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Department of Microbiology
Master´s thesis • 45 hec • Second cycle, A2E
Biotechnology - Master's Programme • Examensarbete/Sveriges lantbruksuniversitet, Institutionen för mikrobiologi, 2015:5 • ISSN 1101-8151
Uppsala 2015

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Credits: 45 hec
Level: Second cycle, A2E
Course title: Independent project/degree project in Biology
Course code: EX0596
Programme/education: Biotechnology - Master's Programme

Place of publication: Uppsala
Year of publication: 2015
Title of series, no: Examensarbete/Sveriges lantbruksuniversitet, Institutionen för mikrobiologi
Number of part of series: 2015:5
ISSN: 1101-8151
Online publication: http://stud.epsilon.slu.se

Keywords: Solute carriers, Amino acid starvation, mTOR Pathway, AAR Pathway, Amino acid transport systems, Membrane transport proteins, Amino acid transporters

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Abstract

Amino acids are the building blocks of proteins. They are essential for cell survival and normal cellular functions as they are the key precursors to numerous cellular pathways and processes. Availability of amino acids exhibit vital role in regulation of gene expression in all mammalian cells. Transporter proteins mediate transport of amino acids across the plasma membrane. In this study, complete amino acid starvation was performed on the hypothalamic cell line 25/2 from mouse to investigate the regulation of gene expression of amino acid transporters. We monitored up and down regulation in transcription of genes encoding solute carriers (Slcs) with a primary focus on amino acid transporters. Amino acid transporters from system A, L, xc, y^+ and system ASC showed a response to amino acid starvation. Members from various solute carrier families that transport amino acids as their preferred substrate were found to be upregulated after 3-5 hours. Moreover, a few orphan genes such as Slc23a3 and Mfsd11 with unknown substrate profile were also found to be upregulated in response to amino acid depletion.

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Abbreviations

4E-BP1	4E-binding protein 1
AA	Amino Acid
AAR	Amino Acid Response
AARE	Amino acid responsive element
APC	Amino acid/polyamine/organocation
AS	Asparagine synthetase
ATF	Activating transcription factor 2
C/EBPs	CCAAT/enhancer binding proteins
CATs	Cationic Amino acid Transporters
CHOP	C/EBP homologous protein
CPA/ AT	Cation/ proton antiporters/ Anion Transporters
DNA	Deoxyribonucleic acid
eIF-2α	Eukaryotic initiation factor 2 alpha
eIF4E	Eukaryotic initiation factor 4E
GCN2	General control nonderepressible 2
Glyt1	Glycine transporter 1
GPCRs	G-protein-coupled receptors
LATs	L-type amino acid transporters
MAPK	Mitogen-activated protein kinase
MCF	Mitochondrial carrier family
MCT	Monocarboxylate transporters
Mfsd	Major Facilitator superfamily domain
mRNA	Messenger ribonucleic acid
mTOR	Mammalian Target of rapamycin
NHE	Sodium Hydrogen Exchanger

NSR	Nutrient Sensing Response
NSRE	Nutrient sensing responsive element
PFAM	Protein Family
RT	Reverse Transcriptase
S6K1	Ribosomal protein subunit 6 kinase 1
SLC	Solute carrier
SNAT	Sodium-coupled Neutral Amino acid transporter
Tm	Melting Temperature
tRNA	Transfer ribonucleic acid

Introduction

Amino acid sensing Pathways

Amino acids play a vital role in gene regulation. Change in the concentration of amino acid has a significant role on protein synthesis (Fafournoux, Bruhat et al. 2000). In mammals there are two well-established amino acid sensing pathways that are responsible for inspecting and reacting to the amino acid availability. These two pathways function inversely to one another in order to change the rate of protein synthesis (Kilberg, Pan et al. 2005). The primary role of these two pathways is to control the mRNA turnover/decay rate for specific genes, the transcription and the protein synthesis (Chaveroux, Jousse et al. 2009).

Amino Acid sensing by mTOR pathway

The mammalian target of rapamycin (mTOR) pathway serves as a sensor for elevated concentration of amino acids and is activated by amino acid supplementation in order to maintain the synthesis of proteins and cell growth (Liao, Majithia et al. 2008). In contrary, GCN2 pathway is activated during the amino acid deficiency and plays its role during the starvation (Palii, Chen et al. 2004, Chaveroux, Jousse et al. 2009). The mechanism by which mTOR kinase senses sufficient amino acid is unknown. However, in the presence of adequate amino acid concentration in the cell mTOR Kinase cascade is activated. Activation of mTOR kinase phosphorylates the p70 ribosomal S6 kinase (S6K1) (Kilberg, Pan et al. 2005, Chaveroux, Jousse et al. 2009) and eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) which is a translator repressor (Hutchinson, Shanware et al. 2011). EIF4E along with its associated proteins (eIF4A helicases) and (eIF4G) forms eIF4F complex that facilitates and promotes protein translation by aiding the interaction with 40S ribosomal subunit. The primary function of 4E-BP1 is to bind eIF4A to prevent the formation of the eIF4A complex and inhibit the protein translation. Phosphorylation of 4E-BP1 deactivates it and prevents it to bind to eIF4E (Liao, Majithia et al. 2008, Laplante and Sabatini 2009). S6 kinase is a translation initiator once phosphorylated. Stimulated S6 Kinase ensures the translation of mRNA at a higher level to synthesize ribosomal protein. In this way the cell growth and protein synthesis rates are kept up at a rate steady with supplement accessibility (Kilberg, Pan et al. 2005).

Amino acid sensing by AAR pathway

The AAR pathway is the second known amino acid sensing pathway that is active in amino acid deficiency. This pathway utilizes an independent stress activated kinase termed as general control nonderepressible (GCN2) kinase to sense amino acid deprivation (Bentivoglio, Del Grosso et al. 1997). Intra or extra cellular amino acid insufficiency results in increased uncharged tRNA that subsequently binds to GCN2 kinase and activates it. In response, GCN2 kinase phosphorylates translation initiation factor (eIF-2α) that suppresses the global protein synthesis (Zhang, McGrath et al. 2002, Kilberg, Balasubramanian et al. 2012), but paradoxically, increases the particular pre existing mRNA translation that includes activating transcription factor 4 (ATF4) (Lu, Harding et al. 2004, Kilberg, Balasubramanian et al. 2012). Activating transcription factor 2 (ATF2) is activated upon its phosphorylation during the amino acid starvation. It has been reported that ATF2 is phosphorylated by a number of kinase pathways termed Mitogen-activated protein kinase (MAPK) and JNK/stress-Activated protein kinase (Averous, Bruhat et al. 2004). Both ATF2 and ATF4 play a primary role in inducing the gene expression during amino acid starvation (Averous, Bruhat et al. 2004).

CHOP a 29KDa protein in Humans is a member of CCAAT/enhancer binding proteins (C/EBPs). It contains a cis-positive amino acid responsive element (AARE) in its promoter (Oyadomari and Mori 2004). AARE is responsible to induce gene expression during amino acid starvation. ATF2 and ATF4 are involved in the regulation of CHOP during amino acid limitation by binding to AARE (Averous, Bruhat et al. 2004). Asparagine synthetase (ASNS) is a protein-encoding gene and is accountable for the biosynthesis of asparagine and glutamine. It is expressed in many mammalian cells and also activated in response to amino acid deprivation (Gong, Guerrini et al. 1991, Hutson and Kilberg 1994). ASNS mRNA level is increased in amino acid starved conditions (Bruhat, Jousse et al. 1997). ASNS gene possesses two cis-elements in its promoter; nutrient sensing responsive element (NSR1 and NSR2). During amino acid starvation these two elements are vital for transcriptional activation and these elements also have sequence and functional similarities with AARE. Activation of ASNS gene in response to amino acid deprivation involves ATF 4 binding to NSRE1 (Siu, Chen et al. 2001, Siu, Bain et al. 2002).

It is evident from the literature that in AAR pathway, GCN2 kinase induces the translation of ATF4 pre-existing mRNA and regulates the gene transcription (Deval, Chaveroux et al. 2009). Genes that possess AARE or similar elements in their promoter site are enhanced transcriptionally in amino acid starvation (Palii, Kays et al. 2009)

Solute Carrier Proteins

SLCs are the largest group of transporters with diverse biochemical properties including coupled transporters, ion driven exchangers; cellular Na^+ gradient dependent and passive transporters; dependent on concentration gradient of the molecule it translocates. The localization of these transporters also varies but the majority is localized in the plasma membrane while some transporters are particularly localized in synaptic vesicles, peroxisomes and mitochondria (Fredriksson, Nordstrom et al. 2008). Almost all SLC families are functionally related (with few exceptions) due to the fact that all of them depend on an ion gradient as a driving force to translocate molecules across the cell membrane. They transport substances include amino acids and oligopeptides, inorganic cations and anions, glucose and other sugars, carboxylate and organic anions, bile salts, acetyl coenzymes A, biogenic amines, essential metals, neurotransmitters, fatty acids and lipids, vitamins, nucleoside, choline, ammonium, urea, thyroid hormone and drugs over the cell membrane (He, Vasiliou et al. 2009, Jacobsson, Stephansson et al. 2010, Hoglund, Nordstrom et al. 2011).

G-protein-coupled receptors (GPCRs) are the largest phylogenetically related membrane protein family with 800 genes coding for proteins in humans (Fredriksson, Lagerstrom et al. 2003, Hoglund, Nordstrom et al. 2011). Solute carriers (Slcs) are the second largest super family of membrane proteins with approximately 55 distinct families coding for over 400 proteins in humans (Vasiliou, Vasiliou et al. 2009, Li and Shu 2014). To assign a particular protein to a specific family it must have at least 20-25% sequence identity to other members of that group. In mammals SLCs are classified into four major groups, α-, β-, γ- and δ based on phylogenetic analysis where proteins in each group share common evolutionary linage. β –cluster, which is the largest cluster of amino acid transporters, includes 3 major families, SLC32, SLC36 and SLC38 (Fredriksson, Nordström et al. 2008).

SLC families in the PFAM classification are divided into three major clans on the basis of sequence profile analysis. Major facilitator superfamily (MFS), Amino acid/polyamine/organocation (APC) and monovalent cation/ proton antiporters (CPA)/anion transporter (AT). MFS superfamily is among the largest clan of membrane transporters in humans. 13 SLC families (SLC2, 15, 16, 17, 18, 19, 21, 22, 29, 33, 37, 43, 45, 46) that are part of α-group, populate MFS clan regarding SLCs. CPA/AT clan include the γ group of the SLC family and contains only 2 SLC families. The APC superfamily includes 9 SLC families (SLC4, 5, 7, 12, 23, 26, 32, 36, 38). This clan mainly contains amino acid transporters and the APC clan contains the entire β family of SLCs. Since the proteins in the APC superfamily are suggested to be most likely homologous, it comprises of a single superfamily (Hoglund, Nordstrom et al. 2011).

Amino Acid Transporters

About 25% of SLC genes transport amino acids as their primary substrate. Until now there are eleven known families that code for amino acid transporters. SLC1, SLC6, SLC7, SLC15, SLC16, SLC25, SLC36, SLC38, and SLC43. Seven of them are expressed in the plasma membrane and account for transport from the extracellular medium into the cytosol, while four of them are intracellular and found in for example lysosomes, mitochondria and synaptic vesicles (Broer 2008, Karunakaran, Umapathy et al. 2008, Stevens 2010). SLC1 transports neutral amino acid and contain the physiologically important high-affinity glutamate transporters. SLC3 is known to encode the heavy subunit of the heteromeric amino acid transporter. SLC7 encodes the glycoprotein transporters/cationic amino acid transporters. SLC15 encodes the proton-oligopeptides symporters. SLC17 is involved in several processes; it is responsible for vesicular storage of glutamate as well as in metabolism and degradation of glycoproteins. SLC32 encodes the vesicular inhibitory amino acid transporters. SLC36 family members are proton/amino acid transporters while SLC38 is involved in Na^+ coupled transport of neutral amino acids. SLC43 represents the Na^+ independent system L like family and mediates transport of heavy neutral amino acids across the cell membrane (Karunakaran, Umapathy et al. 2008, He, Vasiliou et al. 2009, Schioth, Roshanbin et al. 2013). There are almost 60 members out these families that are

known amino acid transporters and about 40 are still orphans that are expected to have amino acids as their primary substrate (Fredriksson, Nordström et al. 2008).

The role of the SLC family of transporters in response to amino acid levels has not been investigated in detail previously. Many of known amino acid transporters are reported to be upregulated during amino acid starvation due to the presence of AAREs due to the fact that expression of these transporter genes are influenced and controlled by AARE (Fernandez, Yaman et al. 2001, Padbury, Diah et al. 2004, Sato, Nomura et al. 2004, Hyde, Cwiklinski et al. 2007).

Amino acid starvation plays a significant role in the regulation of mRNA expression of members from Slc7 family. Slc7a1 (CAT-1), member of the Slc7 family, has high affinity for cationic amino acids (Verrey, Closs et al. 2004) and belongs to system y^+(Hatzoglou, Fernandez et al. 2004). SL7a1 utilizes L-arginine, L-lysine and L-ornithine as substrate (White and Christensen 1982). It has been shown that first exon of CAT-1 gene has AARE which is a responsible for gene regulation of CAT-1 transporter during amino acid deprivation (Fernandez, Lopez et al. 2003). Upon total amino acid starvation, a very significant increase in the protein levels of Slc7a1 has been reported (Fernandez, Yaman et al. 2001).

Similarly, another SLC7 family member; Slc7a11 (xCT) belongs to heterodimeric amino acid transport system xc- which is an anionic Na^+ independent amino acid transport system. It facilitates the exchange of glutamate and cysteine (Liu, Blower et al. 2007). The 5´ flanking region of Slc7a11 (xCT) gene has two AARE like elements in the promoter region (Sato, Nomura et al. 2004). During deprivation of cysteine, arginine and leucine, ATf2 and ATf4 bind to AARE element and induce the transcription of Slc7a11 (xCT) gene (Sato, Nomura et al. 2004).

The amino acid transport system A facilitates Na^+ dependent transport of short chain neutral amino acids, which include glutamine, serine and alanine (Burkhalter, Fiumelli et al. 2007). Na^+ coupled amino acid transporter2 (SNAT2, SLC38a2) is a member of system A (Mackenzie and Erickson 2004, Burkhalter, Fiumelli et al. 2007). Presence of AARE in the

first intron of human and mouse Slc38a2 results in increased expression during amino acid starvation (Palii, Thiaville et al. 2006, Hyde, Cwiklinski et al. 2007). GCN2 kinase lead to phosphorylation of eif2α mediates the induced expression of Slc38a2 (Gaccioli, Huang et al. 2006).

Regulation of gene expression in complete or partial amino acid starvation has been previously studied on a number of different cell lines. Members from Slc7 family have shown to be transcriptionally induced in response to amino acid starvation using various cell lines; Induction in Slc7a1 has been reported when C6 glioma cells from rat were starved with amino acids (Lopez, Wang et al. 2007). In mouse NIH3T3 cell lines, induction in expression level of Slc7a11 that belongs to system xc- has been shown (Sato, Nomura et al. 2004). Slc7a5 expression is induced during amino acid starvation in rat hepatic cell line (Padbury, Diah et al. 2004). In Slc38a2 (SNAT2), transcription is upregulated due to AARE in human HepG2 hepatoma cells (Palii, Chen et al. 2004). Similarly in HeLa cells, Slc38a2 mRNA level induction (Gaccioli, Huang et al. 2006) and in human BeWo cells, the expression of Slc38a2 is enhanced (Novak, Quiggle et al. 2006).

Aim

In this research project, we have used the immortalized embryonic mouse hypothalamic cell line N25/2. These cells were starved for amino acids for different periods of time. 1, 2, 3, 5 and 16 hours of starvation were performed in order to study the response of these neuronal cell lines to amino acid deprivation. The primary objective of this project was to study the regulation of gene expression of those genes that encode for solute carriers (Slcs) with a primary focus on genes encoding amino acid transporters. Both known and orphan amino acid transporters and their response to complete amino acid starvation were the focus of our study.

Material and Methods

Complementary DNA (cDNA) synthesis

RNA from the hypothalamic cell lines of the mouse brain was provided by Robert Fredriksson (supervisor) to generate cDNA using Invitrogen SuperScript® III Reverse Transcriptase Kit. Quantification for each RNA sample was performed and the volume in (µl) for each RNA sample was calculated with final concentration of 1000 ng for the reaction. In brief, 20 µl of reaction volume contained 10 µl of Master mix, 2 µl of RT enzyme (200 unit equals to 1 µl), 1000 ng of RNA (final concentration) and H_2O was calculated separately for each sample. Final reaction volume was 20 µl except first two samples (1 h, 1h-aa1) where the final volume was 40 µl.

Table 1. Concentration for each RNA sample and final volume for RT reaction (final conc. of 1000 ng/sample) is given in this table. Volume of H_2O, RT enzyme and Master mix are also mentioned in the final columns of the table.

RNA Samples	RNA conc ng/µl	RNA in µl for 1000 ng	cDNA Final conc ng/µl	H_2O in µl	RT Enzyme (µl)	Mastermix (µl)
1h (2x vol)	105.21	9.50	25	18.5	20	2
1hr-aa1 (2x vol)	52.25	19.14	20.9	8.86	20	2
2h1xaa1	306.11	3.27	50	4.73	10	2
2h-aa1	264.42	3.78	50	4.22	10	2
3h1xaa1	209.63	4.77	50	3.23	10	2
3h-aa1	276.29	3.62	50	4.38	10	2
5h1xaa11	222.38	4.50	50	3.5	10	2
5h-aa11	278.83	3.59	50	4.41	10	2
5h1xaa12	293.2	3.41	50	4.59	10	2
5h-aa12	190.1	5.26	50	2.74	10	2
5h1xaa13	247.61	4.04	50	3.96	10	2
5h-aa13	288.74	3.46	50	4.54	10	2
5h1xaa14	510.62	1.96	50	6.04	10	2
5h-aa14	525.43	1.90	50	6.1	10	2
16h1xaa1	548.23	1.82	50	6.18	10	2
16h-aa1	251.29	3.98	50	4.02	10	2

RNA samples for reverse transcription were subjected to thermal cycler for cDNA synthesis with the following conditions shown in Table 2.

Table 2. Reverse Transcription PCR protocol.

Heated lid	112°C	
Temperature	25°C	10 min
Temperature	50°C	30 min
Temperature	85°C	5.00 min
Store	4°C	Infinite

In order to avoid RNA contamination in the newly generated cDNA samples, RNAses were added to each cDNA sample tube that degrade any RNA present in the sample. 0.9ul of RNAase H from Invitrogen SuperScript®III Reverse Transcriptase Kit was added and samples were subjected to thermocycler with following conditions in Table 3.

Table 3. PCR Protocol for RNase H addition to cDNA samples.

Heated Lid	112°C	
Temperature	37°C	20 min
Store	4°C	Infinite

Primer Optimization

In order to achieve best melting temperature (T_m) and efficiency to amplify the target cDNA primers optimization was carried out using qPCR. For each primer pair to be optimized, a gradient from 62C-52°C was used. Reactions were run with Taq DNA Polymerase from (BIOTOOLS B&M Labs, S.A).

The PCR mixture was composed of the following components represented in Table 4. cDNA used in primer optimization was generated from RNA isolated from mouse brain.

Table 4. Components of real time qPCR for primer optimization.

No	Component	Volume (µl)	Final Concentration
1	H_20	13.5	
2	10 X buffer	2.00	1X
3	dNTPs	0.20	2µM
4	50 mM $MgCl_2$	1.60	4 mM
5	Primer Fwd	0.05	0.25 µM
6	Primer Rev	0.05	0.25 µM
7	DMSO	1.00	5%
8	10,000X SYBR (Invitrogen)	0.50	0.1X
9	Taq DNA Polymerase 1U/ µl	0.80	0.80 Units
10	cDNA	1.00	5 ng/ µl
	Total	20.00	

List of primers shown in Table 5 were successfully optimized and used further in this project.

Table 5. List of optimized primers using qPCR with appropriate temperatures.

Number	Primer name	Oligo-nucleotide sequence 5'-3'	Optimized Temperature
1	mmSLC7A11 F	TGGAACTGCTCGTAATAC	54°C
	mmSLC7A11 R	GTTCAGGAATTTCACATTGA	
2	mmSLC6A9 F	TTTCCCATACCTCTGCTA	52.8°C
	mmSLC6A9 R	AAAGCTCCATGAAGAAGA	
3	mmSLC7A1 F	AATTATCATCTTAACAGGACTG	55.9°C
	mmSLC7A1 R	GACCAGGACATTGATACA	
4	mmSLC40A1 F	CTTTGCTGTTGTTGTTTG	52.8°C
	mmSLC40A1 R	GAGAGGAACCGAAGATAG	
5	mmSLC23A3 F	TCTTCAACTTCAACTCACAT	54°C
	mmSLC23A3 R	ACAAAGGCAGAGATGAAC	
6	mmSLC25A33 F	AGTTCCTCTGGCTTCTTTG	58.4°C
	mmSLC25A33 R	TCCTGATGACCTCGTGTG	
7	mmSLC38A7 F	TAGCCATTGCGGTCTATAC	61.3°C
	mmSLC38A7 R	GCTCCTTCGACATCACAG	
8	mmSLC9A9 F	TGATATTGATAGTGGAACTGTCT	54.1°C
	mmSLC9A9 R	CTTGGTCGGTGATGTTGA	
9	mmMfsd11 F	CTATGTTTGTCAGTGGTTTG	55.9°C
	mmMfsd11 R	AGATGCTGTGTAGAAGGA	
10	mmSLC25A36 F	ACCTGTGCCACAACCATA	62°C
	mmSLC25A36 R	ATCCATAGCCTTCTTCTTGAAC	
11	mmSLC16A9 F	CCCAATATCTACTTTCTGTTT	54.1°C
	mmSLC16A9 R	CGTCGCTGTGTATAATAAG	
12	mmMfsd2a F	CTATGTCAAGCTCATTGC	54.1°C
	mmMfsd2a R	GAAGTCCAAGGTATAGGT	
13	mmSLC43A2 F	GTTTATGCACAGTGTGTT	54.1°C
	mmSLC43A2 R	AAGATGGAGGTATAGAGG	
14	mmSLC25A10 F	GATTTGGTCAATGTCAGGAT	58.4°C
	mmSLC25A10 R	CAGGGCATGAGAGTAGTT	
15	mmSLC16A2 F	TTTCCCTTCCTCATCAAA	52.8°C
	mmSLC16A2 R	GTAAGTGAGTGAGAGCAG	
16	mmSLC25A1 F	AAGTTCATCCATGACCAG	54.1°C
	mmSLC25A1 R	GTTCCCGAATAATCTCTC	
17	mmActn1 F	CCGAGTTGATTGACTATGGA	55.9°C
	mmActn1 R	GAACCTCTCTGCCACATC	

Quantitative Real time PCR

Each qRT-PCR was carried out in a total volume of 12.5 µl using BR SYBR® Green SuperMix for IQ™ Systems, (Quanta Biosciences Germany).

Components and volume of the qPCR are shown in Table 6.

Table 6. Components of quantitative real time PCR using qPCR kit from Quanta Biosciences

Components	Volume (µl)
Mastermix	6.25
Primer Fwd	0.05 (0.25 µM Final conc.)
Primer Rev	0.05 (0.25 µM Final conc.)
H_2O	5.15
cDNA	1.00 (5 ng/ µl)

Amplification and detection were carried out by Bio-Rad iQ5 Real-Time PCR Detection Systems (Bio-Rad Laboratories Sweden) using the following protocols (Table 7 and 8).

Standard cycling Mode (Primer T_m > 60 °C)

Table 7. Real time QPCR protocol suitable for primer temperature more than and equal to 60°C

Step	Temperature	Duration	Cycles
Initial	50 °C	2 min	Hold
Activation	95 °C	2 min	Hold
Denature	95 °C	15 sec	
Anneal/Extend	60 °C	1 min	40

Standard cycling Mode (Primer T_m < 60 °C)

Table 8. Real time QPCR protocol suitable for primer temperature less than and equal to 60°C.

Step	Temperature	Duration	Cycles
Initial	50 °C	2 min	Hold
Activation	95 °C	2 min	Hold
Denature	95 °C	15 sec	
Anneal/Extend	55- 60 °C	15 sec	40
Extend	72 °C	1 min	

Real time melt curve and threshold cycle (Ct-values) were analyzed using MY IQ5 software (Bio-Rad laboratories Sweden). Melting curve was analyzed for each sample by comparing it to the positive control (cDNA of mouse brain) and negative control (water) to confirm the amplification of only target gene and to avoid the primer dimers formation.

Each sample of cDNA was run in triplicates. Efficiency for each primer pair was determined using LinRegPCR software. Significant outliers were removed using the web-based software Graphpad from http://graphpad.com/quickcalcs/Grubbs1.cfm.

SD and mean for the triplicates were calculated. In order to ensure the reliability of the results a cutoff of maximum 0.99 Ct was used among the triplicates and values exceeding this limit were removed as outliers. Three internal housekeeping genes were also run to normalize the level of gene expression to that of mActn, Beta Tubulin and GAPDH. Geometric means of all three housekeeping genes were calculated using GeNorm software to obtain the normalization factor and finally the relative mRNA expression level was calculated by subsequently normalizing each gene of interest to the geometric mean of the house keeping genes.

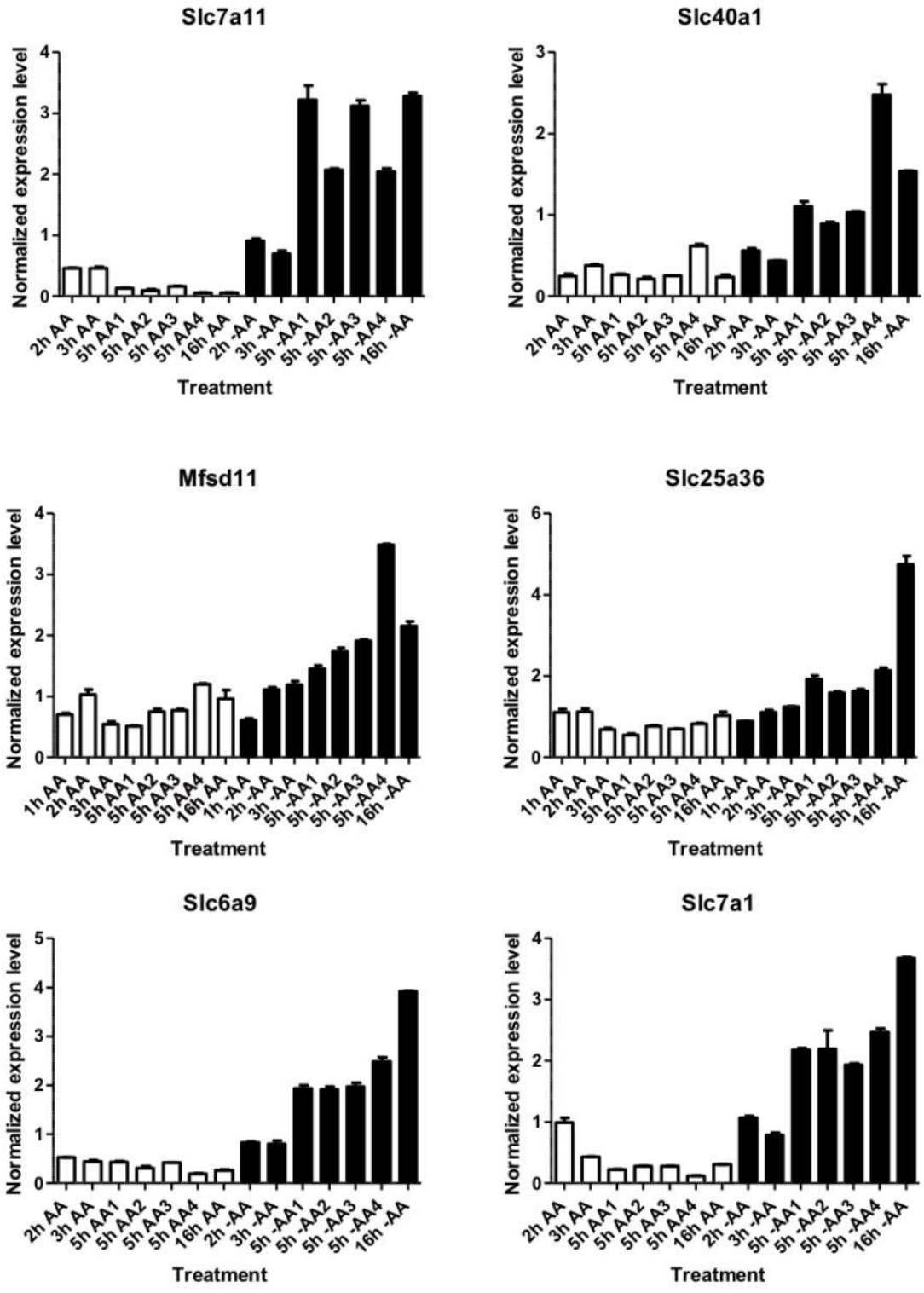

Figure 1a. Upregulated genes.

Quantitative real time PCR data representing relative expression of genes encoding various transporters. X-axis represents the treatment time and treatment medium (1h, 2h, 3h, 5h represent number of incubation hours, AA in the X-axis represent medium with amino acid and –AA represent medium lacking amino acids) while y-axis represents relative expression. Error bars on each column represent standard deviation. White bars in the graphical data show normalized gene expression for the genes from medium containing amino acid. Upregulated genes are shown in black bars.

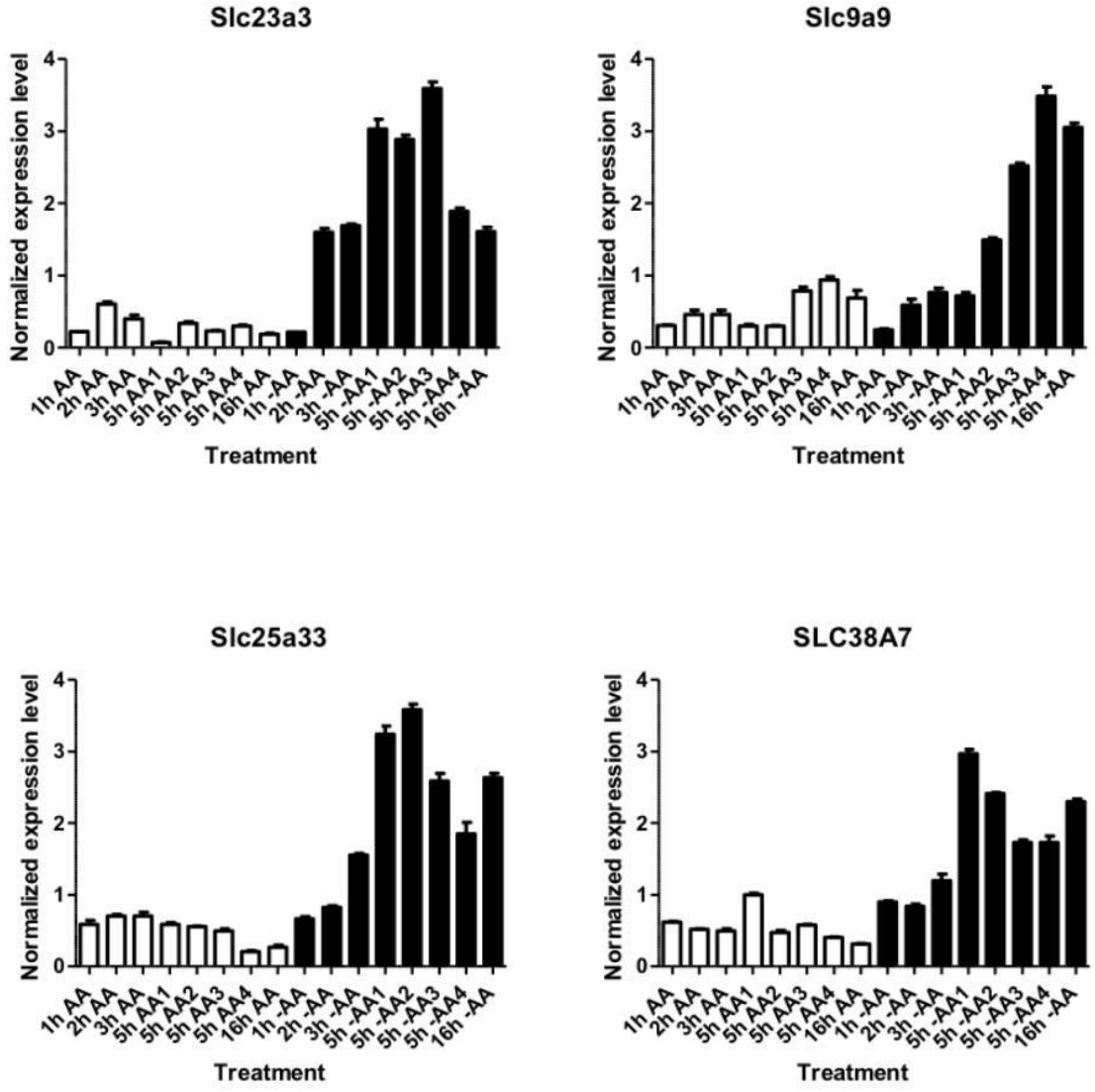

Figure 1b. Upregulated genes.

Quantitative real time PCR data representing relative expression of genes encoding various transporters. X-axis represents the treatment time and treatment medium (1h, 2h, 3h, 5h represent number of incubation hours, AA in the X-axis represent medium with amino acid and –AA represent medium lacking amino acids) while y-axis represents relative expression. Error bars on each column represent standard deviation. White bars in the graphical data show normalized gene expression for the genes from medium containing amino acid. Upregulated genes are shown in black

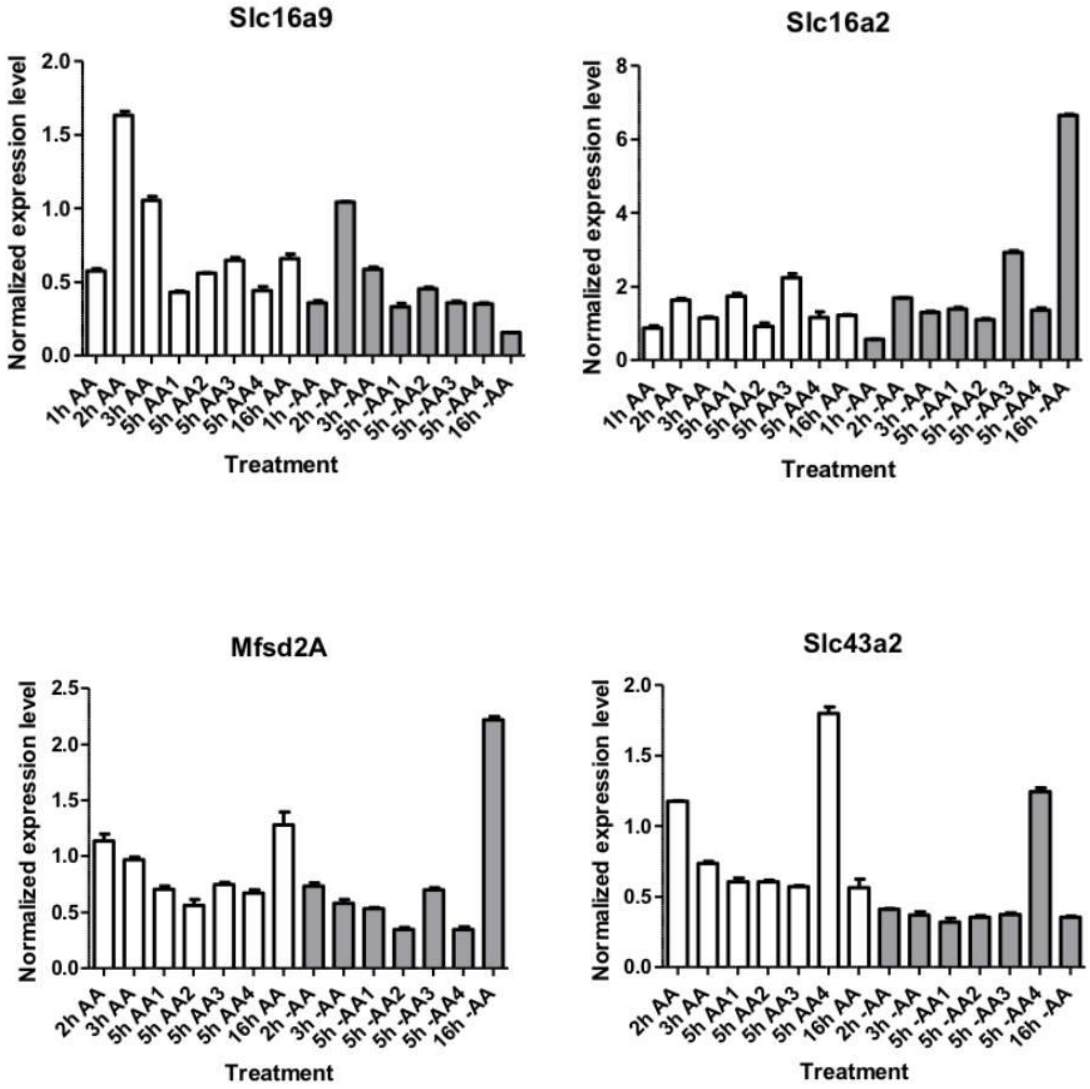

Figure 1c. Downregulated genes.

Quantitative real time PCR data representing relative expression of genes encoding various transporters. X-axis represents the treatment time and treatment medium (1h, 2h, 3h, 5h represent number of incubation hours, AA in the X-axis represent medium with amino acid and –AA represent medium lacking amino acids) while y-axis represents relative expression. Error bars on each column represent standard deviation. White bars in the graphical data show normalized gene expression for the genes from medium containing amino acid. Downregulated genes are shown in gray

Results

Hypothalamic cell lines N25/2 from mouse were used in this study. Starvation experiments on these cell lines were performed by Robert Fredriksson (Supervisor) and provided for this project. A complete amino acid starvation was performed on one set of cell lines and incubated in amino acid free medium for 1, 2, 3, 5 and 16 hours. Another set of same cell lines was incubated in medium containing amino acids to be used as control for the same duration (Hellsten et al. 2014, unpublished data).

Figure 1a, 1b and 1c show the graphical representation of relative expression from quantitative real time PCR. Black bars in figure 1a and 1b indicate the genes that were upregulated while the gray bars in figure 1c the indicate genes that were downregulated in this study. White bars show the gene expression from the medium with amino acid availability.

Two members of Solute carrier family 7, Slc 7A1 and Slc7A11 were upregulated in response to amino acid starvation. The genes were highly upregulated at 5 hours and 16 hours for both members of solute carrier family 7.

Two members of solute carrier family 25 were also upregulated. Slc 25a33 was highly upregulated after 5 hours of starvation and Slc25a36 was highly upregulated after 16 hours of starvation.

Slc38a7 was also upregulated in response to amino acid starvation. Significant upregulation of this gene was observed after 5 and 16 hours of amino acid starvation.

Slc 6a9, Slc9a9, 23a3, Mfsd11 and Slc40a1 were shown to be upregulated in response to amino acid starvation.

Slc 16a9 and Slc16a2 were downregulated with the increasing time of amino acid starvation however Slc 16a2 showed a dramatic upregulation after 16 hours of starvation. Mfsd2 was also downregulated until 5 hours of starvation but was upregulated when the starvation time was extended to 16 hours. Expression of Slc43a2 was downregulated in response to amino acid starvation.

Discussion

Slc7a1 and Slc7a11

Slc family 7 of transporters is divided into two subfamilies, cationic amino acid transporters (CATs, slc7a1-slc7a4) and L type amino acid transporters (LATs, Slc7a5-Slc7a11)(Verrey, Closs et al. 2004). In this project two members of Slc 7 family were upregulated. Slc7a1 (CAT1) belongs to system y^+ (Hatzoglou, Fernandez et al. 2004) and its expression was significantly induced according our results. It has been reported in the literature that Slc7a1 contains AARE in its promoter site. During amino acid starvation ATF2 and ATF4 bind AARE and induce its expression. This gene has been shown to be upregulated previously in response to amino acid deprivation (Fernandez, Yaman et al. 2001, Lopez, Wang et al. 2007).

Slc7a11 (xCT) encodes the heterodimeric amino acid transport system xc- together with SLC3a2. It serves as cysteine/glutamine exchanger (Sato, Tamba et al. 2000). Slc7a11 being the light chain of system xc- and is responsible for the transport activity while the Slc3a2 serves, as a heavy chain of the system is responsible for the expression of system xc- (Verrey, Closs et al. 2004, Liu, Blower et al. 2007).

Slc7a11 has shown to be upregulated in HepG2/C3A cells that were deprived of cysteine. Previously it showed response to cysteine deprivation (Lee, Dominy et al. 2008). In our study the medium was deprived of all the amino acids including cysteine. Slc7a11 has been reported to have an AAR element in its promoter (Lee, Dominy et al. 2008) and is probably induced in the same manner as Slc7a1. The regulation of expression of both Slc7a1 and Slc7a11 is under the control of the CHOP gene.

Slc38a7

The Slc38 family contains sodium coupled neutral amino acid transporters and members of this family belong to system A and N (Hagglund, Sreedharan et al. 2011, Broer 2014). Most of the members of this family have not been shown to be responsive towards changing concentrations of amino acids. However Slc38a2 that belongs to system A has been induced in different cell types upon amino acid starvation in the past (Gaccioli, Huang et al. 2006, Novak, Quiggle et al. 2006, Hyde, Cwiklinski et al. 2007). In our study Slc38a7 (SNAT7) has been upregulated in response to amino acid starvation. Slc8a7 has been assigned to system N recently and has not been shown to respond to amino acid changing concentrations in previous studies, however its substrate profile is wide and it resembles both Slc38a1 and Slc38a2 (Mackenzie and Erickson 2004) (Hagglund, Sreedharan et al. 2011)) and is probably regulated in a similar manner as Slc38a2. However, Slc38a2 contains AARE in its promoter (Palii, Chen et al. 2004) while Slc38a1 and Slc38a7 have not been confirmed to be under the control of an AARE element until now.

Slc23a3

Slc23a3 is one of the four members of Solute carrier family 23 that comprises of Na^+ dependent ascorbic acid transporters. Only two members are yet characterized as L-ascorbic transporters (Takanaga, Mackenzie et al. 2004). Slc23a3 is a member of amino acid-Polymine-organoCation (APC)-superfamily clan of Pfam. Members of APC-superfamily clan encode amino acid transporters (Hoglund, Nordstrom et al. 2011). Most of the transporters from APC superfamily clan are reported to be upregulated in response to amino acid starvation. Slc7a5 (Padbury, Diah et al. 2004), Slc7a1 (Fernandez, Yaman et al. 2001), Slc7a11 (Sato, Nomura et al. 2004) and Slc38a2 (Hyde, Cwiklinski et al. 2007) .

SLC6a9

Slc6a9 (Glyt1) is the Glycine transporter (Zafra, Aragon et al. 1995). Glycine is a non-essential amino acid. It is an important inhibitory and excitatory neurotransmitter and mediates both inhibitory and excitatory synapses in central nervous system of vertebrates (Zafra, Aragon et al. 1995, Fultang, Howard et al. 2014). Expression of Glyt1 is localized mainly in glial cells. Glyt1 is responsible for the clearance of glycine from the sites of

synapses. Alteration in Glyt1 activity in response to various factors disturbs the hemostasis of the glycinergic system, which is involved in both inhibitory and excitatory neurotransmission (Morioka, Abdin et al. 2008). Expression of Slc6a9 (Glyt1) was induced in response to amino acid starvation according to our results. Glyt1 is reported to have AARE in within the 5'UTR that include exon 1 and exon 2. Interaction of ATF4 to these response element sites is responsible for the induction in expression of Glyt1 during amino acid starvation (Fultang, Howard et al. 2014).

Slc9a9

Slc9a9 is an isoform (NHE9), belonging to the Na^+/H^+ exchanger solute carrier family 9 (NHE) and is ubiquitously expressed. The primary role of NHEs is to modulate pH of intracellular organelles and endosome and prevent the cell from internal acidification (Counillon and Pouysségur 2000). pH homeostasis is an important phenomenon in mammalian cells since many basic systems function at specific optimal PH. It also regulates the normal and pathological gene expression; moreover, proteins, functioning requires specific optimal pH(Malo and Fliegel 2006).

Slc9a9 (NHE9) is an endosomal cation/proton antiporter (Kondapalli, Hack et al. 2013). It is exclusively expressed in brain, including cortex and the hippocampal regions where it has been localized to perinuclear space related to recycling endosome (Llongueras, Kondapalli et al. 2014). The transporter is suggested to control cargo trafficking and degradation in addition to mediate the luminal pH of endosome (Llongueras, Kondapalli et al. 2014) (Kondapalli, Prasad et al. 2014). It has been reported that gain of function or increase in NHE9 levels is linked with alkalization of endosomal luminal pH. Overexpression of NHE9 might lead to oncogenic signalling (Kondapalli, Llongueras et al. 2015). Upregulation in expression level of Slc9a9 in our results might suggest that over expression of Slc9a9 is triggered due to alkalinized pH level in response to amino acid starvation.

Slc25a33 and Slc25a36

Solute carrier family 25 in humans encodes 53 transport proteins. It is also known as mitochondrial carrier family (MCF). Slc25a33 and Slc25a36 are the two-mitochondrial pyrimidine nucleotide transporters. Due to high level of sequence identity between Slc25a33 and Slc25a36 genes it has been suggested that both genes share similar functions (Di Noia, Todisco et al. 2014). Mitochondria are known to be the powerhouse of the cell due to their primary role as ATP generator that provides energy for the cell. It also serves as a biosynthetic hub for the cell by providing substrates and precursors for the anabolic pathways. According to Johnson et al 2014; amino acid starvation upregulates the amino acid consuming process of respiration, amino acid catabolism and protein synthesis in mitochondria. Amino acid starvation also resulted in increased mitochondrial transcripts that would ultimately increase the protein synthesis (Johnson, Vidoni et al. 2014). Slc25a33 and Slc25a36 are the pyrimidine (deoxy)nucleotide transporters and are crucial for the synthesis of mitochondrial RNA and DNA by providing precursors as well as their breakdown and removal of products of these processes (Di Noia, Todisco et al. 2014) . Both of these genes were upregulated in response to amino acid deprivation in our study. During amino acid starvation the need for elevated protein synthesis in mitochondria might trigger the upregulation of Slc25a33 and Slc25a36 genes.

Mfsd2 and Mfsd11

A number of genes in this project belong to major facilitator family (MFS) and we show that they display changes in expression level in response to amino acid starvation. On the basis of Pfam database, MFS family is the largest clan of membrane proteins with 13 known SLCs families that are phylogenetically related (Hoglund, Nordstrom et al. 2011). In our results orphan members of MFS family; Mfsd2 and Mfsd11 were regulated in response to amino acid starvation. Mfsd11 that belongs to MFS cluster was upregulated however; Mfsd2 was shown to be downregulated.

Slc16a9 and Slc16a2

Slc16a9 (MCT9) and Slc16a2 (MCT8) that belong to solute carrier Monocarboxylate transporters family (Kristensen, Andersen et al. 2011) were found to be downregulated in our study in response to amino acid starvation. Slc16a9 (MCT9) is an orphan with unknown substrate profile. However SLc16a2 is a known transporter and has high affinity to thyroid hormone. Slc16a2 mediates transport of iodothyronines T2, rT3, T3 and T4 (Kristensen, Andersen et al. 2011). Down-regulation of Slc16a2 in response to amino acid starvation suggests that its expression is effected by cellular amino acid level. However the mechanism and relevance of its response to amino acid starvation is yet to be elucidated.

Slc40a1

Slc40a1 also known, as Ferroportin-1 is an iron-regulated transporter and is responsible for iron homeostasis within the cell by exporting iron from inner cellular environment (Donovan, Brownlie et al. 2000). Ferritin is a cytosolic protein that stores bulk of iron in the form of $Fe(OH)_3$ (about 4500 atoms of iron) and releases it in a controlled manner(Ollinger and Roberg 1997). Ferritin is suggested to serve as a buffer system that detects iron deficiency and iron overload. It has been reported that oxidative stress triggers release of ferrous iron from ferritin and has to be exported from the cytosol (Ollinger and Roberg 1997). Slc40a1 is an iron transporter and exports released iron out of the cell. Cells encounter various number of stress conditions during amino acid starvation. One of the critical stress conditions is oxidative stress that triggers excessive release of iron from Ferritin (Ollinger and Roberg 1997). Induction in Slc40a1 gene expression in our results is suggested to be in response to excessive iron release from Ferritin under amino acid deprivation stress. It could be speculated that Slc40a1 is upregulated to export excessive iron from the cellular space.

Slc43a2

SLC43 represents the Na^+ independent system L like family and mediates the transport of heavy neutral amino acids across the cell membrane (Karunakaran, Umapathy et al. 2008, He, Vasiliou et al. 2009, Schioth, Roshanbin et al. 2013). Slc43a2 (LAT4) is a uniporter and functions as a facilitated diffuser of branched chain amino acids, methionine and

phenylalanine (Guetg, Mariotta et al. 2015). Slc43a2 was down regulated in response to amino acid starvation though it is an amino acid transporter. According to literature a number of other amino acid transporters utilize large neutral amino acids (LNAAs) including methionine and phenylalanine as their preferred substrate. Those include B^0AT-1 (SLC family 6a19), LAT-1 (SLC family 7a5), and members from Slc38 family; SNAT-1 (SLC family 38a1), and SNAT-2 (SLC family 38a2) (Zeng, Li et al. 2011). Since most of them have shown to be upregulated in response to amino acid levels according to literature, SLc43a2 expression level could have been down regulated to maintain the balance of these amino acids in the cellular environment during amino acid starvation conditions.

Conclusion

In this research project we observed upregulation in the expression of genes encoding amino acid transporters. Slc 7a1, Slc7a11, Slc6a9 are known amino acid transporters and were found to be upregulated in our study. These transporters have previously shown to be upregulated in response to amino acid starvation in other cell lines. Slc38a7 is the member of solute carrier family 38 and was upregulated in our study that previously did not show response to amino acid starvation. Only one member, Slc38a2, from this family has previously been reported to be upregulated in other cell lines in response to amino acid starvation. Various members from APC family have also been upregulated including an orphan member, Slc23a3 that belongs to APC superfamily. Mfsd11, which is an orphan member from MFS superfamily, was also upregulated. However one known amino acid transporter, Slc43a2, was downregulated in our study. Highest upregulation can be seen between 3 to 16 hours of study in expression of genes that encode these transporters. However, it is important to mention that the observed up and down regulation in the expression of genes encoding amino acid transporters in this research project is only based on samples from one biological replicate. A number of parallel experiments using multiple biological replicates have to be performed to verify our results and to achieve reliable data.

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Acknowledgement

I would like to express my gratitude to Professor Helgi Schiöth who provided me the opportunity to wok in his reputed laboratory. I would like to thank Associate professor Robert Fredriksson who had been a great mentor and teacher throughout my research period in this laboratory. I highly appreciate him for his supervision, guidance and all the cooperation in this time. He is the best supervisor anyone can wish for. I would like to thank all the members, post docs, PhDs and master students who had been so cooperative and for maintaining such a cool environment in the lab.

Finally, I would like to thank my coordinator Volkmar Passoth from SLU, who has always helped me and guided me in all this time.

Thank you very much everyone.

Tauseef Ahmad.