Solute Carriers in Metabolism

Regulation of known and putative solute carriers in the central nervous system

EMILIA LEKHOLM

UPPSALA
UNIVERSITET

Dissertation presented at Uppsala University to be publicly examined in B21, Biomedicinskt Centrum (BMC), Husargatan 3, Uppsala, Thursday, 30 November 2017 at 09:30 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Docent David Drew (Stockholms Universitet, Department of Biochemistry and Biophysics).

Abstract
Lekholm, E. 2017. Solute Carriers in Metabolism. Regulation of known and putative solute carriers in the central nervous system. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1380. 44 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0104-4.

Solute carriers (SLCs) are membrane-bound transporter proteins, important for nutrient, ion, drug and metabolite transport across membranes. A quarter of the human genome codes for membrane-bound proteins, and SLCs make up the largest group of transporter proteins. Due to their ability to transport a large repertoire of substances across, not just the plasma membrane, but also the membrane of internal organelles, they hold a key position in maintaining homeostasis affecting metabolic pathways. Unfortunately, some of the more than 400 identified SLCs are still not fully characterized, even though a quarter of these are associated with human disease. In addition, there are about 30 membrane-bound proteins with strong resemblance to SLCs, of which very little is known. The aim of this thesis is to characterize some of these putative SLCs, focusing on their localization and function in the central nervous system. Since many of the known SLCs play a vital part in metabolism and related pathways, the response to different nutritional conditions has been used as a key method. MFSD14A and MFSD14B, characterized in Paper I, are putative SLCs belonging to the Major Facilitator Superfamily (MFS) and found to be neuronal, differentially expressed in the mouse central nervous system and transiently upregulated in mouse embryonic cortex cultures due to amino acid deprivation. They were also altered in areas of the mouse brain after starvation as well as after high fat diet. In Paper II, the effect on gene regulation due to complete amino acid starvation was monitored in a mouse hypothalamic cell line and 47 different genes belonging to SLCs, or putative SLCs, were found to be affected. Of these, 15 genes belonged to already known amino acid transporters, whereas 32 were putative SLCs with no known function or SLCs not known to react to amino acids. The three SV2 proteins, SV2A, SV2B and SV2C, were studied in Paper III using human neuroblastoma cell lines. The high metabolic state of cancers often result in an upregulation and alteration of transporter proteins, and alterations of the SV2 proteins were found following different treatments performed in this study. Paper IV focused on putative SLCs of MFS type and their role in glucose metabolism. Mouse embryonic cortex cultures were subjected to glucose starvation and the gene expression of 19 putative transporters were analyzed. All but four of the putative transporters were affected either at 3h or 12h of glucose deprivation. In conclusion, several SLCs and putative SLCs studied in this thesis are strongly affected by alteration in metabolism, either due to amino acids or glucose or both. This makes the putative SLCs dynamic membrane-bound proteins, possibly transporters, highly affected by nutritional status and most likely regulated to maintain homeostasis.

Keywords: Solute Carriers, transporter, amino acid starvation, glucose metabolism, MFS, SV2

Emilia Lekholm, Department of Neuroscience, Functional Pharmacology, Box 593, Uppsala University, SE-75124 Uppsala, Sweden. Department of Pharmaceutical Biosciences, Box 591, Uppsala University, SE-75124 Uppsala, Sweden.

To my fantastic family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I   **Lekholm, E**., Perland, E., Eriksson, M.M., Hellsten, S.V., Lindberg, F.A., Rostami, J., Fredriksson, R. (2017) Putative membrane-bound transporter MFSD14A and MFSD14B are neuronal and affected by nutrient availability. *Front Mol Neuroscience*, 10(11)

II   Hellsten, S.V., **Lekholm, E**., Ahmad, T., Fredriksson, R. (2017) The gene expression of numerous SLC transporters is altered in the immortalized hypothalamic cell line N25/2 following amino acid starvation. *FEBS Open bio*, 7(2):249–264

III   **Lekholm, E**., Ceder, M.M., Forsberg, E., Schiöth, H., Fredriksson, R. (2017) Treatment of human neuroblastoma cell lines with growth supplements, ATRA or VIP alters synaptic vesicle glycoprotein 2 (SV2) expression. *Submitted Manuscript*

IV   **Lekholm, E**[*]., Ceder, M.M[*]., Klaesson, A., Perland, E., Schweizer, N., Fredriksson, R. (2017) Glucose deprived mouse embryonal cortex cultures respond by altering MFS transporter expression and localization. *Manuscript*

[*]Equal contribution

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Additional Papers

V   Perland, E., **Lekholm, E**., Eriksson, M.M., Bagchi, S., Arapi, V., and Fredriksson, R. (2016) The putative SLC yransporters Mfsd5 and Mfsd11 are abundantly expressed in the mouse brain and have a potential role in energy homeostasis. *PloS one*, 11(6):e0156912

VI   Roshanbin, S., Lindberg, F.A., **Lekholm, E**., Eriksson, M.M., Perland, E., Ahlund, J., Raine, A., Fredriksson, R. (2016) Histological characterization of orphan transporter MCT14 (SLC16A14) shows abundant expression in mouse CNS and kidney. *BMC neuroscience* 17(1):43

VII Perland, E[*]., Hellsten, S.V[*]., **Lekholm, E**., Eriksson, M.M., Arapi, V. and Fredriksson, R. (2017) The novel membrane-bound proteins MFSD1 and MFSD3 are putative SLC transporters affected by altered nutrient intake. *J Mol Neurosci*. 61(2):199-214

VIII Hellsten, S.V., Eriksson, M.M., **Lekholm, E**., Arapi, V., Perland, E., Fredriksson, R. (2017) The gene expression of the neuronal protein, SLC38A9, is altered in mouse brain following in vivo starvation and high-fat diet. *Plos One*, 12(2):e0172917

IX   Hoeber, J[*]., Köning, N[*]., Trolle, C[#]., **Lekholm, E**[#]., Zhou, C., Pankratova, S., Åkesson, E., Fredriksson, R., Aldskogius, H., Kozlova E.N. (2017) A combinatorial approach to induce sensory axon regeneration into the dorsal root avulsed spinal cord. *Stem Cells Dev*, 26(14):1065-1077

X   Ceder, M.M., **Lekholm, E**., Hellsten, S.V., Perland, E., Fredriksson, R. (2017) The putative membrane-bound protein UNC93A responds highly to starvation in mice. *Accepted in Front Mol Neuroscience*

[*] And [#] equal contribution

Contents

Abbreviations

AARE	Amino acid responsive element
AD	Alzheimer´s disease
APC	Amino acid/polyamine/organocation
ATF4	Activating transcription factor 4
ATRA	All-*trans*-retinoic acid
ATP	Adenosine triphosphate
BBB	Blood brain barrier
CLN	Ceroid-lipofuscinosis, neuronal
CNS	Central nervous system
CPA/AT	Cation:proton antiporter/anion transporter
DAB	3,3'-Diaminobenzidine
DAT	Dopamine transporter
DMT	Drug/metabolite transporter superfamily
eIF2α	Eukaryotic translation initiation factor 2
ELISA	Enzyme-linked immunosorbent assay
GCN2	General control nonderepressible 2
GLUT	Glucose transporter, SLC2 family
HFD	High fat diet
HGNC	Human genome nomenclature committee
HMM	Hidden Markov model
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IT	Ion transporter
MFS	Major facilitator superfamily
MFSD	Major facilitator superfamily domain containing
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Norepinephrine transporter
PD	Parkinson´s Disease
Pfam	Protein family database
PPP	Pentose phosphate pathway
RNA-seq	RNA sequencing
SERT	Serotonin transporter
SD	Standard deviation
SLC	Solute carrier
SNAT	Sodium-coupled neutral amino acid transporter

SPNS	Sphingolipid transporters
SSRI	Selective serotonin reuptake inhibitor
SV2	Synaptic vesicles glycoprotein 2
SVOP/SVOPL	SV2 related proteins
qRT-PCR	Quantitative real time PCR
RNA	Ribonucleic acid
TCDB	Transporter classification database
TMS	Transmembrane segment
Unc-93	Uncoordinated-93 protein
VGLUT	Vesicular glutamate transporter
VIP	Vasoactive intestinal peptide

Introduction

The Cell - The basic membrane-bound unit that contains the fundamental molecules of life and of which all living things are composed.

Encyclopedia Britannica

A cell would not be a cell without its protective membrane, separating it from the outside environment. Membranes are composed of lipids, amphipathic molecules arranged in layers, and they make up not only the outside plasma membrane, but also surround intracellular organelles, Figure 1. Membranes are dynamic structures, growing with the cell and under constant reorganization. The plasma membrane needs to maintain its integrity, while simultaneously allowing for interaction between the inside and outside compartments. This is true for single cell organisms as well as more complex life forms such as humans. Signals and solutes need to be conveyed into and out of the cells, and between membrane-bound compartments within the cell. This is facilitated by proteins. More than a quarter of the human genome codes for membrane-bound proteins [1], proteins that are either connected to, or integrated into, the membrane. The most prevalent membrane-bound proteins in the human genome are receptors [1, 2], proteins capable of causing an internal cellular response upon external signals. Receptor proteins carry a signal through the membrane but do not allow molecules to pass through. The second largest group of membrane proteins is the transporters. These integral membrane proteins provide a direct passage between the external and internal environment. They are vital for the survival of cells as they allow import of nutrients and energy sources into the cytosol and internal compartments. The integral membrane proteins also allow for the outward transport of signaling molecules, amino acids, sugars, excretion of end products from different metabolic reactions, as well as expulsion of toxins. In addition, they maintain the ion gradients and solute concentrations necessary for many biological processes [3]. Transporter proteins can be divided into groups based on their mode of transport: active or passive. Active transport utilizes energy in the form of ATP to drive translocation of molecules against their electrochemical gradient. Both ion pumps and ATP-binding cassette transporters (ABC-transporters) are active transporters. Passive transporters, such as channels and solute carriers (SLCs), do not use ATP, Figure 2A. These rely on electrochemical gradients to transport molecules across membranes. Ion and water channels are regulated by gating,

which allows the channels to be either open or closed. However, SLCs do not possess such gating but are thought to undergo a conformational changes to allow solutes to pass through the inner pore of the protein [4]. The SLCs are the largest group of transporter proteins in humans [1], with 430 members divided into 52 families [5], and new members are continuously being added.

The research included in this thesis is about SLCs and focuses, specifically, on putative SLCs.
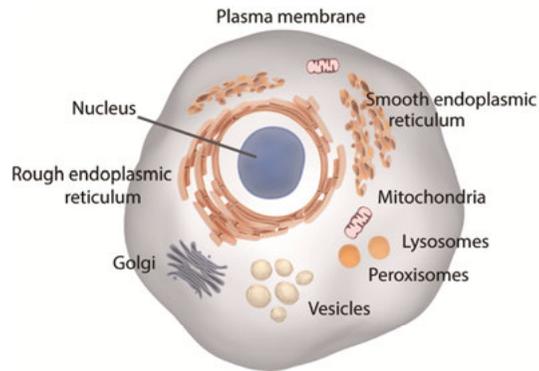

Figure 1. The eukaryotic cell, and its internal organelles, is surrounded by a plasma membrane. The membrane ensures the compartmentalization of different environments inside the cells, which is important for many biological processes. The membrane needs to remain intact but also allow for passage of solutes, metabolites and waste. The transport of solutes across the membranes is performed by transporter proteins that are expressed both on the outer plasma membrane, as well as on intracellular organelles. Credit to Somersault 18:24 (www.somersault1824.com) for figure components, shared under creative commons license.

Solute Carriers

Even though SLCs are the largest group of transporters in the human genome, they are underrepresented in research [6], and as much as 30% of membrane proteins thought to be transporters are still orphans in that their localization and function are unknown [5]. SLCs are non-ATP-driven transporters, but many can still move substrates against their electrochemical gradient by coupling their transport to other solutes. SLCs can be either uniporters, symporters or antiporters as illustrated in Figure 2B. Uniporters, as the name implies, transport one solute at a time down their concentration gradient and are considered completely passive. Symporters and antiporters

use the electrochemical gradient of one substance or ion to transport another substrate against its concentration gradient. Symporters move both substances in the same direction, whereas antiporters, also called exchangers, couple the electrochemically favorable transport and the desired transport in opposite directions. SLCs can transport solutes both into and out of cells or cellular compartments, depending on the concentration gradients of the substrate or on the concentration gradient of a coupled substance. In effect, this means that the transport capability of SLCs is highly connected to the amount of substrate present in different compartments and on electrochemical gradients established by other ATP-driven transporter proteins.
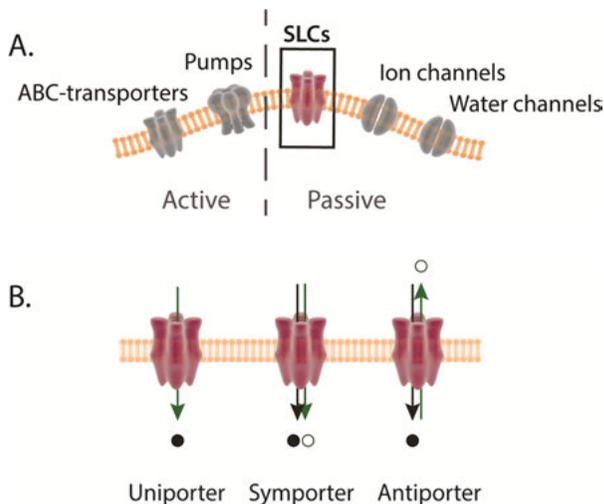

Figure 2. Transporters span the plasma membrane, and enable the transport of solutes through the membrane. They can be situated in the outer plasma membrane, or on the plasma membrane of internal organelles. (A) Transporters can be divided into active or passive depending on their mode of transport. The active ABC-transporters and pumps use ATP directly, whereas the passive channels and SLCs do not. (B) SLCs can transport substances through the membrane in different ways. Uniporters transport one substrate down its concentration gradient (indicated by a green arrow). Symporters and antiporters couple the movement of one substrate down their concentration gradient (green arrows) in order to move another against their concentration gradient (indicated by black arrows). Symporters move both substrates in the same direction, while antiporters move one substrate in by moving another out. Credit to Somersault18:24 (http://www.somersault1824.com/) for figure components, shared under creative commons license.

SLCs are categorized into families based on function, homology, and phenotype [7], and the protein sequences in each SLC family have at least 20% amino acid sequence identity to other members [8, 9], although some excep-

tions do appear in the classification. The names of the human SLC genes are annotated according to systems set by the Human Genome Nomenclature Committee (HGNC), where the root SLC1-52 is given to a family and the members numerical names, divided into sub-families by A, B and so forth. Essentially, this groups SLCs into families based on the conserved regions within the proteins, which often correlates with the substrates they transport [10]. SLCs are capable of transporting amino acids, glucose and other sugars, inorganic cations and anions, neurotransmitters, fatty acids, metals, vitamins, toxins and drugs, listed in Table 1, based on SLC tables found at http://slc.bioparadigms.org and [5, 8, 11].

Table 1. Human SLC families, number of family members, main transported substrate and Pfam clan classification.

SLC family	Members	Main substrate	Pfam clan
SLC1	7	Glutamate and amino acids	No clan
SLC2	14	Sugars	MFS (CL0015)
SLC3	2	Amino acids	Tim barrel (CL0058)
SLC4	10	Bicarbonate	APC (CL0062)
SLC5	12	Sugar, fatty acids	APC (CL0062)
SLC6	20	Neurotransmitters	APC (CL0062)
SLC7	13	Amino acids	APC (CL0062)
SLC8	4	Ions	No clan
SLC9	13	Ions	CPA/AT (CL0064)
SLC10	7	Bile salt	CPA/AT (CL0064)
SLC11	2	Ions	APC (CL0062)
SLC12	9	Ions	APC (CL0062)
SLC13	6	Sulfate/carboxylate	IT (CL0182)
SLC14	8	Urea	No clan
SLC15	5	Oligopeptides	MFS (CL0015)
SLC16	14	Monocarboxylate	MFS (CL0015)
SLC17	9	Vesicular glutamate	MFS (CL0015)
SLC18	4	Vesicular amines	MFS (CL0015)
SLC19	3	Folate/Thiamine	MFS (CL0015)
SLC20	2	Phosphate	No clan
SLCO	12	Ions	MFS (CL0015)
SLC22	23	Ions	MFS (CL0015)
SLC23	3	Ascorbic acid	APC (CL0062)
SLC24	5	Ions	No clan
SLC25	53	Mitochondrial carrier	No clan
SLC26	11	Ions	APC (CL0062)
SLC27	6	Fatty acids	ANL (CL0378)
SLC28	3	Nucleosides	No clan
SLC29	4	Nucleosides	MFS (CL0015)
SLC30	10	Zinc	No clan
SLC31	2	Copper	No clan
SLC32	1	Vesicular amino acids	APC (CL0062)
SLC33	1	Acetyl-CoA	MFS (CL0015)
SLC34	3	Phosphate	No clan
SLC35	31	Nucleoside/Sugar	DMT (CL0184)
SLC36	4	Amino acids	APC (CL0062)
SLC37	4	Sugar/Phosphate	MFS (CL0015)

SLC family	Members	Main substrate	Pfam clan
SLC38	11	Amino acids	APC (CL0062)
SLC39	14	Metal ions	DMT (CL0184)
SLC40	1	Basolateral iron	MFS (CL0015)
SLC41	3	Magnesium	No clan
SLC42	3	Rh ammonium	No clan
SLC43	3	Amino acids	MFS (CL0015)
SLC44	5	Choline-like	No clan
SLC45	4	Sugars	MFS (CL0015)
SLC46	3	Folate	MFS (CL0015)
SLC47	2	Multidrug/Toxin	MviN, MATE (CL022)
SLC48	1	Heme	No clan
SLC49/MFSD7	4	FLVCR	MFS (CL0015)
SLC50	1	Sugar	MtN3-like (CL0141)
SLC51	2	Steroid-derived molecules	No clan
SLC52	3	Riboflavin	No clan
MFSD#	17		MFS (CL0015)
SV2	3	Galactose[1]	MFS (CL0015)
SVOP/SVOPL	2		MFS (CL0015)
SPNS	3		MFS (CL0015)
UNC-93	2		MFS (CL0015)
TMEM104	1		APC (CL0062)
OCA2	1		IT (CL0182)
CLN3	1		No clan

Information collected from SLC tables (http://www.bioparadigms.org) and Perland *et al.* 2017 [5] unless otherwise stated. [1]Substrate information from Madeo et al. [12]

All recognized transporter proteins, regardless of species origin, are also collected in the Transporter Classification Database (TCDB) and categorized into superfamily systems [13]. In addition, the Protein Family Database (Pfam) aims to cluster all proteins into families and clans based on homology [14]. SLCs fall into some of these clans, indicated in Table 1. Unsurprisingly, many SLCs are highly conserved and they are present in both bacteria and eukaryotes [15], a strong indication of their importance. Mammals have a higher number of members in many SLC families, perhaps reflecting the added need of precisely regulated transport in the larger central nervous system [15].

Due to the difficulty in overexpressing and crystalizing these rather large integral membrane proteins, there are few solved crystal structures of mammalian SLCs and the exact structure of many human SLCs remain unknown. However, there are many structures of bacterial SLC homologues available [16-19], which provide some insight into both the general structure and conformational changes during transport. SLCs are typically composed of one large protein with α-helices passing through the membrane with less struc-

tured loops connecting the membrane spanning regions. The structure of SLC42A3, the human Rhesus glycoprotein, RhCG ammonium transporter, was solved in 2010 [20], and since then, solved structures for human glucose transporters, GLUT1 (SLC2A1) [21] and GLUT3 (SLC2A3) [22] have been published. Both GLUT1 and GLUT3 belong to the Major Facilitator Superfamily (MFS) Pfam clan (Pfam CL0015) and most members of this clan have 12 membrane spanning helices. There are a number of prediction tools available that draw information about the crystal structures to build models for proteins with no solved structure. MFSD14A (studied in Paper I and IV) also belongs to the MFS Pfam clan and does, according to prediction software programs, contain 12 transmembrane spanning regions, shown in Figure 3A. It folds into a similar structure as other sugar transporters, Figure 3B.
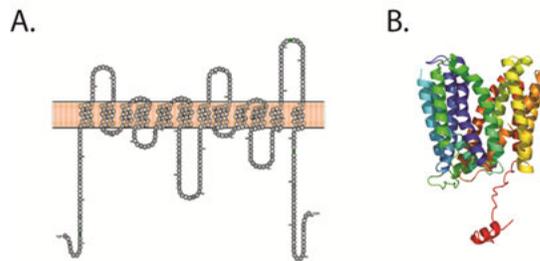

Figure 3. Prediction of secondary and tertiary structure of human MFSD14A protein, a putative transporter belonging to the MFS Pfam clan. (A) Secondary structure for MFSD14A was visualized using Protter [23], indicating 12 transmembrane regions. (B) Using Phyre2 for protein modeling [24], a 3D model for MFSD14A was built based on a template of *E. coli* yajr transporter, c3wdoA, with a 100% confidence and 17% identity.

Localization and Regulation of Solute Carriers

SLCs are found in many different membranes of cells: the outer plasma membrane [8, 25], vesicles [26-28], mitochondria [29, 30], Golgi [31, 32], Endoplasmic reticulum [33], and lysosomes [34], as shown in Figure 1. However, as membranes are dynamic, the localization of the membrane-bound SLCs, by nature, also becomes dynamic. Synaptic vesicles are especially dynamic structures and this is where the Synaptic vesicle glycoprotein 2 (SV2) family [35], putative SLCs belonging to the MFS, are located. They

are also transiently expressed on the plasma membrane as the vesicles undergo fusion with the outer membrane. Here they can act as receptors for drugs [36, 37] and toxins [38, 39]. The location of a transporter is crucial for its biological role, and alterations in location can be used as a means of regulation but are also the source of disorders. Thioredoxin-interacting protein (TXNIP), a protein that increases in concentration in response to raised blood glucose levels, can suppress glucose import by binding to GLUT1 and cause internalization of the transporter [40], hence inhibiting its contact with glucose in the extracellular space. Additional substances can also bind to transporters to alter the expression, or transport kinetics. TXNIP causes not only internalization of GLUT1 but also a reduction of GLUT1 mRNA levels [40], leading to less expression of GLUT1. Furthermore, both ATP [41] and caffeine [42] bind to GLUT1 and inhibit glucose uptake. The dopamine transporter (DAT), a member of the SLC6 family and responsible for transporting the neurotransmitter dopamine from the extracellular space, is regulated on many levels. DAT undergoes at least four posttranslational modifications that result in changed interaction between DAT and other proteins, alters transport kinetics, and dictates the distribution of DAT on the plasma membrane [43]. Altered dopamine levels are implicated in many disorders such as Parkinson´s, attention deficit disorder and substance abuse [43].

Many SLCs can transport the same substrate, and the large quantity of different transporter families can, at first glance, seem superfluous. However, the different and specific localization of SLCs ensures that the substrates are transported into different cell types and organelles. For example, most of the SLC2 family members (GLUTs) all transport glucose but do so with different affinity and are expressed on different cell types [44]. A similar organization exists for the many amino acid transporters [45]. Different organs and cells are also surrounded by different concentrations of solutes, and since SLCs are passive, this greatly alters their transport capability. In addition, as coupled transporters rely on additional substances, the electrochemical gradient and concentration of the coupled substance also play a role in the efficacy of the transport. Many different SLCs and putative SLCs are co-expressed on the same cell [46], and the combined transport profile of all transporters, including that of ATP-dependent transporters, will affect the transport profile of that cell.

Solute Carriers and Metabolism

Amino acids, glucose, fatty acids and nucleotides are used as building blocks when cells grow and divide. Fluctuations in external sources of nutrients is mitigated by internal physiological responses, such as limitation of non-essential protein synthesis and reutilization of existing resources [47]. A

central regulatory pathway coordinating nutrient levels with cell metabolism is the mechanistic target of rapamycin, mTOR, of which there are two distinct protein complexes, known as mTOR Complex 1 (mTORC1) and Complex 2 (mTORC2) [48]. When activated by cues of sufficient levels of oxygen, growth factors, amino acids, energy and glucose, mTORC1 induces pathways leading to protein, lipid, and nucleotide synthesis. When insufficient concentrations of nutrients are present or signals of stress increase, mTORC1 becomes inactive and allows for pathways involved in the breakdown of cells, such as autophagy, to become active instead. mTORC1 regulates cell growth and metabolism whereas mTORC2 is more involved in proliferation and survival, and is more tightly coupled to the presence of growth factors for activation [48]. In protein synthesis, one amino acid cannot compensate for the absence of another, therefore, cells need to detect the lack of any amino acid to prevent failure in peptide-chain synthesis [49]. During protein synthesis, the ribosome incorporates amino acids, delivered by transfer RNAs (tRNAs), into peptides. Each tRNA carries one specific amino acid to the ribosome, and during shortage of one, or several amino acids, free uncoupled tRNAs accumulate. This is sensed by the protein general control nonderepressible 2 (GCN2) [49] which has a high affinity for free tRNAs. GCN2 activates eukaryotic translation initiator factor 2α (eIF2α), and together with other signals, inhibits translation of mRNA and hence protein synthesis [49]. However, it also causes increased translation of a few selected mRNAs. One in particular, the activating transcription factor 4 (ATF4) is initiated during conditions of amino acid starvation and stress [50]. ATF4 is a transcription factor, which works as a master regulator of genes involved in metabolism, nutrient uptake, and apoptosis [51]. All mammalian cells have to obtain essential amino acids (threonine, methionine, phenylalanine, tryptophan, valine, isoleucine, leucine, and lysine) from external sources since they cannot be synthesized like non-essential amino acids [52]. Some very specific amino acids (leucine, glutamine and arginine) are also potent activators of the mTORC1 signaling pathway [52, 53]. In addition, epigenetic modification of DNA and histones are dependent on the amino acids serine and methionine [54]. At least two-dozen amino acid transporters have been identified, expressed in different tissues and in a development-specific manner [45, 55]. SLC38A9, a lysosomal amino acid transporter, interacts with the mTORC1 complex and conveys arginine availability directly to the complex, and therefore, also functions as a sensor [56, 57].

Even though the brain only accounts for 2% of the total body mass, it uses an impressive 25% of the glucose and 20% of the oxygen [58]. The action potential generated neurotransmitter release in neurons creates an enormous displacement of ions and neurotransmitters which needs to be replenished, a process requiring large amounts of energy [59, 60]. Glucose is the main en-

ergy source for the adult brain, and while it can be supplemented by lipids and ketone bodies, it can never be substituted. Transport of glucose has been evolutionarily important. The sugar transporter family is the largest MFS family (TC no. 2.1.1) according to the TCDB across species and is present in the three domains of life: bacteria, archaea and eukaryote [61]. Its members transport glucose, fructose, mannose, galactose, arabinose, xylose, maltose, lactose and many more sugar derivatives [62, 63]. Forty-six percent of the 252 *Slc* genes present in the brain are involved in energy production and consumption, based on both known and proposed functions and substrates of human and rodent *Slc* genes [64]. There are three known families of glucose transporters in the human genome SLC2, SLC5 and SLC50 [25, 44] and one putative sucrose transporter family SLC45 [65, 66]. SLC2 and SLC45 are both members of the MFS Pfam clan (CL0015), while SLC5 belongs to the APC Pfam clan (CL0062) and SLC50 belongs to the MtN3-like Pfam clan (CL0141). However, as the substrate of many human putative SLCs proteins are still not known, some of these might also be sugar transporters.

Glucose metabolism involves not only glycolysis, the process by which glucose is broken down to yield lactate and/or pyruvate and energy in the form of ATP, but also other pathways. In the pentose phosphate pathways (PPP) glucose is used to generate NADPH and ribose-5-phospate for nucleotide biosynthesis. In the hexosamine pathway, an amine group is added to the hexoses to provide a sugar donor for the glycosylation of proteins. Glycogenesis generates glycogen for glucose storage. Finally, in the serine biosynthesis pathway, amino acids are generated and followed by the one-carbon metabolism cycle required for NADPH synthesis that is important for purine and glutathione biosynthesis and also for methylation [44, 67, 68]. All of these are important for proper regulation of energy use, for generation of new building blocks, and for protection against oxidative stress. SLCs transporting sugars are hence implicated in all of these different pathways.

Solute Carriers in Disease

Because SLCs are involved in uptake and efflux of many different solutes and metabolites in different tissues, it is not surprising that they play a role in both the cause of, and the treatment of disease. Solute carriers modulate drug levels by affecting their absorption, distribution, metabolism, and elimination [69, 70]. The blood brain barrier (BBB) shields the brain from pathogens and toxins by providing a protected environment for the central nervous system (CNS). The correct formation of the BBB is very important, and *Mfsd2a*, an orphan putative transporter belonging to the MFS, has been found to be a key regulator in this formation [71]. A functional BBB protects the brain but becomes an obstacle for treating trauma, disorders and diseases

of the CNS. One well characterized transporter in the CNS is SLC6A4 (SERT) which is responsible for reuptake of serotonin from the synaptic cleft, and the target for selective serotonin reuptake inhibitors (SSRI) used to treat depression [72]. Interestingly, both polymorphisms and gene regulation by methylation of SLC6A4 have a role in not only the severity of depression and other linked disorders [73, 74] but also on the effectiveness of treatment by SSRI [75]. SV2A has been found to be able to transport galactose in a yeast model [12], however, this has not been seen in mammals. Instead, the trafficking of synaptotagmin, a calcium sensing protein, and regulation of synaptic vesicle release are its main functions [76-78] and a mutation in the SV2A gene has been linked to epilepsy [79]. A new class of anti-epileptic drugs targets SV2A and has been found to have positive effects on hard to treat patients [37].

Many forms of cancer have up-regulated aspects of their glucose metabolism and an increased intake of glucose to maintain their higher metabolism. In addition to increased carbohydrate metabolism, the protein, lipid, and nucleic acid syntheses are also affected. As SLCs are the major transporters of these molecules, their expression is often altered in cancer cells, and can therefore be exploited in both identification and treatment of cancer [54, 80]. SLC2A1 (GLUT1) is up-regulated in almost all cancer forms, and feeds glucose into the aerobic glycolysis present in many cancer types, a fact that is exploited for cancer diagnosis [52]. Inhibition of GLUT1 expression results in reduced tumorgeniticy in cell cultures [81]. In addition to an increase in glucose transport, several SLCs transporting amino acids are up-regulated in cancer cells, e.g. SLC1A5, SLC7A5, SLC7A11, and SLC6A14 [52]. The tight interconnectivity of SLCs with each other for transport becomes apparent in cancer cells as certain pathways are accelerated. SLC7A5 transports leucine, a potent activator of mTOR signaling and heavily involved in cancer growth. SLC7A5 is an exchanger and needs an amino acid to transport out to accommodate entry of leucine into the cells. As such, its up-regulation is tightly linked with SLC1A5, a Na^+-coupled transporter for alanine, serine, cysteine, and glutamine [82]. Glutamine enters the cancer cells via SLC1A5, and is then transported out via SLC7A5 coupled to the entry of leucine. The inhibition of SLC1A5 caused inhibition of mTOR due to the inhibition of SLC7A5. SLC6A14 is similarly coupled to the function of both SLC7A5 and SLC7A11 due to its capability to transport glutamine into tumor cells and enabling the influx of cysteine important for the glutathione status in cancer cells [83]. In addition, neither up-regulation of SLC7A5 nor SLC7A11 would benefit cancer proliferation unless SLC3A2 was also up-regulated. While SLC7A5 and SLC7A11 perform the transport, SLC3A2 functions as a chaperone for correct localization in the plasma membrane [84].

As altered metabolism and disease states go hand in hand, and the transporters are key players in the transport of metabolites, some of the orphan transporters and putative SLCs included in this thesis could prove themselves important. The more information that is available, the easier it will become to understand the pathways that make up a healthy brain, and which pathways do not.

Aims

Currently, 30% of SLCs are considered orphans in that their transport function and localization are unknown. The aim of this thesis has been to characterize some of these putative transporter proteins, as well as to look at already known SLCs under different conditions. In order to narrow down the scope of the investigative work, focus has been placed on the localization and expression in the central nervous system and, in particular, on effects coupled to metabolism.

Paper I

The aim of this paper was to provide a basic histological characterization of two putative SLC transporters, MFSD14A and MFSD14B, belonging to the Major Facilitator Superfamily (MFS) Pfam clan. Their prevalence in mice tissue, with a focus on the central nervous system, was studied both at gene and protein level. As many transporters are capable of transporting nutrients, the gene expressions of these putative transporters were monitored in cell cultures deprived of several amino acids as well as in mice subjected to starvation or high fat diet. These two transporters, MFSD14A and MFSD14B, have previously not been studied in detail.

Paper II

Here the aim was to identify SLCs and putative SLCs that respond to amino acid starvation. The correct sensing and regulation of amino acids in cells are paramount for accurate response to external nutrient availability, and many amino acid transporters are known to respond to amino acid starvation. As the function and regulation of many SLCs and putative SLCs are still unknown, the mouse hypothalamic cell line, N25/2, was cultured in media deprived of all amino acids, and subsequently the gene expression was monitored by Genechip, Microarray, for 28270 mouse genes. Gene regulation could occur in a time dependent manner, and so RNA samples were taken at 1, 2, 3, 5, and after 16 hours of deprivation. The hypothalamus is a well-established nutrient sensing area in the brain, and regulation of SLCs here could have wide-spread implications. A cell line, rather than primary cells,

was chosen, as many cell lines have an increased expression of SLCs, and the aim of the screen was to identify as many targets as possible.

Paper III

Due to the increased metabolism in cancer cells the expression and regulation of SLCs is disturbed. The aim of this paper was to monitor three putative transporters belonging to the MFS Pfam clan, SV2A, SV2B and SV2C, in two different human neuroblastoma cell lines. The SV2s have the capability of binding drugs and toxins, and the expression of SV2A has been linked with several cancer forms [85, 86]. SV2A has also been indicated to be a galactose transporter [12]. This possible dual role as transporter and receptor is intriguing as it could serve as a means for cancer identification and treatment. In this paper, gene and protein expression alterations after treatment with growth supplements (B-27 and N-2), all-*trans*-retinoic acid (ATRA) and vasoactive intestinal peptide (VIP), was studied. The treatments were chosen with the aim of eliciting maturation in the neuroblastoma cell lines.

Paper IV

Previous results, obtained from the amino acid starved mouse hypothalamic cell line and mouse brain samples from starved mice, indicate that many SLCs and putative transporters belonging to the MFS are affected by amino acid and energy levels [31, 46, 87-90]. To explore this further, a large screening of their regulation during glucose deprivation was done. In this study, mouse primary cortex cultures were used, as the interplay between neurons and astrocytes is important in glucose homeostasis. Gene expression changes of 19 putative SLCs belonging to the MFS were analyzed, and some were supplemented with protein expression data. As little is known about these putative transporters, the aim was to begin mapping out which transporters respond to glucose deprivation, and which do not. In addition, in order to gain information about their possible transport function, the sequences of the MFS proteins studied were examined for conserved domains as well as homology to bacterial MFS. Many of the bacterial MFS are characterized, and could shed light on functions of the MFS expressed in mammals.

Methods

"All cell biologists are condemned to suffer an incurable secret sorrow: the size of the objects of their passion. … But those of us enamored of the cell must resign ourselves to the perverse, lonely fascination of a human being for things invisible to the naked human eye"

- L.L. Larison Cudmore

Homology and Phylogenetic Analysis

As SLCs with common structural regions could share function and transport mechanism, evolutionary relationship and similarity of the putative transporter proteins with the known SLCs could provide insight into their possible function. Through homology modeling of SLCs of MFS type, many putative transporters were found, such as MFSD14A and MFSD14B which were studied in Paper I. Since phylogenetic relationships can offer insight into the transport mechanism and substrate, homology modeling can be a useful tool. In paper IV, sequences of bacterial orthologues for the putative transporters give indications about function.

Gene Expression Analysis

To map out where and to what extent the putative transporters are expressed, quantitative real time PCR (qRT-PCR) was used. All cells in an organism contain the same genetic makeup, but depending on the type of cells, different genes are actively used. qRT-PCR allows for the amplification and detection of a mRNA corresponding to a target gene, compared to other genes in the same sample or between samples. The laboratory work for this method is nowadays straightforward, as many companies provide ready-made reagents for RNA extraction from tissues and cells and purification columns for RNA. The RNA is then converted into complimentary DNA (cDNA) and combined with primers flanking segments of the target gene, nucleotides, a fluorescent detection dye for double stranded DNA, and Taq polymerase. The resulting fluorescent signal correlates to the mRNA levels for the target gene in the tissue or cells that the RNA was extracted from. One important

aspect of, and a source of error in, this method is to find stable genes to normalize the target gene expression against. These stable genes, called housekeeping genes, need to be assessed for each new experiment as these can differ. The stability of several housekeeping genes were evaluated using the GeNorm protocol [91] in the work included in this thesis, and the most stable were chosen as normalization factors for the results.

Gene expression was studied using qRT-PCR in Paper I, III and IV, where the targets were already known. However, in Paper II, the genes of interest were not known beforehand, so a high-throughput screening method, Microarray, was used instead, allowing for parallel detection of many thousand genes. Here, mRNA is converted to cDNA, biotinylated and allowed to bind to a GeneChip prepared with DNA probes corresponding to the genes present in the organism of the study. In Paper II, 28 270 mouse genes were present on the GeneChip. The amount of cDNA bound to each DNA probe on the GeneChip was detected by fluorescence [92]. The data was then verified by qRT-PCR for a selected number of targets.

Protein expression

Immunostaining

Immunohistochemistry (IHC) techniques using antibodies is a way to visualize protein localization. This method has been used in Paper I, III, and IV with different variations of detection method and sample to visualize the protein expression of the transporters studied. The usefulness of this method is dependent on the specificity of the antibodies, and evaluation has been carried out using SDS-PAGE and Western Blot, allowing separation of a protein sample based on size, blotted onto a membrane, and then detected by antibodies. With this method, it is possible to visualize if the antibody binds several proteins of different sizes or only one and compare the resulting size of the protein band with the expected size of the target protein. Different antibodies, binding to different target sequences in the same proteins, can also be used and the staining patterns compared. This was done in Paper III, as clear separation by SDS-PAGE for the highly glycosylated SV2 proteins is notoriously difficult. Immunostaining assays can be performed either on larger tissue samples, or on cell cultures. IHC on larger tissue, such as brain samples, provides an overview of the staining pattern and can give useful information about possible function of the target. Many areas of the brain are intimately connected with physiological, behavioral, and higher order cognitive functions, and many CNS diseases are frequently associated with distinct regions [64]. An overview of localization of orphan transporters can be a good first step in deciphering its function. For cellular localization, im-

munocytochemistry (ICC) on mouse primary cortex cultures were utilized in Paper I and IV. Cell cultures provide a single cell resolution. The detection of antibody-target binding can be done either by colorimetric staining (using DAB) or by fluorescence. In both of these detection methods, an amplification step, utilizing secondary antibodies, is added to boost the signal. Using fluorescence, many different targets can be visualized in the same sample at the same time, as different fluorophores can be used in parallel and kept apart by their difference in emitted wavelength upon excitation. This allows simultaneous visualization of, for example, the cell nucleus, the cell membrane, and the putative transporter.

Enzyme-linked Immunosorbent Assay

A way to quantify a specific protein in a sample is to use an Enzyme-Linked Immunosorbent Assay (ELISA). Here, the detection is carried out with the use of antibodies, and multiple antibodies can be used to both capture the protein in the sample and for detection. The proteins are in their native state, as the method is performed in solution. For this assay to work, both the antibody and the protein concentration have to be optimized to fall within a linear range of the fluorescent signal of the combination. For quantitative results, a standard curve of pure target protein is added to each plate. In paper III, differences in protein levels of SV2A and SV2C between control and treated cells were analyzed, providing relative expression values.

Metabolism Experiments

In vitro

SLCs are important for metabolism, and many are thought to be involved in sugar or amino acid transport or sensing. Using models in which energy availability is altered can be a starting point in finding important putative SLCs. The gene regulation of transporters and putative transporters as a result of amino acid deprivation, amino acid starvation, or glucose deprivation could provide useful information about the pathways in which they play a role, and possibly, what substrate they transport. For metabolism experiments, both mouse primary cortex cultures and cell lines have been used. Primary cultures, although more demanding to cultivate and inherently more variable, provide a comprehensive system because different cell types are present in the cultures, and represent a more biologically relevant model. Mouse primary cortex cultures have been subjected to amino acid deprivation in Paper I and glucose deprivation in Paper IV. However, not all transporters are expressed in levels high enough for detection in mouse primary cortex cultures, and therefore, other cell cultures were also used. Cancer cell

lines are easier to culture, usually more resilient, and express more transporters. A mouse hypothalamus cell line was used in Paper II to provide as much data as possible about SLCs and putative transporters involved in, or affected by, amino acid starvation. However, cell lines have an altered energy metabolism compared to non-dividing cells, and the results might not always represent the mechanism in a healthy cell culture, or organism. The biology of cancer cells is interesting in and of itself, as expression and regulation of transporters and other targets can have implications for detection and treatment of various cancers. In Paper III, two human neuroblastoma cell lines were used in the study of three specific MFS proteins. Here, effects of nutrient availability were not studied directly, but rather the focus was on changes in specific targets, SV2A, SV2B and SV2C, after treatments to slow down proliferation of the cancer cells. Many metabolic pathways would accompany such a switch though.

In vivo

To gain insight into how the putative transporters are affected in a complete intact biological system, their gene regulation was also studied in samples taken from mice. All animal work involving mice was approved by the local ethical committee in Uppsala and in unity with the guidelines of the European Communities Council Directive. To minimize the number of animals used in this investigative research, diet experiments were set up, and the RNA samples from these were used in several projects [31, 87, 89, 90]. Adult male mice were either subjected to 24 h starvation with access to water, or 8 weeks of high fat diet (HFD). The different nutritional states were chosen as two extremes, and as biological differences between the individual mice were not the aim of the panel the samples were pooled and used in the analysis. In Paper I, the focus was on gene expression changes in the CNS, and samples from selected brain regions were investigated. As nutrient availability affects the whole body, the results seen in gene regulation in the CNS can be due to a direct affect in the CNS or due to alterations in peripheral organs involved in metabolism. This makes the results biologically more relevant than gene regulation alterations in a cell culture, but also more complex and harder to interpret. In addition, both starvation and high fat diet affect not only energy metabolism, but also induce stress and inflammation [93].

Both *in vitro* and *in vivo* work is important to gain an overall picture of relevant factors for the expression changes of the putative transporters.

Results Summary

In-depth results and discussion can be found in the corresponding papers and manuscripts whereas the summary below is a more concise version. Some of the proteins studied are included in more than one paper, and for these, the different papers aid in completing a biological puzzle of the novel proteins in different biological situations. I hope that others will continue to build on this, and that someday, the full function of these transporters will be known.

Paper I

MFSD14A and MFSD14B, two putative transporter proteins, had not previously been characterized, so I set out to find their localization in mouse tissue and their biological relevance through experiments focusing on metabolism. Both proteins are similar to SLCs, and share a branching node with SLC15 (Proton oligopeptide cotransporter family), 19 (Folate/thiamin transporter family), 22 (Organic cation/anion/zwitterion transporter family), 29 (Facilitative nucleoside transporter family), and 43 (Type II Na+-phosphate cotransporter family). However, MFSD14A and MFSD14B were on a separate branch, and do not have enough sequence identity to be included into any of those families. Using qRT-PCR, their gene expression patterns in the brain and some peripheral tissues were screened. Both transporters could be found expressed in most tissues included, indicating an important and general function. Focusing on the central nervous system, DAB-IHC was performed for both proteins to get a sense of their localization. Here, clear differences between the two transporters could be seen in both of the morphology of the stained cells, the localization and the abundance. To investigate which cell types express the transporters, fluorescent IHC was used, and a neuronal localization was found for both transporters. Going deeper, I looked at the cellular localization using mouse cortex primary cultures. Again, neuronal co-localization was found for both transporters and an intracellular placement. Using different antibodies for internal organs, MFSD14A was seen to co-stain with the Golgi marker. MFSD14B was found more around the Endoplasmic reticulum (ER). They were both seen to respond to altered nutrient states, both amino acid deprivation *in vitro* and food starvation in mice. Their gene expression was up-regulated in the striatum of mice subjected to a high fat diet. This provided the first histological data of these

two putative transporters, and their co-localization to neurons in the mouse central nervous system. They were found to be present both in embryonic primary cultures and in the adult mouse brain. Their altered gene regulation in the diet experiments implicates a role in energy sensing or metabolism.

Paper II

Amino acid sensing and transport is vital for cells, and to investigate the effect of amino acid starvation on SLCs and putative SLCs, the mouse hypo-thalamic cell line N25/2 was used to screen for gene regulation. Of the 28 270 genes included in the mouse GeneChip Microarray, 86.2% were found in the cell line. At 5 h of starvation, a total of 1001 genes were up-regulated and 848 genes were down-regulated. Of these, we found 15 genes belonging to amino acid transporter families. From these, 13 genes were up-regulated (*Slc7a11, Slc6a9, Slc7a1, Slc1a4, Slc7a5, Slc1a5, Slc38a7, Slc38a1, Slc3a2, Slc38a2, Slc25a26, Slc15a4,* and *Slc16a10*) and only 2 down-regulated (*Slc3a1 and Slc43a2*). Many of these contain characterized amino acid responsive elements, and our results corroborate the results from other cell lines. Notably, 18 genes coded for non-amino acid transporters but belonged to transporters of thiamine (vitamin B1), iron, sugar, vitamin, ions, fatty acid, UTP, pyrimidine nucleotide, and hormones. In addition, expression levels of 14 genes belonging to orphan or atypical SLCs were also altered by amino acid starvation. Among these were many MFS members, such as *Mfsd1, Mfsd2a, Mfsd7b,* and *Mfsd11*. Because amino acid starvation was performed for 1, 2, 3, 5 and 16 h, a time line of alteration could also be seen. Most gene alterations could be seen after 3 and 5 h, although many were up-regulated already after 2 h, such as *Slc23a3*. Since the Microarray gave results for more than just genes coding SLCs, general effects from the amino acid starvation could be monitored. Using Gene Ontology (GO), annotations for biological processes and molecular functions were analyzed, and unsurprisingly, genes involved with Metabolic processes (GO:0008152) were down-regulated after 1 h. Gene involved in Cell cycle (GO:0007049), Regulation of cell cycle (GO:0051726), Cell proliferation (GO:0008283) were affected early, at 2 h and 3 h, while many genes involved in Metabolic process (glycoprotein biosynthesis, macromolecules, sterol, cholesterol, amine, and lipid metabolic processes) were affected after 5 h and continued to be affected at 16 h. All of the data from the Micorarray was uploaded in the NCBI-GEO database with accession number GSE61402 and will hope-fully be useful for other researchers.

Paper III

Two human neuroblastoma cell lines, IMR-32 and SiMa, were subjected to three different treatments; B27/N2 supplement, all-*trans*-retinoic acid (AT-RA) and vasoactive intestinal peptide (VIP). The treatments were chosen to cause maturation of the cancer cell lines. The effects on gene expression of *SV2A, SV2B,* and *SV2C*, previously not monitored under these conditions, were analyzed using qRT-PCR. Immunostaining, for visualization of the proteins in the cell lines, and ELISA, for the relative quantification of the SV2 proteins, were also performed. The cell lines responded differently to all treatments and individual differences were seen between the cell lines as well. The SiMa cells treated with B27/N2 supplement had a decreased gene expression of *SV2B,* together with markers for cholinergic and inhibitory neurons. Protein expression of SV2A, SV2C as well as SNAP25 was altered after the treatment. Gene expression alterations were not found following ATRA treatment, whereas protein alterations for SV2A, SV2C and SNAP25 were found. VIP treatments did not affect the SiMa cells. For IMR-32 cells, gene expression changes could be seen after treatments with B27/N2 supplements and with VIP, while effects on protein levels were seen following all treatments. After B27/N2 treatment, *SV2B* was increased, as well cholinergic neuron types. After VIP treatment, *SV2C* was increased together with an increase of dopamine transporter *DAT (SLC6A3)*, indicating a switch to a dopaminergic neuron type. Protein expression of both SV2C and SNAP25 was also seen in VIP treated IMR-32 cells. A connection between SV2B and cholinergic neurons has been implicated in AD, and the co-regulation of *SV2B* and *ChAT* found here was intriguing. Likewise, a connection between SVC and dopaminergic neurons has been implicated in PD, and found to co-regulate in the IMR-32 cells.

Paper IV

Since a number of putative transporters were seen to be affected in the diet panels and also in the amino acid starvation experiment performed in Paper II, the effect of glucose deprivation was tested. Glucose regulation and homeostasis is highly important, apparent in disorders where glucose dysregulation is seen such as obesity and diabetes type 2 [94]. Dysregulation of glucose metabolism is also one of the earlies signs of Alzheimer´s disease [95]. As both astrocytes and neurons are important for glucose sensing and regulation in the central nervous system [58, 67], mouse primary cortex cultures were used to monitor gene expression of putative SLCs belonging to the MFS clan. The cells were subjected to glucose deprivation for 3 h and 12 h and gene expression changes for many of the putative transporters were found. *Mfsd1, Mfsd2a, Mfsd4a, Mfsd5, Mfsd9, Mfsd10, Mfsd11, Mfsd13a,*

Mfsd14a, Mfsd14b, Sv2a, Sv2b, Svop, Spns2, and *Unc93a* showed up or down-regulation while only four genes, *Mfsd2b, Mfsd6, Mfsd8,* and *Cln3*, were unaffected by the deprivation. The majority of the MFS were down-regulated after 3 h, and up-regulated after 12 h. For this study other more general targets were also monitored in the cultures, to study the overall metabolism and stress levels in the cell cultures. Metabolism effects were monitored by *Gapdh, Rheb, Gsk3b,* and *Slc25a3,* while stress responses were looked at using *Nrf2, p53,* and *Gclc.* The majority of these targets were also down-regulated after 3 h in the glucose deprived cells compared to the controls. Up-regulation after 12 h was seen for the metabolism markers, *Gapdh, Rheb* and *Slc25a3,* while the stress markers, *Gsk3b, Nrf2, p53, Gclc,* were unaffected. That so many MFS were altered after glucose deprivation was surprising, especially since the media still contained a substantial amount of glucose (1 g/L D-Glucose, as compared to 4.5 g/L D-Glucose). We hypothesized that the complete media change that was done at the start of the deprivation experiment would be even more stressful for the glucose deprived group. This turned out to not be true, as the glucose deprived group showed less expression of the stress targets that we monitored. To complement the mRNA expression changes, protein alterations were analyzed using ICC. To avoid introducing bias in the analysis, Cell Profile was used to quantify the changes in fluorescent intensity and position. Not all targets were analyzed for this, since for some targets, no adequate antibodies were found, or their expression in the primary cultures was too low. From the ICC data, the MFS studied were found to be expressed in neurons, and for some of the putative transporters, alterations in their protein expression, visualized by fluorescent intensity changes, were found. This implies that many of the MFS included in this study are highly dynamic and regulated due to a decrease in glucose. Since most of the MFS studied here are expressed in neurons, their regulation could be a vital part in understanding neurological diseases where glucose homeostasis plays a key role.

Conclusions

Many of the putative transporters included in this work bear strong phylogenetic resemblance to SLCs, and could be included in current SLC families in the future, or make up new families. The MFS proteins (Pfam CL0015) studied here were all expressed in neurons, and the focus has been on their expression and alteration within the central nervous system. As all papers, with the exception of Paper III, have dealt with nutrient availability, the collective results from several papers provide more information about the role of the putative SLCs in metabolism, than each paper by itself. Here I will highlight some of the putative transporters studied in several papers.

MFSD14A, found to be abundantly expressed in neurons in the mouse central nervous system, was studied both in Paper I and IV. Deprivation of glycine, alanine, asparagine, glutamine, histidine, isoleucine, leucine, serine, and valine in mouse primary cortex cultures produced an up-regulation in gene expression after 3 h, but no difference compared to controls at 7 or 12 h (Paper I). In contrast to this, *Mfsd14a* was highly up-regulated in primary cortex cultures after 12 h of glucose deprivation, while no significant change could be seen after 3 h (Paper IV). This implies an involvement in pathways involved in energy metabolism more closely connected to glucose. MFSD14B, also found to be expressed in neurons, although with a different localization pattern, behaved in a similar manner in mouse cortex primary cultures as MFSD14A after amino acid deprivation (Paper I). However, after 3 h of glucose deprivation, *Mfsd14b* was up-regulated and even more so after 12 h (Paper IV). This indicates that MFSD14B is also involved in glucose metabolism, although in a different way, or in a different subset of neurons, than MFSD14A.

Studying the differences and similarities in gene regulation of putative transporters during complete amino acid starvation in Paper II and after glucose deprivation in Paper IV, gives insight into whether their regulation is due to general metabolic effects, or more specific substrate effects. The N25/2 hypothalamic mouse cell line used in Paper II was completely deprived of amino acids, while the mouse cortex primary cells used in Paper IV had all metabolites at normal levels, except for glucose. *Mfsd1, Mfsd2a* and *Mfsd11* were studied in both papers. *Mfsd1* was up-regulated after both amino acid starvation and glucose deprivation, indicating that it is altered due to general

energy and nutrient levels, rather than specifically due to amino acids or glucose. *Mfsd11* was also up-regulated after both amino acid starvation and 12 h of glucose deprivation, indicating a general role in metabolism *Mfsd2a*, however, was down-regulated after amino acid starvation, and slightly down-regulated after 3 h of glucose deprivation. *Mfsd2a* is a transporter for docosahexaenoic acid, an omega-3 fatty acid [96], and its vital role in the BBB has been established [97]. Its down-regulation during complete amino acid starvation and moderate and transient affect during glucose deprivation was not surprising.

The SV2 proteins, SV2A, SV2B and SV2C studied in Paper III, were affected by the treatments in different ways in the two different human neuroblastoma cell lines. SV2 proteins have been found to be vital for calcium mediated neurotransmitter release, important for functional neurotransmission [77, 98, 99], and also linked to several disease such as cancer [86], epilepsy [37, 79], Alzheimer´s disease [100], Parkinson´s disease [101], and the receptors for botulinum toxin widely used for both medicinal and cosmetic purposes [39]. Their regulation and expression in human neuroblastoma cell lines could therefore be of interest both as cancer identifiers and as therapeutic targets. These proteins, being putative SLCs, were also included in Paper IV. *Sv2a* and *Sv2b* were both down-regulated after 3 h of glucose deprivation, while no further change could be seen after 12 h. SV2A has been found to transport galactose in a yeast model [12], however, a role in sugar transport or sensing might not be their most important biological function as both *Sv2a* and *Sv2b* showed only transiently affected by the altered glucose concentrations.

That so many putative SLCs are dynamic in their gene regulation was interesting and somewhat surprising. Their regulation must have a biological relevance that is important for the cells, and animals, during conditions of lowered amino acid and sugar availability. They could be involved in the direct transport of the missing substance from the extracellular space, or provide transport between internal compartments to redistribute the available resources, as in autophagy. The transporters could also be transporting necessary coupling substrates for other transporters. This could be the reason for the alterations seen after amino acid starvation in many SLCs not thought to transport amino acids. From the experiments done here, it is not possible to distinguish the two functions, and more research will have to be done.

Perspectives

In order to completely understand the biological roles of the putative transporters included in this thesis, their substrate profile have to be elucidated. If they are indeed transporters, their substrate and transport mechanism could shed light on exactly how they are involved in, and affected by, alterations in nutritional status in the central nervous system. Since many transporter proteins are expressed on the same cell, and many of the putative transporters studied here also have been found to co-localize [46], it could prove difficult to perform uptake assays in cell cultures. In addition, some of the putative transporters are intracellularly expressed, such as MFSD14A and MFSD14B in Paper I, and this complicates matters further. Therefore, more controlled systems could be a more effective way to identify transport capability and substrate. A classic choice in the study of transporters is to overexpress the protein in *X. laevis* oocytes and then perform uptake assays or efflux assays using radiolabeled substrates. As an example, this has been done for members of the SLC38 family of amino acid transporters, SLC38A8 [102] and SLC38A10 [103]. A challenge in the study of many putative transporters is that even the general type of substrate is unknown. In the SLC38 family, many members had already been characterized as amino acid transporters, and provide indication of which substrate types could be transported by new members with similar structure. Hence, an even simpler transport system in which many different substrates at different concentrations, together with different cofactors could be screened would be advantageous. Utilizing a thermal shift assay it might be possible to screen putative transporters for substrate interactions. The method relies on the concept that binding to a ligand or substrate will stabilize the protein during heat treatment. A difference between transporters bound to a substrate and transporters without ligand will then produce a thermal shift [104, 105]. Since the SV2 proteins are known to function as receptors for drugs [37, 106], it is plausible that some of the orphan MFS proteins could also be receptors and hence found using thermal shift assay.

For more precise functional studies, gene knock out (KO) studies would be the most informative experiment. As many of the proteins are highly evolutionarily conserved [46], a model organism such as *D.melanogaster* could be a good starting point in search for phenotypes. However, the transported substrate, transport mechanism, and localization will still be valuable information to have in the interpretation of phenotypes. MFSD14A has been

knocked out in mice, resulting in viable but infertile mice [107]. Even with this clear phenotype, the exact function of MFSD14A could still not be pin-pointed.

I hope that others can build on the work included in this thesis, and that the function of these putative transporters will be found. As many are evolution-arily conserved and abundantly expressed, their importance is clear, and the knowledge of what their function is could prove to be important in under-standing aspects of metabolic pathways.

Acknowledgements

Last but not least, I want to express my gratitude and appreciation to all the people that have helped me along the way.

First, I want to express my gratitude towards Robert Fredriksson, my main supervisor, who gave me the opportunity to make this thesis possible. Thank you for allowing me to work independently and for the many stimulating discussions. Thank you also for your patience during times when my eagerness for perfection has not matched the reality of research. Your calm attitude has been a source of inspiration. I also want to express my gratitude towards my co-supervisors, Helgi Schiöth, for giving me the opportunity to start many PhD in your group, and for pushing me to become better, and to Sonchita Bagchi, for all the support. I also want to thank Michael Williams, who took time to help with *D.melanogaster* work early in my PhD and who has always had good advice to give about research.

I want to extend a special and warm thank you to all my fantastic colleagues, collaborators and friends. Emelie Perland – thank you for introducing me to everything, for making me feel so welcome, and for all the fun hours in the lab. It has been a luxury to have you as a colleague, collaborator, and now friend. I am happy that we can stay in touch even now that you have left us Dr. Perland. Mikaela Eriksson – what would I do without you? Thank you for your enthusiasm for everything, for your collaboration on many papers, for your awesome ability to load qPCR plates perfectly, for many hours of scientific discussion and for your humor. Karin Nordenankar, your calm and positive attitude has been fantastic to have in the group. Thank you for helping me with my manuscripts and thesis, and for always having time to talk about work, children and life. And for all the gifs!! Sofie Hellsten – thank you for your collaboration on many papers, your impressively fast work, kind attitude and very entertaining stories. Sahar Roshanbin – thank you for making me feel part of the group when I first started, for interesting discussions (not so much about work) and humor. I wish we could have worked on more projects together! Thank you Nadine Schweizer and Rekha Tripathi for the work we did together and your kind support during my final stressful months. Thank you Frida Lindberg for help with the furry ones, and for training inspiration. I will start, any day now. Thank you Tanya Aggarwal for the office dancing! Thank you also to the many colleagues at the depart-

ment of Neuroscience, Anders, Pleunie, Giulia, Ataur, Marta, and Jörgen. Thank you to Jan Hoeber for interesting discussions, collaboration and general support. Thank you to Axel Klaesson for you expertise in Cell Profiler and fun collaboration. I would also like to thank all the students that have done projects in the lab and that have helped out in my research. Thank you Johanna, Fatemah, Mona, Jinar, Elin, Mohamed, Robin and a special thank you to Erica Forsberg – your energy and positive attitude is contagious. Thank you for all your hard work and for producing beautiful ICC images. You will go far. Remember to breathe a little (advice I am bad at following myself). Thank you also to Malin Kylberg and Åsa Liljegren Sundberg and my colleagues at Q-Med, Galderma, for allowing me to pursue a PhD and for the support.

I also want to thank my friends who have had my back in life and during these somewhat stressful years. A special thank you to Monica Lundström who is the reason I started studying Molecular Biology in the first place. Thank you for critically reading my thesis and for your fantastic support in all aspects of my life. You are wise and inspirational. Thank you also to Camilla Lundgren – for making the university years fabulous, for the many adventures and for listening to me rant about experiments going wrong. Thank you also to Marie Salomonsson, Lina Lundeborg, and Sandra Westman for your insightful thought about life, support and friendship and to "Gänget" for being a fantastic group of people! Andrea, Mirja, Anna, Petra S, Petra H, Caroline, Rebecca, Niklas, Linus, Stefan, Robert, Christofer, Fredrik – thank you for all the non-work related discussions and general awesomeness. Thank you also to Victoria for reading my thesis and for providing perspective in life.

To my family: Thank you Lena and Elof for inspiring me to pursue a PhD and for prompting an interest in science. Thank you to my older siblings, Matilda and Erik for your support. Thank you to my sister Alexandra for being one of the smartest and strongest people I know. I which I was smarter and could come up with something that would fix everything. Thank you also to Micke and Casper for fun play dates. And lastly, to my rock, inspiration and love of my life, Ville Lekholm - thank you for your support, kind words and everything. And thank you for the best collaboration ever on the one project that really matters -.Edvard. Edvard – you can´t read yet, so for you I have a puzzle on the next page.

For Edvard

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