). We also tested further three antibodies that were negative in ELISA: the antibody 1-MSH-3F2-1-4 showed cross-reactivity in ELISA with the PC-2 medium. In WB, we found that 1-MSH-3F2-1-4 does correctly recognize the POMC precursor, but also a low molecular weight protein that runs with the front, explaining therefore the unspecific stain of the ELISA negative control. In contrast the antibody 1-MSH-2B1-1-1, which also had some ELISA cross-reactivity was able to bind to Hu-POMC specifically in WB. Finally, the antibody 2-MSH-4G11-1-1 was incapable to identify POMC in WB in line with the ELISA results.

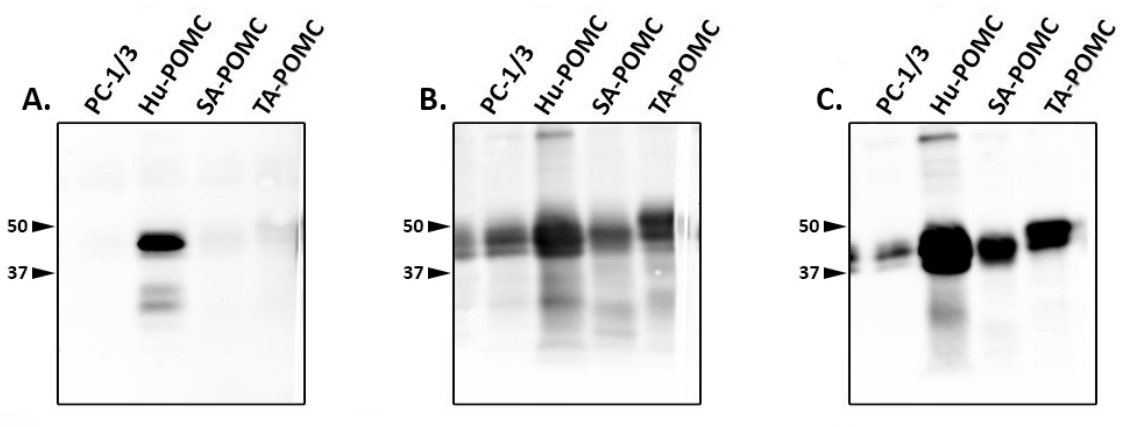

Figure 9. A. *The membrane was revealed with antibody SC-57018 raised against human ACTH.* **B.** *The membrane was revealed with antibody A-1A12 raised against human ACTH.* **C.** *Positive control, the membrane was revealed with anti-HA antibody.*

The membrane revealed with antibody SC-57018 recognized only Hu-POMC as well as its degradation products. This is consistent with the fact that this custom antibody was raised against human ACTH. Despite the fact that antibody A-1A12 was raised against human ACTH, the membrane revealed with this antibody was not specific to any of the species used and recognized all Hu-, SA-, and TA-POMC, PC-1/3 was also recognized but this is most likely due do spillage of the supernatant loaded in the Hu-POMC lane.

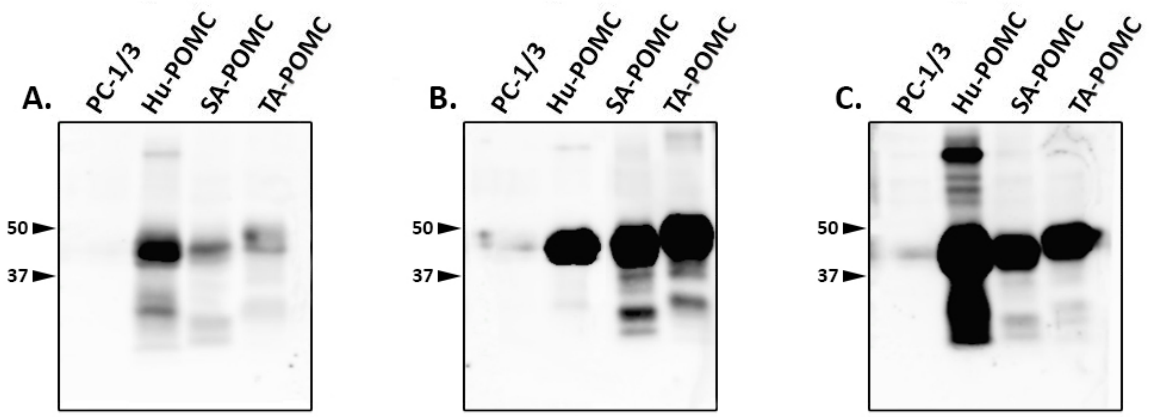

Figure 10. A. *The membrane was revealed with antibody A-2A3 raised against human ACTH.* **B.** *The membrane was revealed with antibody 1-ACTH-1E3-4-1 raised against owl ACTH.* **C.** *Positive control, the membrane was revealed with anti-HA antibody.*

The membrane revealed with antibody A-2A3 also recognized all POMC but Hu-POMC more specifically than SA- and TA-POMC, which is consistent with the fact that it was also raised against human ACTH. Antibody 1-ACTH-1E3-4-1 was raised against owl ACTH and recognized all POMCs as well, with higher affinity for the owl POMCs and recognized degradation products better in the owl samples than the human one.

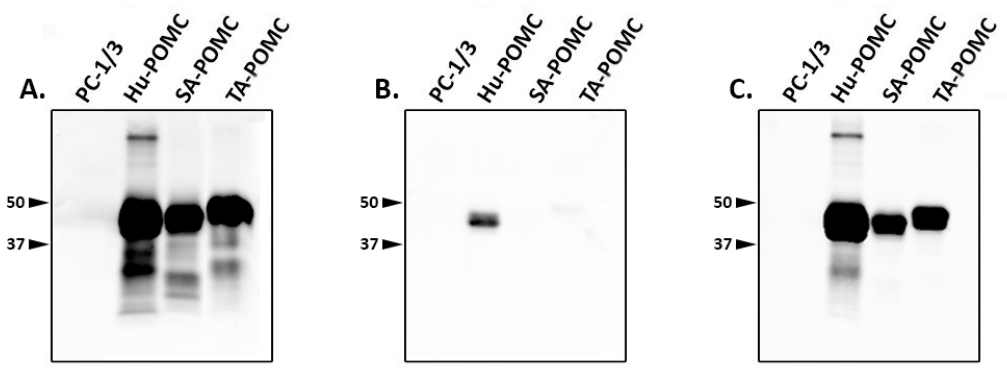

Figure 11. A. *The membrane was revealed with antibody 1-ACTH-2G9-4-1 raised against owl ACTH.* **B.** *The membrane was revealed with antibody 1-MSH-2B1-1-1 raised against Tyto alba* β*-MSH.* **C.** *Positive control, the membrane was revealed with anti-HA antibody.*

Antibody 1-ACTH-2G9-4-1 was raised against owl ACTH and recognized each POMC as well as their degradation products. Antibody 1-MSH-2B1-1-1 was raised against owl β-MSH and recognized almost Hu-POMC. We can see a light band in the TA-POMC line as well. The better recognition of human POMC is likely due to the higher POMC amount in this lane compared to the Tyto *alba* POMC lane. We cannot see degradation products of POMC indicating that the degradation products might not contain the β-MSH part of the protein.

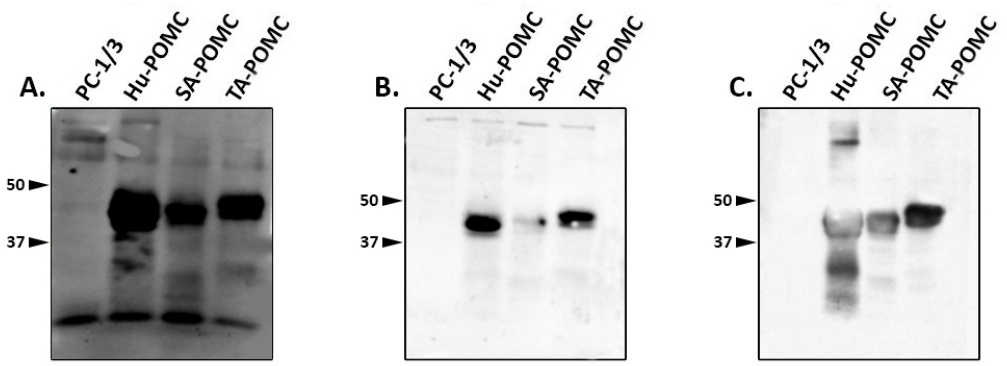

Figure 12. A. *The membrane was revealed with antibody 1-MSH-3F2-1-4 raised against Tyto alba* β*-MSH.* **B.** *The membrane was revealed with antibody 2-MSH-3A1-1-1 raised against Strix aluco* β*-MSH.* **C.** *Positive control, the membrane was revealed with anti-HA antibody.*

Antibody 1-MSH-3F2-1-4 was raised against *Tyto alba* β-MSH and recognized each POMC but with low sensitivity since the exposure had to be raised high in order to see the bands clearly. Antibody 2-MSH-

3A1-1-1 was raised against Strix *aluco* β-MSH and recognized Hu-POMC and TA-POMC better than SA-POMC.

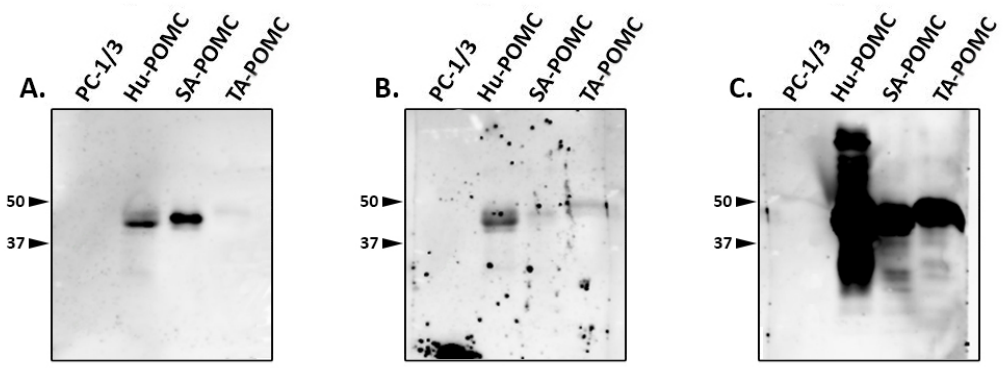

Figure 13. A. *The membrane was revealed with antibody 5-MSH-1G6-1-H1 raised against Strix aluco* β*-MSH.* **B.** *The membrane was revealed with antibody 3-MSH-3B6-4-4 raised against owl* γ*-MSH.* **C.** *Positive control, the membrane was revealed with anti-HA antibody.*

Antibody 5-MSH-1G6-1-H1 was raised against Strix *aluco* β-MSH and recognized SA-POMC and Hu-POMC specifically. Antibody 3-MSH-3B6-4-4 was raised against owl γ-MSH and recognized both human and owl POMCs with higher specificity for human and overall low affinity since revealing the membrane required high exposure.

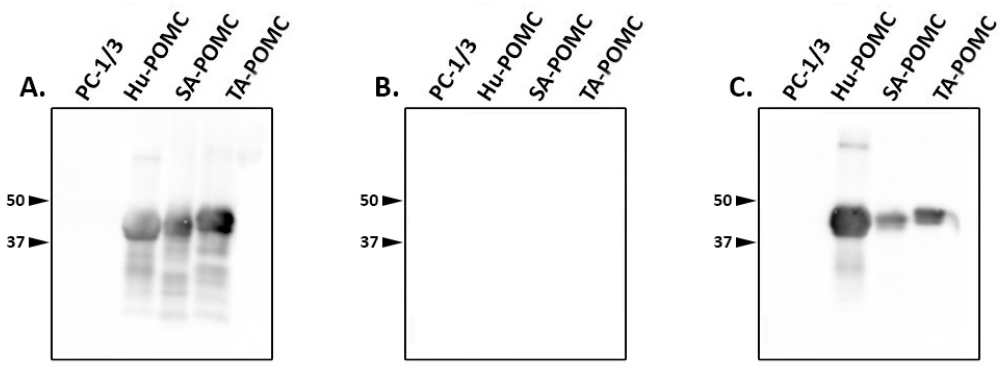

Figure 14. A. *The membrane was revealed with antibody 6-MSH-1B12-F8-5 raised against owl γ-MSH.* **B.** *The membrane was revealed with antibody 2-MSH-4G11-1-1 raised against Strix aluco* β*-MSH.* **C.** *Positive control, the membrane was revealed with anti-HA antibody.*

Antibody 6-MSH-1B12-F8-5 was raised against owl γ-MSH and recognized both human and owl POMCs. Antibody 2-MSH-4G11-1-1 raised against Strix *aluco* β-MSH which was taken randomly did not recognize any of the POMCs. Overall, we successfully characterized the panel of POMC antibodies in our hands.

Our analyses identified through both ELISA and western blotting several antibodies that can react with POMC of different species in over-expressed system. In table 3, we summarize our results. Whether the antibodies are sensitive enough to detect endogenous POMC in the according cell lines and tissues remains to be tested.

	SA-POMC	**TA-POMC**	**Hu-POMC**	**PC-2**
Santa Cruz SC-57018 (clone O2A3)	-	-	+	-
A-1A12 (Anne White)	+	+	+	-
A-2A3 (Anne White)	-	-	+	+/-
1-ACTH-1E3-4-1	+++	+++	+++	-
1-ACTH-2G9-4-1	+++	+++	+++	-
1-MSH-2B1-1-1	-	-	+	+/-
1-MSH-3F2-1-4	+	+	+	+
2-MSH-3A1-1-1	-	-	+	-
5-MSH-1G6-1-H1	+++	-	++	-
3-MSH-3B6-4-4	+++	+++	+++	-
6-MSH-1B12-F8-5	+++	+++	+++	-
2-MSH-4G11-1-1	-	-	-	-

Table 3. Western blot summary of the antibodies' affinity against the different POMCs

The table summarizes both ELISA and WB analyses. ELISA –WB discrepancies: For 1-MSH-3F2-1-4 antibody, the bands are at the expected molecular weight, so it most probably detects the correct protein even though in ELISA the PC-2 medium background was stronger than the POMC signal. It is possible that PC-1/3 does not contain specific impurities present in PC-2 media or the cross reacting components of the medium are denatured in Western blot and are no longer recognized by the antibody. The other discrepancy found was the detection of Hu-POMC by the SC-57018 antibody using western blotting procedure as opposed to ELISA, which gives a signal below the negative control.

D. USE OF POMC ANTIBODIES TO INVESTIGATE POMC MATURATION

In our lab, we are interested in POMC maturation and how this could be affected by the unusual insertion of Poly-Ser found in Tyto *alba*. In a first attempt, we tried to follow the multiple cleavages using N- and C- terminal tags to POMC. However, this approach is limited since internal fragments, which correspond to MSHs, are lost. To overcome this problem, we decided to use our newly characterized antibodies. Briefly, an *in vitro* digestion was performed using supernatants from human POMC overexpressing HEK cells and incubating them with either supernatants from PC-2 overexpressing HEK cells or water. Samples were collected after incubation for different times (t=0 , t=6h, t=4 days) and immediately frozen at -20C. Western blot analysis was carried out using antibodies directed against HA and β-MSH using the custom antibody 5-MSH-1G6-1-H1. Results show that we do not detect any PC-2 specific cleavage of POMC going beyond unspecific degradation, potentially due to the non-optimal pH in the supernatant (see your pH profile for PC-2 with the AMC-peptide, optimal at around pH 4.5, non detectable at pH 7). Also, we would need PC-1/3 to cleave POMC first in order to provide the substrate for PC-2, we would need to co-overexpress POMC with PC-1/3 and PC-2 in HEK cells, so that the cleavage can happen in the cell in the acidic secretory granules. Once released to the supernatant, the pH is too high, ex vivo cleavage is therefore very difficult to realize in supernatants, where we cannot optimize buffer conditions. Results showed that impurities present in the POMC conditioned medium are sufficient to degrade the proopiomelanocortins, impeding any further analysis by PC-2 processing (Fig. 15). This experiment suggests that POMC purification is a pre-requisite needed prior any further analysis.

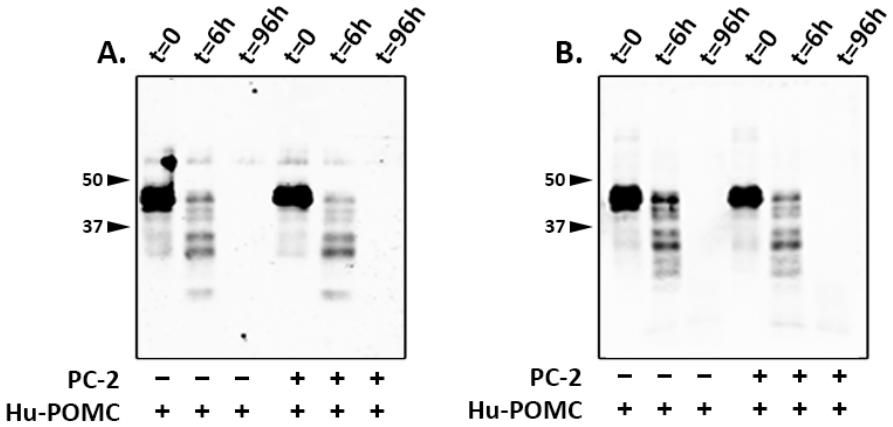

Figure 15. A. *The membrane was loaded with Hu-POMC incubated with or without PC-2 at t=0, t=6h, and t=96h and revealed with antibody 5-MSH-1G6-1-H1* **B.** *The same was done in this membrane but it was revealed with anti-HA*

antibody.

E. IMMUNOPRECIPITATION OF POMC

Since our attempts of POMC purification using anti-HA/anti-V5 agarose beads were unsuccessful and given that this is a key step required for our project, we decided to test an antibody directed against the internal ACTH epitope, rather than N-or C-terminal tags which might have been burried in natively folded POMC . In order to purify SA-POMC, we selected the antibody 1-ACTH-1E3-4-1, and anti-HA as negative control, and coupled it to Protein A Dynabeads. A third eppendorf tube contained no antibodies and was used as negative control. Immunoprecipitation followed the protocol described in materials and methods.

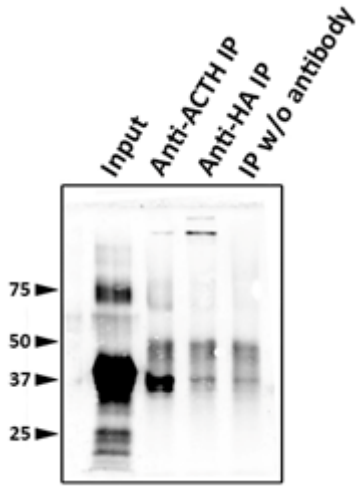

Figure 16. Magnetic beads immunoprecipitation. *Dynabeads magnetic beads were linked to antibody 1-ACTH-1E3-4-1, anti-HA antibody, and without antibodies. SA-POMC was loaded before and after immunoprecipitation.*

Immunoprecipitation of SA-POMC using anti-ACTH antibodies coupled to the magnetic beads allowed us to successfully purify the protein. In contrast, again the anti-HA antibody was very inefficient, further demonstrating that POMC cannot be purified targeting N-terminal tags (Fig. 16). Despite the fact that POMC was found to be a difficult protein to purify via terminal tags, we identified the ACTH epitope as suitable for efficient pull-down of this protein. Further experiments are needed for protocol optimization.

DISCUSSION AND PERSPECTIVES

POMC is the precursor of several hormones with a plethora of different functions. Understanding how POMC is matured and investigating the mechanism behind the generation of the melanocortin products is highly valuable to shed light into POMC biology in normal and pathological conditions.

Here, we produced and fully characterized recombinant forms of POMCs as well as the two enzymes – PC-1/3 and PC-2, known to be responsible for POMC cleavage into its active peptide hormones. In addition, novel anti-POMC antibodies have been tested and shown to be effective in ELISA and WB analyses.

POMCs, PC-1/3, and PC-2 supernatants were obtained by transient transfection in mammalian cells using the EPFL platform for protein production. Proteins were produced at high levels and PC-1/3 and

PC-2 were highly active according to the expectations. Interestingly, we found that POMC *per se* at high concentrations can activate PC-1/3 but not PC-2. We can speculate that the substrate is able to dock to the enzyme and to induce a conformational change favorable for activity. In this case, it is conceivable that PC-1/3 activation can be regulated by the presence of the substrate, thus avoiding that the enzyme cleaves wrong substrates.

We tried to purify POMC proteins by affinity purification, taking advantage on the C-terminal or N-terminal tags (V5 and HA). The two approaches did fail since we recovered the entire POMC in the flowthrough. We hypothesize that the failure may be due to propensity of POMC to self-associate to form oligomers. This is in line with Cawley et al. [13]. In this scenario, the terminal tags may be hindered within the oligomeric structures. Further approaches may be successful to attain protein purification. Using antibodies raised against central portion rather than C- and N- terminal of the protein could be a better approach to purify POMC, as demonstrated by the magnetic beads affinity purification using custom anti-ACTH antibody 1-ACTH-1E3-4-1. Indeed, previous studies on recombinant POMC showed successful purification through affinity immunoprecipitation using anti γ-MSH antibodies [14]. Size exclusion chromatography could be another option to purify POMC since this technique would separate the different components of the solution based on their size. In other terms, no need of recognition of specific epitopes by external antibodies is required.

The inability to purify large quantities of supernatant (very low amount of custom antibodies are available) did impair the analysis of POMC processing since gross degradation does occur when POMC supernatants are incubated at 37°C with no extra addition of enzymes. Nonetheless, we decided to use POMC supernatants in ELISA and WB, based on the low protein background observed in the Coomassie gels. ELISA tests were performed to assess the specificity of an array of antibodies that were previously raised. Among the several samples, we found antibodies capable to specifically recognize different parts of POMC, including the three melanocortin stimulating hormones (MSH) α, β, and γ, with potential applications in diagnostic as well as tools in biological tests. We are aware that the approach herein used has limitations. Indeed, we will need to assess the response of the selected antibodies against endogenous levels of POMC in ELISA tests. The sensitivity of our antibodies is extremely important, of course, especially for medical applications. POMC detection in blood is currently used in order to diagnose ectopic ACTH-secreting tumors, and it is mainly detected through immunoradiometric assay. If our antibodies are sensitive enough (high affinity binding), we may bypass the use of radioactive material. Being able to detect POMC with ELISA without the use of a radioisotopic technique would

reduce the cost of the diagnostic procedure and the impact of the radioactive markers to the users and to the environment.

Surprisingly, we observed that many custom antibodies are reactive also in WB. So far, rare examples of anti-POMC antibodies have been reported with very low affinity. Thus, our antibodies represent a valuable novel tool for POMC biology. Also in this case, the sensitivity of the antibody has to be assessed.

Finally, the antibody library was useful to try to solve the problem of POMC purification. As proof-of-concept, we showed that the anti-POMC antibodies but not anti-HA/V5 can be used for affinity purification of POMC. This is in line with our hypothesis that POMC oligomerizes, thus making only specific parts of the protein accessible to the antibody. The large antibody database available could provide a way to have a first insight of the conformational structure of the oligomers by testing if the antibodies raised against different portion of POMC can bind to the oligomer.

Further application of antibodies raised against different hormones of the melanocortin system could potentially be used diagnostically and therapeutically in order to prevent over stimulation of the melanocortin receptors locally and remotely in cases of metastatic melanoma [15]. Anti α-MSH antibodies, although not present in the antibody library, could be potentially useful in order to treat anorexia nervosa by lowering the anorexigenic stimulation caused by α-MSH on the MC4R. However, this application is limited by the need of the antibody to cross the blood brain barrier [16]. As said earlier the detection of POMC through specific antibodies could help diagnose ectopic ACTH-dependant Cushing's syndrome caused by an occult ectopic tumor that tends to elevate POMC plasma concentration as opposed to pituitary secretion of ACTH that presents with lower plasma concentration of POMC [17].

Overall, the studies here presented help in identifying novel anti-POMC antibodies, working in ELISA and WB analyses. We used them as tools to set a new protocol for POMC purification. Highly purified protein will be extremely valuable for also the investigation of POMC processing and crystal structure.

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