The gene expression of numerous SLC transporters is altered in the immortalized hypothalamic cell line N25/2 following amino acid starvation

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Amino acids are known to play a key role in gene expression regulation, and in mammalian cells, amino acid signaling is mainly mediated via two pathways, the mammalian target of rapamycin complex 1 (mTORC1) pathway and the amino acid responsive (AAR) pathway. It is vital for cells to have a system to sense amino acid levels, in order to control protein and amino acid synthesis and catabolism. Amino acid transporters are crucial in these pathways, due to both their sensing and transport functions. In this large-scale study, an immortalized mouse hypothalamic cell line (N25/2) was used to study the gene expression changes following 1, 2, 3, 5 or 16 h of amino acid starvation. We focused on genes encoding solute carriers (SLCs) and putative SLCs, more specifically on amino acid transporters. The microarray contained 28 270 genes and 86.2% of the genes were expressed in the cell line. At 5 h of starvation, 1001 genes were upregulated and 848 genes were downregulated, and among these, 47 genes from the SLC superfamily or atypical SLCs were found. Of these, 15 were genes encoding amino acid transporters and 32 were genes encoding other SLCs or atypical SLCs. Increased expression was detected for genes encoding amino acid transporters from system A, ASC, L, N, T, xc-, and y+. Using GO annotations, genes involved in amino acid transport and amino acid transmembrane transporter activity were found to be most upregulated at 3 h and 5 h of starvation.

It is vital for cells to have system for sensing amino acid levels in order to regulate protein and amino acid synthesis and catabolism [1]. In mammalian cells, amino acid signaling is, in principal, mediated via two pathways and the amino acid availability plays a key role in the regulation of gene expression [2]. The mammalian/mechanistic target of rapamycin complex 1 (mTORC1) pathway and the amino acid responsive (AAR) pathway can control the protein synthesis by either upregulate or downregulate it, depending on the levels of amino acids [3]. The mTORC1 pathway is activated when the cell has sufficient amino acid levels and function as a sensor for adequate amino acid concentrations, in order to maintain protein synthesis and cellular growth [4]. The AAR pathway, on the other hand, is activated when the cell has limited access to amino acids, which results in inhibition of general protein synthesis [5]. When the AAR pathway

Abbreviations
AAR, amino acid responsive; AARE, amino acid responsive element; AS, asparagine synthetase; ATF, activating transcription factor; CHOP, CCAAT/enhancer-binding protein homologous protein; eIF2\textalpha, eukaryotic initiation factor 2\textalpha; GCN2, general control nonderepressible 2; MFS, major facilitator superfamily; mTORC1, mammalian target of rapamycin complex 1; NSRE, nutrient-sensing responsive element; SLC, solute carrier.
is activated, the general control nonderepressible 2 (GCN2) kinases are activated by binding to uncharged tRNA, which accumulate during deprivation [3,6]. In turn, these kinases inactivate the eukaryotic initiation factor 2α (eIF2α) by phosphorylation resulting in inhibition of protein translation [7]. The activation transcription factor 4 (ATF4), and to some extent ATF2, are transcriptionally upregulated by the inhibition of protein synthesis and these factors play key roles in the regulation of gene expression [8,9]. These transcription factors bind elements, termed amino acid responsive element (AARE), nutrient-sensing element 1 (NSRE1) or NSRE2, which are short sequences of nucleotides, and genes holding these elements are upregulated [5,6,10,11]. The first responsive elements identified were found in the genes encoding CCAAT/enhancer-binding protein homologous protein (CHOP) and asparagine synthetase (AS) [11–13], which are induced during amino acid deprivation [14,15]. The solute carrier (SLC) superfamily is the largest family of transport proteins in mammals, with 456 members [16] divided into 52 families in human [17]. Out of these SLCs, over 60 have been found to transport amino acids, and in addition, another 40 orphans are closely related to known amino acid transporters, suggesting there could be over 100 amino acid transporters in human [18]. The SLCs are ATP-independent uniporters, symporters, or antiporters, and further divided into different transport systems (e.g. system A, L, N, and xc-) depending on transport mechanism and substrate profile [19]. Transporters are thought to be important regulators in nutrient sensing and signaling [20] and amino acid transporters have been suggested to function as transceptors, transporters with both transport and receptor functions [21]. They have the capacity to regulate the intracellular amino acid concentrations, and in addition, also sense alterations in extracellular amino acid levels [21]. Genes encoding amino acid transporters, with characterized responsive elements, from several SLC families, have previously been found to be induced upon amino acid starvation, e.g. Slc7a1 [22], Slc7a5 [23], Slc7a11 [24], Slc1a4 [25,26], Slc1a5 [26], Slc3a2 [26], and Slc38a2 [27]. The regulation has been studied in different cells, deprived of one or several amino acids, e.g. in mouse NIH3T3 cells, the system xc- activity and Slc7a11 mRNA were increased [24], in rat hepatic WB cells the Slc7a5/Slc3a2 expression and activity was induced [23], in rat C6 glioma cells Slc7a1 was upregulated [28] and Slc38a2 was found to be induced in both human HepG2 hepatoma cells [29] and human trophoblast BeWo cells [30]. However, how SLC encoding genes respond to amino acid starvation has not previously been studied on a larger scale.

In this study, the immortalized mouse embryonic hypothalamic cell line N25/2 was deprived of all amino acids for 1, 2, 3, 5, or 16 h. Hypothalamus has a well-established role in sensing amino acid levels [31,32] and therefore we chose to deprive a hypothalamic cell line of amino acids. The aim was to, on a large scale, study the regulation of genes encoding amino acid transporters and putative amino acid transporters from the SLC superfamily or atypical SLCs, using microarray analysis.

Materials and methods

Culturing of the immortalized hypothalamic cell line N25/2

The immortalized mouse embryonic hypothalamic cell line, N25/2, (mHypoE-N25/2, CEDARLANE, Burlington, ON, Canada) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco®, Life technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), (Gibco®, Life technologies), 1% penicillin-streptomycin, liquid (Gibco®, Life technologies), and 1% Fungizone® Antimycotic (Amphotericin B) (Gibco®, Life technologies) at 37 °C in a humidified atmosphere of 5% CO2, 95% air. Cells were grown to 70–90% confluence in Nunclon surface dishes 150 × 20 mm (Thermo Scientific, Waltham, MA, USA).

Amino acid deprivation of the immortalized hypothalamic cell line N25/2

Medium for the experiment was prepared with Earle’s balanced salt solution (EBSS) (Gibco®, Life technologies), 1 mm sodium pyruvate 100 mm (Gibco®, Life technologies), 4X MEM vitamin solution (100X) liquid (Gibco®, Life technologies). Neither the control medium nor the starved medium was supplemented with FBS. Following amino acids were added to the EBSS medium containing amino acids, 0.4 mm glycine, 0.4 mm l-arginine, 0.2 mm l-cystine, 4.0 mm l-glutamine, 0.2 mm l-histidine, 0.8 mm l-isoleucine, 0.8 mm l-leucine, 0.8 mm l-lysine, 0.2 mm l-methionine, 0.4 mm l-phenylalanine, 0.4 mm l-serine, 0.8 mm l-threonine, 0.08 mm l-tryptophan, 0.4 mm l-tyrosine, and 0.8 mm l-valine (Sigma-Aldrich, St. Louis, MO, USA), the same amino acid concentrations as in the commercially available DMEM medium. The complete DMEM medium was removed and replaced with EBSS medium lacking amino acids or EBSS medium supplemented with amino acids. The cells were treated in the different media for 1 h (n = 1), 2 h (n = 1), 3 h (n = 1), 5 h (n = 4), or 16 h (n = 1) before RNA was extracted with RNeasy Midi Kit (Qiagen, Hilden, Germany), following the manufacture’s protocol.
Microarray analysis of gene expression

The RNA concentration was measured with ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA, USA). 250 ng of total RNA from each sample was used to produce amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the Ambion WT Expression Kit (P/N 4425209 Rev C 09/2009) and Affymetrix GeneChip® WT Terminal Labeling and Hybridization User Manual (P/N 702808 Rev 3, Affymetrix Inc., Santa Clara, CA, USA). GeneChip® ST Arrays (GeneChip® Mouse Gene 1.0 ST Array) were hybridized for 16 h in a 45 °C incubator, and rotated at 60 rpm. According to the GeneChip® Expression Wash, Stain and Scan Manual (PN 702731 Rev 3, Affymetrix Inc.), the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip® Scanner 3000 7G. Analysis of the gene expression data was carried out in the freely available statistical computing language R (http://www.r-project.org) using packages available from the Bioconductor project (www.bioconductor.org). The raw data was normalized using the robust multi-array average (RMA) method first suggested by Li and Wong in 2001 [33,34]. In order to search for the differentially expressed genes between the X samples and the Y samples group an empirical Bayes moderated t-test was then applied [35], using the ‘limma’ package [36]. To address the problem with multiple testing, the P-values were adjusted using the method of Benjamini and Hochberg [37]. The quadruplicates at 5 h of starvation (n = 4); singlets in each treatment group was run at a time followed by microarray analysis, while 5-h triplicates together with singlets from the other incubation times (1, 2, 3, and 16 h) were run and analyzed with microarray at a different time. The microarray data from 5 h were combined and analyzed together as one set of data of quadruplicates. The array was performed at the Array and Analysis Facility, Science for Life Laboratory at Uppsala Biomedical Center (BMC), Husargatan 3, 751 23 Uppsala, Sweden. The microarray data can be found in the NCBI-GEO database with accession number GSE61402.

qPCR analysis of gene expression

The RNA concentration was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and cDNA was synthesized using the SuperScript® III Reverse Transcriptase Kit (Invitrogen, Waltham, MA, USA) following the manufacture’s protocol before diluted to a concentration of 5 ng µL⁻¹. The cDNA samples were analyzed using qPCR on MyiQ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). All primers were designed with beacon designer v.8 (Premier Biosoft, Palo Alto, CA, USA), and sequences can be found in Table 1. Housekeeping genes used for normalization were mouse mβ-Actin, mβ-Tubulin, and mGlyceraldehyde 3-phosphate dehydrogenase. The qPCR reactions for all primer pairs except Slc38a7 and Slc23a3 were run in a total volume of 12.5 µL with 5 ng cDNA using BR SYBR® Green SuperMix for IQ™ Systems (Quanta Biosciences, Gaithersburg, MD, USA) because of requirement of cDNA amplification. For Slc38a7 and Slc23a3, SYBR® Select Master Mix kit (Applied Biosystems™, Waltham, MA, USA) was used for qPCR reaction in a total volume of 20 µL with 5 ng of cDNA. The amplification was performed under following conditions; for primers with annealing temperature ≥60 °C; initial denaturation 95 °C for 2 min followed by 40 cycles of: denaturation at 95 °C for 15 s, annealing/elongation at 60 °C for 1 min. For primers with annealing temperature ≤60 °C; initial denaturation 95 °C for 2 min, followed by 40 cycles of: denaturation at 95°C for 15 s, annealing at 55–60 °C for 15 s, and elongation at 72°C for 1 min. In both cases, the cycling was followed by melt curve performance starting at 55 to 95 °C with steps of 0.5 °C. The experiment was performed in triplicates. Water was used as a negative control and cDNA from a whole mouse brain was included on each plate as a positive control. For Slc16a2, Slc40a1, and Mfsd2a, samples from 16-h starvation were analyzed using 40ng cDNA per qPCR reaction combined with 0.05 µL of each primer (100 pmol µL⁻¹), 2 µL 10X DreamTaq buffer (Thermo Fisher Scientific), 0.2 µL of 25 mm dNTP mix (Thermo Fisher Scientific), 1 µL DMSO, 0.5 µL SYBR Green (Invitrogen), and 0.08 µL of Dream Taq (5U µL⁻¹, Thermo Fisher scientific). The volume was adjusted to 20 µL with sterile water. qPCR was run using initial denaturation for 30 s at 95 °C, 50 cycles of 10 s at 95 °C, 30 s at 52–55 °C (optimal temperature depending on primer), and 30 s at 72 °C. A melting curve was performed starting at 55 °C for 81 cycles at 10-s interval and a temperature increase of 0.5 °C per cycle. All q-PCR
were run in quadruplicates and a negative control was included on each plate.

qPCR data analysis and relative expression calculations

The MyIQ software (Bio-Rad Laboratories) was used to obtain the qPCR threshold cycle Ct-values and melt curve data. The melting curves were compared to the positive and negative control to verify that only one product was amplified. The triplicates for the raw Ct-values were compared and outliers were excluded if the difference was greater than 0.99 between the Ct triplicates. The efficiency for each primer pair was determined using LinRegPCR v7.5. The average qPCR primer efficiency and standard deviation for each primer was calculated after outliers were removed using Grubbs test (GraphPad Software, San Diego, CA, USA). The delta Ct-method was used to transform the mean of raw Ct-values into relative quantities with standard deviations. Geometric means of all three housekeeping genes were calculated and used for normalization. Unpaired t-tests (*p≤0.05, **p≤0.01, ***p≤0.001) were performed using GraphPad Prism 5 between the control cells and the starved cells.

Results

Amino acid starvation of the immortalized hypothalamic cell line N25/2

The hypothalamic cell line N25/2 was starved of all amino acids for 1, 2, 3, 5, and 16 h. Shorter times were chosen because this would possibly enable detection of changes in expression of genes involved in an earlier response. A starvation time of 16 h was chosen based on previous studies in other cell lines [25,38], where similar times resulted in marked expression level alterations of amino acid transporters. The microarray GeneChip had 28 270 probes and 86.2% of the genes had detectable expression in the cell line (i.e. value of expression >5). About 1849 genes were significantly (adj. P-value <0.01) up- or downregulated at 5 h of amino acid starvation compared with controls, and of these, 1001 genes were upregulated and 848 genes were downregulated. Among these, 47 transcripts encoding SLCs or atypical SLCs were found. We provide expression levels for all genes on the array and the data can be found in the NCBI-GEO database with accession number GSE61402.

Principal component analysis

The gene expression for the entire array, at all times, was analyzed in a principal component analysis (PCA) plot (Fig. 1). The nonstarved cells cluster to the left in the figure, while the amino acid deprived cells shift to the right, with more shift with increased time of deprivation.

Type of SLC transporters and putative transporters

The 47 SLC or atypical SLC genes found, were divided into four groups based on what type of transporters they encode (Fig. 2). Among these, 15 genes encoded amino acid transporters from the SLC family, 10 genes encoded orphan SLCs, 4 genes encoded atypical orphan

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Table 1. Primers used for the qPCR reactions.

| Primer     | Forward/Reverse |
|------------|-----------------|
| Slc7a11    | tgg aac tgc taa tgc gaag cag gaa ttt cac att ga |
| Slc4a1     | ctt tgc tct gtt ttc ggg acg cca aga tag |
| Mfsd11     | cta tgg ttt bca gtt tgc tgg aga gga |
| Slc25a36   | acc tgt gcc aca acc ata atc cat aag ctt ctt gaa |
| Slc6a9     | ttt ccc ata cct tgt caa aca gct cca tga aqa aqa |
| Slc7a1     | aat tat cat ccc agg actg/gac cag gac att gat aca |
| Slc23a3    | tct tca act cca act cac at/aca aag gca gag atg aac |
| Slc9a9     | tga ttt tgg tgg aac tgtct/cct ggt cgg tga tgt tga |
| Slc25a33   | aat tcc ggc ttc tttt/tcc tga cag ctt gtt |
| Slc38a7    | tgc tct tgg cgg ctt atac/gct cct tgt aca tca cag |
| Slc16a9    | ccc ccc ttt ctt ctt ctt ctt gag ttt ctt ctt cgg cgt |
| Slc16a2    | ttt aat tgt cat ctc tgt ctt gaa gtc caa ggt aat gtt |
| Mfsd2a     | ttt tgt gca cag tgt gtt ctt gga tga tag aga |
| Slc43a2    | cct ttc tgg cca gaa ctt cgc ctt gat ggc ata gga |
| mβ-Actin   | agg gct cct ctt ctc cag tat ctc ctt cgt ggt aag tgc |
| mβ-Tubulin | gcc ttc tgt gtt ccc acc gcc tgc ttc acc acc acc ttc |
| mGAPDH     | gaa tta gcc cct tgg aga gga tga ggg gcg |
SLCs, and 18 genes encoded nonamino acid SLC transporters, e.g., transporters for thiamine, iron, sugars, vitamins, ions, fatty acids, UTP, pyrimidine nucleotides, and hormones. In Table 2, a summary of the amino acid transporter encoding genes are presented. In Table 3, the genes encoding orphan SLCs, atypical orphan SLCs and nonamino acid SLC transporters are listed.

GO annotations to cluster gene categories

The genes were analyzed using Gene Ontology (GO) annotations for biological process, Table 4, and molecular function, Table 5, using EASE version 2.0. An EASE score ≤0.01 was considered significant, and a maximum of 10 categories for each time were considered. This analysis was performed to pinpoint when the gene clusters related to amino acid transport were regulated. About 1849 genes from each starvation time were used in the GO analysis. The genes from the incubation times 1, 2, 3, and 16 h, with singlets in each treatment group, were sorted by the absolute value of the difference in log 2 expression value between the control and starved cells, and the top 1849 genes were extracted.

After 1 h, only one gene category belonging to biological process, ‘cell communication’, was upregulated and many genes involved in metabolic processes were downregulated (Table 4). At this time, only one category of molecular function was downregulated, ‘nucleic acid binding’ (Table 5).

At 2 h of amino acid starvation, genes from the groups ‘regulation of cell cycle’ and ‘cell proliferation’ were found to be up- and downregulated in approximately equal proportions. Two of the largest groups of upregulated genes belong to ‘intracellular signal transduction’ and ‘cell proliferation’. Two groups of genes involved in transcription from RNA polymerase II promoter were also upregulated. Among the downregulated genes, a large number belong to groups involved in DNA-dependent transcription. The genes involved in anatomical structure morphogenesis and regulation of cellular processes and biological processes were also downregulated (Table 4). From the molecular function analysis, many GTPase genes were upregulated and the two largest groups of upregulated genes were involved in protein and DNA binding. Many different groups of genes involved in binding were downregulated along with groups of chemokine, cytokine and chemoattractant activity (Table 5).

After 3-h starvation, a large number of transporters were found to be upregulated. For example, genes encoding transporters for carboxylic acid, organic acid, amino acid, and amine transport were upregulated. The largest group of upregulated genes belongs to the category ‘regulation of transcription, DNA-dependent’ and also genes from the category ‘regulation of cell cycle’ were upregulated. One large group of downregulated genes were genes involved in metabolic processes.
The genes from the categories ‘biosynthetic process’, ‘cell proliferation’, and ‘cell cycle’ were also downregulated (Table 4). Regarding molecular function, genes involved in transferase activity and ‘nucleic acid binding’ were downregulated. Genes involved in transmembrane transporter activity and transcription factor activity were upregulated (Table 5).

At 5 h, numerous genes involved in the category ‘metabolic process’ were found to be upregulated, together with genes coding for transporters involved in organic and carboxylic acid transport. Genes involved in sterol, cholesterol, and alcohol metabolism were all downregulated (Table 4). Upregulated gene categories belonging to molecular function were groups of genes involved in transmembrane transporter activity. Several genes involved in binding were also upregulated as well as genes involved in transcription and translation. Numerous binding genes were also downregulated, as well as genes involved in transferase, kinase and protein tyrosine/threonine phosphatase activity (Table 5).

After 16 h of amino acid deprivation five different groups of metabolic processes were upregulated, ‘metabolic process’, nucleobase-containing compound, RNA, tRNA, and cellular amino acid metabolic process. ‘RNA processing’, ‘RNA modification’, and ‘tRNA modification’ were also upregulated. Groups of genes involved in processes with lipids, cholesterol, and sterol were downregulated. The three largest downregulated groups of genes were ‘multicellular organismal development’, ‘organ morphogenesis’, and ‘anatomical structure morphogenesis’ (Table 4). From molecular function analysis, different groups of transmembrane transport activity were upregulated along with groups involved in transferase activity. The largest group of genes that were upregulated belonged to ‘nucleic acid binding’. Many groups of binding proteins were downregulated and also oxidoreductase, cytokine, and growth factor activity (Table 5).

**Heat map of the gene expression for SLCs and atypical SLCs**

A heat map over the alterations in gene expression (1–16 h) for all 47 genes encoding SLCs or atypical SLCs...
### Table 3. Up- and downregulated genes encoding nonamino acid-transporting SLCs, orphan SLCs, or atypical SLCs. Data from 5 h of amino acid starvation. The information about substrate/system is from SLC tables, http://slc.bioparadigms.org/.

| Clan                              | Pfam family | Gene      | Substrate                  | Adj. P-value | log2Fold Change | Probe ID   |
|-----------------------------------|-------------|-----------|----------------------------|--------------|-----------------|------------|
| **MFS**                           | FPN1        | Slc40a1   | ferrous iron               | 2.1E-03      | 1.6             | 10354374   |
| **APC superfamily**               | Xan_ur_permease | Slc23a3  | O                          | 2.6E-04      | 1.5             | 10355717   |
| **MFS**                           | Sugar_tr    | Slc2a12   | glucose                    | 1.8E-06      | 1.2             | 10368229   |
|                                   | MFS_1       | Slc25a33  | UTP                        | 2.8E-05      | 1.2             | 10518726   |
| **CPA/AT transpoter family**      | Na_H_exchanger | Slc9a9   | Na⁺, K⁺, H⁺                 | 1.5E-05      | 1.0             | 10587854   |
| **Drug/metabolite transporter**    | Cation_efflux | Slc30a1  | O                          | 3.0E-05      | 1.0             | 10352777   |
| **MFS**                           | MFS_1       | Slc16a14  | O                          | 2.3E-04      | 0.7             | 10356240   |
| **MFS**                           | Folate_carrier | Slc19a2 | thiamine                   | 5.7E-04      | 0.7             | 10351259   |
|                                   | Mito_carr   | Slc25a37  | Fe²⁺                       | 1.4E-04      | 0.6             | 10421172   |
| **MFS**                           | MFS_1       | Slc8a9    | Na⁺, K⁺, H⁺                 | 1.5E-05      | 1.0             | 10352777   |
|                                   | Mito_carr   | Slc25a36  | pyrimidine                 | 1.2E-03      | 0.5             | 10479979   |
| **DMT**                           | Cation_efflux | Slc30a4 | O                          | 2.3E-04      | 0.4             | 10487021   |
| **MFS**                           | MFS_1       | Slc17a5   | sialic acid, other          | 1.5E-03      | 0.4             | 10595189   |
|                                   | Mito_carr   | Slc25a30  | O                          | 8.9E-04      | 0.4             | 10421648   |
| **APC superfamily/STAS domain**   | Sulfate_transp | Slc26a11 | Cl⁻, HCO₃⁻, SO₄²⁻, oxalate | 6.3E-03      | 0.4             | 10382133   |
| **DMT**                           | Cation_transp | Slc26a2 | SO₄²⁻, oxalate, Cl⁻         | 7.9E-03      | –0.3            | 10459183   |
| **MFS**                           | Folate_carrier | Slc19a3 | thiamine                   | 3.9E-03      | 0.3             | 10356145   |
| **MFS**                           | MFS_1       | Mfsd11    | O                          | 3.5E-05      | 0.7             | 10382852   |
| **DMT**                           | TPT         | Slc35e1   | O                          | 9.9E-04      | –0.3            | 10579724   |
| **DMT**                           | UAA         | Slc35b1   | O                          | 3.8E-03      | –0.3            | 10380524   |
| **APC superfamily/STAS domain**   | Sulfate_transp | Slc26a2 | SO₄²⁻, oxalate, Cl⁻         | 7.9E-03      | –0.3            | 10459183   |
| **ANL superfamily**               | AMP-binding | Slc27a4   | LCFA, VLCFA                 | 1.4E-03      | –0.4            | 10470751   |
| **APC superfamily/Phosphotransferase/anion transport protein** | HCO3_cotransp | Slc4a3   | Cl⁻, HCO₃⁻                 | 7.1E-03      | –0.4            | 10347697   |
| **MFS**                           | MFS_1       | Slc16a2   | T2, rT3, T3, T4             | 2.5E-04      | –0.5            | 10606186   |
|                                   | Mito_carr   | Slc25a1   | citrate, isocitrate, malate, PEP | 4.0E-04   | –0.5            | 10438262   |
| **DMT**                           | Zip         | Slc39a10  | Zn                         | 3.9E-05      | –0.6            | 10354389   |
| **MFS**                           | Sugar_tr    | Slc2a1    | glucose, galactose, mannose, glucosamine | 3.0E-04 | –0.7            | 10507594   |
|                                   | MFS_1       | Slc25a10  | malate, phosphate, succinate, sulfate, thiosulfate | 2.0E-05 | –0.7            | 10383395   |
| **MFS**                           | Mito_carr   | Slc25a35  | O                          | 3.1E-03      | –0.8            | 10377372   |
| **MFS**                           | MFS_1       | Mfsd2a    | O                          | 4.1E-04      | –0.9            | 10516064   |
| **MFS**                           | MFS_1       | Slc16a9   | O                          | 9.2E-06      | –0.9            | 10363860   |
Table 4. Up- and downregulated gene categories in response to amino acid starvation across GO biological processes. For each GO term, the number of genes up- or downregulated in response to amino acid starvation is presented.

| Time (h) | Ontology ID biological process | Gene category | Upregulated | Downregulated |
|----------|--------------------------------|---------------|-------------|---------------|
| 1        | GO:0007154 Cell communication   |               | 91          |               |
| 1        | GO:0008152 Metabolic process    |               | 224         |               |
| 1        | GO:0006139 Nucleobase-containing compound metabolic process | | 101 | |
| 1        | GO:0016070 RNA metabolism       |               | 22          |               |
| 2        | GO:0051726 Regulation of cell cycle |          | 23          | 19            |
| 2        | GO:0007049 Cell cycle           |               | 31          |               |
| 2        | GO:0035556 Intracellular signal transduction | | 36 | |
| 2        | GO:0007264 Small GTPase-mediated signal transduction | | 15 | |
| 2        | GO:0006357 Regulation of transcription from RNA polymerase II promoter | | 15 | |
| 2        | GO:0009894 Regulation of catabolic process | | 5 | |
| 2        | GO:0008283 Cell proliferation    |               | 35          | 35            |
| 2        | GO:0019222 Regulation of metabolic process | | 8 | |
| 2        | GO:0006366 Transcription from RNA polymerase II promoter | | 17 | |
| 2        | GO:0000278 Mitotic cell cycle   |               | 15          |               |
| 2        | GO:0006355 Regulation of transcription, DNA-dependent | | 60 | |
| 2        | GO:0006351 Transcription, DNA-dependent | | 62 | |
| 2        | GO:0050794 Regulation of cellular process | | 15 | |
| 2        | GO:0050789 Regulation of biological process | | 15 | |
| 2        | GO:0009653 Anatomical structure morphogenesis | | 41 | |
| 2        | GO:0007266 Rho protein signal transduction | | 5 | |
| 3        | GO:0046942 Carboxylic acid transport | | 7 | |
| 3        | GO:0015849 Organic acid transport | | 7 | |
| 3        | GO:0006365 Amino acid transport | | 6 | |
| 3        | GO:0015837 Amine transport | | 6 | |
| 3        | GO:0006355 Regulation of transcription, DNA-dependent | | 49 | |
| 3        | GO:0051726 Regulation of cell cycle | | 16 | |
| 3        | GO:0008152 Metabolic process | | 221 | |
| 3        | GO:0008283 Cell proliferation | | 43 | |
| 3        | GO:0007049 Cell cycle | | 32 | |
| 3        | GO:0009101 Glycoprotein biosynthetic process | | 9 | |
| 3        | GO:0009058 Biosynthetic process | | 47 | |
| 5        | GO:0008152 Metabolic process | | 231 | |
| 5        | GO:0006412 Translation | | 20 | |
| 5        | GO:0009058 Biosynthetic process | | 57 | 53 |
| 5        | GO:0006139 Nucleobase-containing compound metabolic process | | 103 | |
| 5        | GO:0009451 RNA modification | | 12 | |
| 5        | GO:0015849 Organic acid transport | | 9 | |
| 5        | GO:0046942 Carboxylic acid transport | | 9 | |
| 5        | GO:0009059 Macromolecule biosynthetic process | | 45 | |
| 5        | GO:0006396 RNA processing | | 22 | |
| 5        | GO:0006413 Translational initiation | | 8 | |
| 5        | GO:0016125 Sterol metabolic process | | 9 | |
| 5        | GO:0008203 Cholesterol metabolic process | | 8 | |
| 5        | GO:0016126 Sterol biosynthetic process | | 6 | |
| 5        | GO:0030036 Actin cytoskeleton organization | | 9 | |
| 5        | GO:0006996 Organelle organization | | 25 | |
| 5        | GO:0006066 Alcohol metabolic process | | 16 | |
| 5        | GO:0030029 Actin filament-based process | | 9 | |
| 5        | GO:0009101 Glycoprotein biosynthetic process | | 10 | |
| 5        | GO:0007010 Cytoskeleton organization | | 21 | |
| 16       | GO:0006139 Nucleobase-containing compound metabolic process | | 106 | |
| 16       | GO:0006396 RNA processing | | 28 | |
| 16       | GO:0016070 RNA metabolic process | | 28 | |
found to be significantly altered at 5 h of starvation was generated (Fig. 3). The genes and experiments were hierarchical clustered and the clustering displayed a clear time-dependent effect with 1, 2, and 3 h clustering separately from the longer times, 5 h and 16 h.

Verification of the microarray data using qPCR analysis

The microarray data were verified using qPCR analysis for some of the genes found to be significantly altered at 5 h of starvation (Fig. 4). The genes analyzed were Slc7a11, Slc40a1, Mfsd11, Slc25a36, Slc6a9, Slc7a1, Slc23a3, Slc9a9, Slc25a33, Slc38a7, Slc16a9, Slc16a2, Mfsd2a, and Slc43a2. The results from the qPCR analysis comply with the results from the microarray, although the gene expression for Slc16a2 was not significantly downregulated at 5 h of starvation using qPCR.

Discussion

Complete amino acid starvation of the immortalized hypothalamic cell line N25/2 was performed and the gene expression alterations at 1, 2, 3, 5, or 16 h of starvation were analyzed using Affymetrix expression microarrays. At 5 h of starvation, expression levels of 1849 genes were significantly altered after adjustments for multiple testing; 1001 genes were upregulated, while 848 genes were downregulated. In this study, we decided to focus our analysis on genes encoding transporters from the SLC family, especially amino acid transporters and or putative amino acid transporters. The 1849 genes identified for each incubation time were analyzed using GO annotations for biological process and molecular function to pinpoint where the gene clusters related to amino acid transport were regulated. The overall analysis showed that early, following 1–2 h of amino acid starvation, there were mainly changes in gene categories involved in basal cellular processes such as nucleic acid binding. GTPase-related genes were also upregulated, whereas chemokine-related genes and genes involved in metabolism were downregulated, suggesting that the cells respond with reduced growth. After 3 h and 5 h of starvation, genes involved in transport and metabolic processes were upregulated, possibly to increase intake and availability of amino acids and other substrates and genes involved in transferase activity and binding were downregulated. After 16 h of deprivation, there was upregulation of genes involved in RNA processes and transport activity and downregulation of genes involved in binding, lipid, sterol and cholesterol metabolism and organ morphogenesis, manifesting the assumption that the cells in general react with reduced growth, increased uptake and biomolecule synthesis. Taken together, genes involved in amino acid transport and amino acid transmembrane transporter activity were mainly upregulated at 3 h and 5 h, but amino acid transmembrane transporter activity was also upregulated following 16 h of amino acid starvation.

Forty-seven genes encoding SLCs were found to be altered in the immortalized hypothalamic cell line at 5 h of starvation and these were divided into amino acid transporter encoding genes (15 genes) and genes...
Table 5. Up- and downregulated gene categories in response to amino acid starvation across GO molecular function. For each GO term, the number of genes up- or downregulated in response to amino acid starvation is presented.

| Time (h) | Ontology ID | Gene category | Upregulated | Downregulated |
|---------|-------------|---------------|-------------|---------------|
| 1       | GO:003676   | Nucleic acid binding | 100         |               |
| 2       | GO:000515   | Protein binding   | 73          |               |
| 2       | GO:0005083  | Small GTPase regulatory/interacting protein activity | 12         |               |
| 2       | GO:003234   | Enzyme regulator activity | 25         |               |
| 2       | GO:003924   | GTPase activity   | 14          |               |
| 2       | GO:003095   | GTPase regulator activity | 12         |               |
| 2       | GO:003677   | DNA binding       | 58          |               |
| 2       | GO:0005100  | Rho GTPase activator activity | 3          |               |
| 2       | GO:003676   | Nucleic acid binding | 90         | 66            |
| 2       | GO:003677   | DNA binding       | 90          |               |
| 2       | GO:0042379  | Chemokine receptor binding | 7          | 7             |
| 2       | GO:0008009  | Chemokine activity | 7           |               |
| 2       | GO:0005488  | Binding           | 203         |               |
| 2       | GO:0042056  | Chemoattractant activity | 7          |               |
| 2       | GO:0016644  | G-protein-coupled receptor binding | 7          |               |
| 2       | GO:0005125  | Cytokine activity | 16          |               |
| 2       | GO:0016757  | Transferase activity, transferring glycosyl groups | 13         |               |
| 3       | GO:000700   | Sequence-specific DNA binding transcription factor activity | 36         |               |
| 3       | GO:0015171  | Amino acid transmembrane transporter activity | 7          |               |
| 3       | GO:0030528  | Transcription regulator activity | 41         |               |
| 3       | GO:0005275  | Amine transmembrane transporter activity | 7          |               |
| 3       | GO:0005342  | Organic acid transmembrane transporter activity | 7          |               |
| 3       | GO:0046943  | Carboxylic acid transmembrane transporter activity | 7          |               |
| 3       | GO:003677   | DNA binding       | 54          |               |
| 3       | GO:0016757  | Transferase activity, transferring glycosyl groups | 18         |               |
| 3       | GO:003676   | Nucleic acid binding | 101        | 62            |
| 3       | GO:0016740  | Transferase activity | 62         |               |
| 3       | GO:0016758  | Transferase activity, transferring hexosyl groups | 11         |               |
| 5       | GO:0015171  | Amino acid transmembrane transporter activity | 9          |               |
| 5       | GO:0005275  | Amine transmembrane transporter activity | 9          |               |
| 5       | GO:0046943  | Carboxylic acid transmembrane transporter activity | 9          |               |
| 5       | GO:0005342  | Organic acid transmembrane transporter activity | 9          |               |
| 5       | GO:0005488  | Binding           | 229         |               |
| 5       | GO:003712   | Transcription cofactor activity | 14         |               |
| 5       | GO:0008134  | Transcription factor binding | 15         |               |
| 5       | GO:003824   | Catalytic activity | 162         |               |
| 5       | GO:003676   | Nucleic acid binding | 94          |               |
| 5       | GO:0045182  | Translation regulator activity | 10         |               |
| 5       | GO:0005515  | Protein binding   | 98          | 19            |
| 5       | GO:003779   | Actin binding     | 19          |               |
| 5       | GO:0008092  | Cytoskeletal protein binding | 23         |               |
| 5       | GO:0016757  | Transferase activity, transferring glycosyl groups | 23         |               |
| 5       | GO:0001385  | Protein tyrosine/threonine phosphatase activity | 18         |               |
| 5       | GO:0016301  | Kinase activity   | 38          |               |
| 16      | GO:003676   | Nucleic acid binding | 96          |               |
| 16      | GO:0008168  | Methyltransferase activity | 12         |               |
| 16      | GO:0016741  | Transferase activity, transferring one-carbon groups | 12         |               |
| 16      | GO:0015171  | Amino acid transmembrane transporter activity | 8          |               |
| 16      | GO:0005275  | Amine transmembrane transporter activity | 8          |               |
| 16      | GO:0046943  | Carboxylic acid transmembrane transporter activity | 8          |               |
| 16      | GO:0005342  | Organic acid transmembrane transporter activity | 8          |               |
| 16      | GO:0008757  | S-adenosylmethionine-dependent methyltransferase activity | 8          |               |
encoding nonamino acid transporters (18 genes), orphan SLCs (10 genes), or atypical SLCs (4 genes). About 13 upregulated (Slc7a11, Slc6a9, Slc7a1, Slc1a4, Slc7a5, Slc1a5, Slc38a7, Slc38a1, Slc3a2, Slc38a2, Slc25a26, Slc15a4, and Slc16a10) and two downregulated (Slc3a1 and Slc43a2) genes encoding amino acid transporters were found, see Table 2. Three members from the SLC7 family, the system y+ encoding gene Slc7a1 (CAT-1) [39], the system L encoding gene Slc7a5 (LAT-1) [40], and Slc7a11 (xCT) from system xc- [41], had induced gene expression in the hypothalamic cell line, and all three were among the top five most upregulated amino acid transporters at 5 h of deprivation. Slc7a1 [28,42], Slc7a5 [23], and Slc7a11 [24] all have been found to respond with increased gene expression following amino acid starvation in several studies. Slc7a11 forms a dimer with the heavy subunit Slc3a2, a cysteine/glutamate exchanger, belonging to system xc- [43]. Slc3a2 also heterodimerizes with Slc7a5, and forms a system L transporter for large neutral amino acids [43]. Slc3a2 has been found to be upregulated in human prostate cancer cells in an

| Time (h) | Ontology ID | Gene category | Upregulated | Downregulated |
|---------|-------------|---------------|-------------|---------------|
| 16      | GO:0015203  | Polyamine transmembrane transporter activity | 6           |               |
| 16      | GO:0015175  | Neutral amino acid transmembrane transporter activity | 4           |               |
| 16      | GO:0008092  | Cytoskeletal protein binding | 29          |               |
| 16      | GO:0003779  | Actin binding | 23          |               |
| 16      | GO:0005509  | Calcium ion binding | 47          |               |
| 16      | GO:0048872  | Metal ion binding | 72          |               |
| 16      | GO:0016491  | Oxidoreductase activity | 50          |               |
| 16      | GO:0008083  | Growth factor activity | 19          |               |
| 16      | GO:0008289  | Lipid binding | 17          |               |
| 16      | GO:0005543  | Phospholipid binding | 9           |               |
| 16      | GO:0005544  | Calcium-dependent phospholipid binding | 6           |               |
| 16      | GO:0005125  | Cytokine activity | 22          |               |

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Fig. 3. A heat map of the 47 regulated genes encoding SLCs or atypical SLCs. The heat map displays the alterations in gene expression for the starved cells compared with controls in the hypothalamic cell line N25/2 at 1, 2, 3, 5, or 16 h of amino acid starvation. The color scale represents the log2 difference for (1, 2, 3, and 16 h) and the log2 fold change value (5 h) between starved and control cells. Green color represents downregulation and red color represents upregulation of gene expression. The genes and experiments were hierarchical clustered. Genes encoding amino acid transporters are highlighted in yellow.
Fig. 4. Gene expression data from the hypothalamic cell line N25/2 using qPCR to verify the microarray data. Normalized expression level ± SD, n = 3, (n = 4 for 16 h samples for Slc16a2, Slc40a1, and Mfsd2a). Unpaired t-tests were performed, *≤0.05, **≤0.01, ***≤0.001 between starved cells and controls. Dark blue bars represent amino acid starved cells and light blue bars represent amino acid-treated control cells. The x-axis represents time of treatment in hours. Note that in some graphs, the data for 1 h of treatment are missing and the scale on y-axis varies in the graphs.
ATF4-mediated way [26] and we also found induced gene expression of Slc3a2 in the hypothalamic cell line. Slc7a5/Slc3a2 together with Slc1a5 are known to be involved in amino acid signaling and physiologically relevant activators in the mTORC1 pathway, where glutamine and leucine translocation is central, without this transport the activation is absent [44,45]. Moreover, the glycine transporter encoding gene, Slc6a9 [46], was found to be the second most upregulated gene at 5 h of starvation in the hypothalamic cell line. Two members from the SLC1 family were found to be induced, the system ASC encoding genes, Slc1a4 and Slc1a5 [47]. This is in concurrence with previous studies, where the expression of Slc1a4 was upregulated in human HepG2/C3A cells following starvation [25] and Slc1a5 was found to be upregulated in prostate cancer cells in an ATF4-dependent way [26]. Furthermore, three genes from the SLC38 family were upregulated. The expression of the system A encoding gene, Slc38a2 was increased, which is in agreement with several studies in different cell types [21,30,38]. More remarkably, there was also induced expression of Slc38a1, as well classified to system A [48]. In a previous study, Slc38a2 mRNA and protein expression were upregulated but no effect was seen on expression of Slc38a1 [30]. However, when SLC1A5 (Slc38a5) was silenced in cancer cells, SLC38A1 (Slc38a1) was found to be upregulated in an amino acid starvation response manner [49]. Furthermore, in our study also the system N encoding gene Slc38a1 was upregulated [50], which has not previously been shown to respond to amino acid levels.

Among the genes encoding nonamino acid transporters, orphan SLCs, or atypical SLCs, 32 genes (18 upregulated, 14 downregulated) were found, see Table 3. About 14 of the genes were orphans, including 4 atypical SLCs, and 18 genes encode transporters for e.g. sugars, ions, hormones, iron, fatty acids, and vitamins. Among the orphan SLC genes, the gene Slc23a3 was the most upregulated one, and in the heat map (Fig. 3), it forms a cluster with the glycine transporter Slc6a9 (GLYT1) [51] and the cationic t-amino acid transporter Slc7a1 [43], indicating that they are regulated in a similar way. The SLC23 family, the Na+ dependent ascorbic acid transporter family, with four members, have so far two characterized L-ascorbic acid transporters [52]. Slc23a3 belongs to the Pfam clan Amino acid-Polymine-organCat ion (APC) superfamily. The APC clan also encloses the amino acid transporter-encoding genes, Slc7a11 [24], Slc7a1 [42], Slc7a15 [23], and Slc38a2 [27], known to be upregulated in response to amino acid starvation. It is therefore possible that Slc23a3 also could encode a transporter with preference for amino acids. In addition, we found 13 genes belonging to the major facilitator superfamily (MFS) clan, encoding putative SLC transporters, Mfsd1, Mfsd2a, Mfsd7b, and Mfsd11 or genes encoding SLC transporters Slc2a1, Slc2a12, Slc16a2, Slc16a9, Slc16a14, Slc17a5, Slc19a2, Slc19a3, and Slc40a1. The MFS family is the largest group of phylogenetically related genes with the SLC superfamily, and at least 13 of the SLC families belong to the MFS clan [53]. Mfsd11 was upregulated and had a similar regulation pattern in the heat map as the amino acid transporter genes, Slc3a2 and Slc38a2. Furthermore, the orphan gene Mfsd2a were among the most downregulated genes at 5 h of starvation, while the two orphan members Mfsd7b and Mfsd11 were upregulated. We found four members, which are phylogenetically closely related [54], from the SLC16 family. Slc16a2 and Slc16a9 were downregulated while Slc16a10 and Slc16a14 were upregulated in the hypothalamic cell line. Slc16a9 and Slc16a14 are orphans, while Slc16a2 (MCT8) is a transporter for thyroid hormones [55], and Slc16a10 (MCT10) is a transporter for thyroid hormones [56] and the aromatic amino acids tryptophan, tyrosine, and phenylalanine, classified to system T [57,58]. It is possibly that Slc16a14 could encode a transporter for amino acids.

The immortalized cell line N25/2 used in this study is in many aspects different from normal neuronal cells. For example, N25/2 cells divide readily while primary neuronal cells do not. Still, the N25/2 cells have retained many neuronal characteristics such as formation of synapse like structures and expression of neuronal markers such as neuro N and synaptic vesicle proteins 2 (SV2). The cell line was also originally created by infection of mouse embryonic hypothalamic cultures with SV40 retroviruses (https://www.cedarlanelabs.com/Products/Detail/CLU110?lob=AllProducts). This would likely result in fewer genetic changes than what is found in a cell line derived from tumors. However, the in vivo validity of the present results needs to be further investigated, preferably in whole animals or in primary neuronal cells. In our large-scale study, we have screened the entire mouse genome for genes responding to amino acid deficiency, and we have not measured any changes on protein level. However, the alterations in gene expression found for several SLC genes needs to be further investigated on protein level, to better reflect cellular function.

The fact that we found several genes, Slc7a1, Slc7a5, Slc7a11, Slc3a2, Slc1a4, Slc1a5, and Slc38a2, upregulated in our study, as previously shown to be induced by amino acid starvation in several cell lines, reinforces the validity of our microarray data.
Moreover, we also found increased gene expression for the amino acid transporter encoding genes Slc6a9, Slc38a1, Slc38a7, Slc25a26, Slc15a4, and Slc16a10, not previously known to respond to altered amino acid levels. In addition, we also found genes encoding orphan SLCs, e.g., Slc23a3 and Slc16a14 among others, which possibly encode transporters with preference for amino acids. Our data therefore suggest that numerous of the genes found to be regulated in this study could be involved in amino acid sensing and signaling pathways and could hold responsive elements.

Conclusions

In this study, 1001 genes were significantly upregulated and 848 genes were significantly downregulated of 28 270 genes in the immortalized mouse hypothalamic cell line N25/2, at 5 h of amino acid starvation. Among these 1849 genes, 47 were SLCs or atypical SLCs. About 15 genes encoding SLC amino acid transporters were found, Slc7a11, Slc6a9, Slc7a1, Slc1a4, Slc7a5, Slc1a5, Slc38a7, Slc38a1, Slc3a2, Slc38a2, Slc25a26, Slc15a4, and Slc16a10 were upregulated while only two genes, Slc3a1 and Slc43a2, were downregulated. At 5-h deprivation, genes encoding amino acid transporters from system A, ASC, L, N, T, xc-, and y+ were upregulated. We also found, according to GO annotations, that the gene clusters involved in amino acid transport and amino acid transporter activity were most upregulated at 3 h and 5 h of amino acid starvation.

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Author contributions

SH wrote manuscript, designed study, performed starvation experiment, analyzed data, and performed statistical analysis. EL performed qPCRs, analyzed qPCRs, and wrote part of manuscript. TA performed qPCRs and analyzed qPCRs. RF wrote manuscript, designed study, analyzed data, and performed bioinformatic analysis.

References

1. Efeyan A, Comb WC and Sabatini DM (2015) Nutrient-sensing mechanisms and pathways. Nature 517, 302–310.
2. Fafournoux P, Bruhat A and Jousse C (2000) Amino acid regulation of gene expression. Biochem J 351, 1–12.
3. Kilberg MS, Pan YX, Chen H and Leung-Pineda V (2005) Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. Annu Rev Nutr 25, 59–85.
4. Liao XH, Majithia A, Huang X and Kimmel AR (2008) Growth control via TOR kinase signaling, an intracellular sensor of amino acid and energy availability, with cross-talk potential to proline metabolism. Amino Acids 35, 761–770.
5. Palii SS, Kays CE, Deval C, Bruhat A, Fafournoux P and Kilberg MS (2009) Specificity of amino acid regulated gene expression: analysis of genes subjected to either complete or single amino acid deprivation. Amino Acids 37, 79–88.
6. Deval C, Chaveroux C, Maurin AC, Cherasse Y, Parry L, Carraro V, Milenkovic D, Ferrara M, Bruhat A, Jousse C et al. (2009) Amino acid limitation regulates the expression of genes involved in several specific biological processes through GCN2-dependent and GCN2-independent pathways. FEBS J 276, 707–718.
7. Zhang P, McGrath BC, Reinert J, Olsen DS, Lei L, Gill S, Wek SA, Vattem KM, Wek RC, Kimball SR et al. (2002) The GCN2 eIF2 alpha kinase is required for adaptation to amino acid deprivation in mice. Mol Cell Biol 22, 6681–6688.
8. Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M and Ron D (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 6, 1099–1108.
9. Averous J, Bruhat A, Jousse C, Carraro V, Thiel G and Fafournoux P (2004) Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation. J Biol Chem 279, 5288–5297.
10. Chen H, Pan YX, Dudenhausen EE and Kilberg MS (2004) Amino acid deprivation induces the transcription rate of the human asparagine synthetase gene through a timed program of expression and promoter binding of nutrient-responsive basic region/leucine zipper transcription factors as well as localized histone acetylation. J Biol Chem 279, 50829–50839.
11. Bruhat A, Averous J, Carraro V, Zhong C, Reimold AM, Kilberg MS and Fafournoux P (2002) Differences in the molecular mechanisms involved in the transcriptional activation of the CHOP and asparagine synthetase genes in response to amino acid deprivation or activation of the unfolded protein response. J Biol Chem 277, 48107–48114.
Amino acid starvation changes expression of Slcs

12 Bruhat A, Jousse C, Carraro V, Reimold AM, Ferrara M and Fafournoux P (2000) Amino acids control mammalian gene transcription: activating transcription factor 2 is essential for the amino acid responsiveness of the CHOP promoter. *Mol Cell Biol* **20**, 7192–7204.

13 Barbosa-Tessmann IP, Chen C, Zhong C, Siu F, Schuster SM, Nick HS and Kilberg MS (2000) Activation of the human asparagine synthetase gene by the amino acid response and the endoplasmic reticulum stress response pathways occurs by common genomic elements. *J Biol Chem* **275**, 26976–26985.

14 Gong SS, Guerrini L and Basilico C (1991) Regulation of asparagine synthetase gene expression by amino acid starvation. *Mol Cell Biol* **11**, 6059–6066.

15 Bruhat A, Jousse C, Wang XZ, Ron D, Ferrara M and Fafournoux P (1997) Amino acid limitation induces expression of CHOP, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels. *J Biol Chem* **272**, 17588–17593.

16 Cesar-Razquin A, Snijder B, Frappier-Brinton T, Isserlin R, Gyimesi G, Bai X, Reithmeier RA, Hepworth D, Hediger MA, Edwards AM et al. (2015) A call for systematic research on solute carriers. *Cell* **162**, 478–487.

17 Hediger MA, Clemencou B, Burrier RE and Bruford EA (2013) The ABCs of membrane transporters in health and disease (SLC series): introduction. *Mol Aspects Med* **34**, 95–107.

18 Fredriksson R, Nordstrom KJ, Stephansson O, Hagglund MG and Schioth HB (2008) The solute carrier (SLC) complement of the human genome: phylogenetic classification reveals four major families. *FEBS Lett* **582**, 3811–3816.

19 Hediger MA, Romero MF, Peng JB, Rolfs A, Takanaga H and Bruford EA (2004) The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins. *Introduction*. *Pflugers Archiv: Europ J Physiol* **447**, 465–468.

20 Hyde R, Taylor PM and Hundal HS (2003) Amino acid transporters: roles in amino acid sensing and signalling in animal cells. *Biochem J* **373**, 1–18.

21 Hyde R, Cwiklinski EL, MacAulay K, Taylor PM and Hundal HS (2007) Distinct sensor pathways in the hierarchical control of SNAT2, a putative amino acid transporter, by amino acid availability. *J Biol Chem* **282**, 19788–19798.

22 Fernandez J, Lopez AB, Wang C, Mishra R, Zhou L, Yaman I, Snider MD and Hatzoglou M (2003) Transcriptional control of the arginine/lysine transporter, cat-1, by physiological stress. *J Biol Chem* **278**, 50006–50009.

23 Padbury JF, Diah SK, McGonnigal B, Miller C, Fogere C, Kuzmiar M and Thompson NL (2004) Transcriptional regulation of the LAT-1/CD98 light chain. *Biochem Biophys Res Commun* **318**, 529–534.

24 Sato H, Nomura S, Maebara K, Sato K, Tamba M and Bannai S (2004) Transcriptional control of cysteine/glutamate transporter gene by amino acid deprivation. *Biochem Biophys Res Commun* **325**, 109–116.

25 Lee JI, Dominy JE Jr, Sikalidis AK, Hirschberger LL, Wang W and Stipanuk MH (2008) HepG2/C3A cells respond to cysteine deprivation by induction of the amino acid deprivation/integrated stress response pathway. *Physiol Genomics* **33**, 218–229.

26 Wang Q, Tiffen J, Bailey CG, Lehman ML, Ritchie W, Fazli L, Metierre C, Feng YJ, Li E, Gleave M et al. (2013) Targeting amino acid transport in metastatic castration-resistant prostate cancer: effects on cell cycle, cell growth, and tumor development. *J Natl Cancer Inst* **105**, 1463–1473.

27 Pali SS, Thiaville MM, Pan YX, Zhong C and Kilberg MS (2006) Characterization of the amino acid response element within the human sodium-coupled neutral amino acid transporter 2 (SNAT2) System A transporter gene. *Biochem J* **395**, 517–527.

28 Lopez AB, Wang C, Huang CC, Yaman I, Li Y, Chakravarty K, Johnson PF, Chiang CM, Snider MD, Wek RC et al. (2007) A feedback transcriptional mechanism controls the level of the arginine/lysine transporter cat-1 during amino acid starvation. *Biochem J* **402**, 163–173.

29 Pali SS, Chen H and Kilberg MS (2004) Transcriptional control of the human sodium-coupled neutral amino acid transporter system A gene by amino acid availability is mediated by an intron element. *J Biol Chem* **279**, 3463–3471.

30 Novak D, Quiggle F and Haafiz A (2006) Impact of forskolin and amino acid depletion upon System A activity and SNAT expression in BeWo cells. *Biochimie* **88**, 39–44.

31 Schwartz GJ (2013) Central leucine sensing in the control of energy homeostasis. *Endocrinol Metab Clin North Am* **42**, 81–87.

32 Anthony TG and Gietzen DW (2013) Detection of amino acid deprivation in the central nervous system. *Curr Opin Clin Nutr Metab Care* **16**, 96–101.

33 Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U and Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249–264.

34 Li C and Wong WH (2001) Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci USA* **98**, 31–36.

35 Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**, 3.

36 Smyth GK (2005) Limma: linear models for microarray data. *Bioinformatics and Computational Biology*
Amino acid starvation changes expression of Slcs

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Solutions Using R and Bioconductor (Gentleman R, Carey V, Dudoit S, Irizarry R and Huber W, eds), pp. 397–420. Springer, New York, NY.

37 Benjamini Y and Hochberg Y (1995) Controlling the false discovery rate – a practical and powerful approach to multiple testing. *J Roy Stat Soc B Met* 57, 289–300.

38 Gaccioli F, Huang CC, Wang C, Bevilacqua E, Franchi-Gazzola R, Gazzola GC, Bussolati O, Snider MD and Hatzoglou M (2006) Amino acid starvation induces the SNAT2 neutral amino acid transporter by a mechanism that involves eukaryotic initiation factor 2alpha phosphorylation and cap-independent translation. *J Biol Chem* 281, 17929–17940.

39 Hatzoglou M, Fernandez J, Yaman I and Closs E (2004) Regulation of cationic amino acid transport: the story of the CAT-1 transporter. *Annu Rev Nutr* 24, 377–399.

40 Prasad PD, Wang H, Huang W, Kekuda R, Rajan DP, Leibach FH and Ganapathy V (1999) Human LAT1, a subunit of system L amino acid transporter: molecular cloning and transport function. *Biochem Biophys Res Commun* 255, 283–288.

41 Sato H, Tambu M, Kuriyama-Matsumura K, Okuno S and Bannai S (2000) Molecular cloning and expression of human xCT, the light chain of amino acid transport system xc. *Antioxid Redox Signal* 2, 665–671.

42 Fernandez J, Yaman I, Mishra R, Merrick WC, Snider MD, Lamers WH and Hatzoglou M (2001) Internal ribosome entry-site-mediated translation of a mammalian mRNA is regulated by amino acid availability. *J Biol Chem* 276, 12285–12291.

43 Verrey F, Closs EJ, Wagner CA, Palacin M, Endou H and Kanai Y (2004) CATs and HATs: the SLC7 family of amino acid transporters. *Pflugers Arch : Europ J Physiol* 447, 532–542.

44 Fuchs BC and Bode BP (2005) Amino acid transporters ASCT2 and LAT1 in cancer: partners in crime? *Semin Cancer Biol* 15, 254–266.

45 Nicklin P, Bergman P, Zhang B, Triantafellou E, Wang H, Nyfeler B, Yang H, Hild M, Kung C, Wilson C et al. (2009) Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell* 136, 521–534.

46 Kim KM, Kingsmore SF, Han H, Yang-Feng TL, Godinot N, Seldin MF, Caron MG and Giros B (1994) Cloning of the human glycine transporter type 1: molecular and pharmacological characterization of novel isoform variants and chromosomal localization of the gene in the human and mouse genomes. *Mol Pharmacol* 45, 608–617.

47 Kanai Y and Hediger MA (2004) The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects. *Pflugers Arch : Europ J Physiol* 447, 469–479.

48 Mackenzie B and Erickson JD (2004) Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. *Pflugers Arch : Europ J Physiol* 447, 784–795.

49 Broer A, Rahimi F and Broer S (2016) Deletion of amino acid transporter ASCT2 (SLC1A5) reveals an essential role for transporters SNAT1 (SLC38A1) and SNAT2 (SLC38A2) to sustain glutaminolysis in cancer cells. *J Biol Chem* 291, 13194–13205.

50 Hagglund MG, Sreedharan S, Nilsson VC, Shaik JH, Almkvist IM, Backlin S, Wrone A and Fredriksson R (2011) Identification of SLC38A7 (SNAT7) protein as a glutamine transporter expressed in neurons. *J Biol Chem* 286, 20500–20511.

51 Guastella J, Brecha N, Weigmann C, Lester HA and Davidson N (1992) Cloning, expression, and localization of a rat brain high-affinity glyoxylate transporter. *Proc Natl Acad Sci USA* 89, 7189–7193.

52 Takanaga H, Mackenzie B and Hediger MA (2004) Sodium-dependent ascorbic acid transporter family SLC23. *Pflugers Arch : Europ J Physiol* 447, 677–682.

53 Hoglund PJ, Nordstrom KJ, Schioth HB and Fredriksson R (2011) The solute carrier families have a remarkably long evolutionary history with the majority of the human families present before divergence of Bilaterian species. *Mol Biol Evol* 28, 1531–1541.

54 Roshanbin S, Lindberg FA, Lekholm E, Eriksson MM, Perland E, Ahlund J, Raine A and Fredriksson R (2016) Histological characterization of orphan transporter MCT14 (SLC16A14) shows abundant expression in mouse CNS and kidney. *BMC Neurosci* 17, 43.

55 Friesema EC, Kuiper GG, Jansen S, Visser TJ and Kester MH (2006) Thyroid hormone transport by the human monocarboxylate transporter 8 and its rate-limiting role in intracellular metabolism. *Mol Endocrinol* 20, 2761–2772.

56 Friesema EC, Jansen J, Jachtenberg JW, Visser WE, Kester MH and Visser TJ (2008) Effective cellular uptake and efflux of thyroid hormone by human monocarboxylate transporter 10. *Mol Endocrinol* 22, 1357–1369.

57 Kim DK, Kanai Y, Matsuou H, Kim JY, Chairengouda A, Kobayashi Y, Enamoto A, Cha SH, Goya T and Endou H (2002) The human T-type amino acid transporter 1: characterization, gene organization, and chromosomal location. *Genomics* 79, 95–103.

58 Kim DK, Kanai Y, Chairengouda A, Matsuou H, Cha SH and Endou H (2001) Expression cloning of a Na+-independent aromatic amino acid transporter with structural similarity to H+-monocarboxylate transporters. *J Biol Chem* 276, 17221–17228.