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Atypical Solute Carriers

Identification, evolutionary conservation, structure and histology of novel membrane-bound transporters

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Abstract
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Solute carriers (SLCs) constitute the largest family of membrane-bound transporter proteins in humans, and they convey transport of nutrients, ions, drugs and waste over cellular membranes via facilitative diffusion, co-transport or exchange. Several SLCs are associated with diseases and their location in membranes and specific substrate transport makes them excellent as drug targets. However, as 30 % of the 430 identified SLCs are still orphans, there are yet numerous opportunities to explain diseases and discover potential drug targets. Among the novel proteins are 29 atypical SLCs of major facilitator superfamily (MFS) type. These share evolutionary history with the remaining SLCs, but are orphans regarding expression, structure and/or function. They are not classified into any of the existing 52 SLC families. The overall aim in this thesis was to study the atypical SLCs with a focus on their phylogenetic clustering, evolutionary conservation, structure, protein expression in mouse brains and if and how their gene expressions were affected upon changed food intake. In Papers I-III, the focus was on specific proteins, MFSD5 and MFSD11 (Paper I), MFSD1 and MFSD3 (Paper II), and MFSD4A and MFSD9 (Paper III). They all shared neuronal expression, and their transcription levels were altered in several brain areas after subjecting mice to food deprivation or a high-fat diet. In Paper IV, the 29 atypical SLCs of MFS type were examined. They were divided into 15 families, based on phylogenetic analyses and sequence identities, to facilitate functional studies. Their sequence relationships with other SLCs were also established. Some of the proteins were found to be well conserved with orthologues down to nematodes and insects, whereas others emerged at first in vertebrates. The atypical SLCs of MFS type were predicted to have the common MFS structure, composed of 12 transmembrane segments. With single-cell RNA sequencing and *in situ* proximity ligation assay, co-expression of atypical SLCs was analysed to get a comprehensive understanding of how membrane-bound transporters interact.

In conclusion, the atypical SLCs of MFS type are suggested to be novel SLC transporters, involved in maintaining nutrient homeostasis through substrate transport.

Keywords: Major facilitator superfamily, solute carrier, transporter, protein expression, mRNA expression, phylogenetic clustering, orthologues, co-expression, subcellular location, nutrition.

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If thou, dear reader, are bored with
these wearisome calculations, take
pity on me, who did them 70 times.

Johannes Kepler

List of Papers

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

I **Perland**, **E**., Lekholm, E., Eriksson, M.M., Bagchi, S., Arapi, V., and Fredriksson, R. (2016). The putative SLC transporters Mfsd5 and Mfsd11 are abundantly expressed in the mouse brain and have a potential role in energy homeostasis. *PLoS One* 11(6), e0156912. DOI: 10.1371/journal.pone.0156912.

II **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. *Journal of Molecular Neuroscience* 61(2), 199-214. DOI: 10.1007/s12031-016-0867-8.

III **Perland**, **E**., Hellsten, S.V., Schweizer, N., Arapi, V., Rezayee, F., Bushra, M., and Fredriksson, R. (2017). Structural prediction of two novel human atypical SLC transporters, MFSD4A and MFSD9, and their neuroanatomical distribution in mice. *Manuscript in review at PLoS One*

IV **Perland**, **E**., Bagchi, S., Klaesson, A., and Fredriksson, R. (Forthcoming Aug. 2017). Characteristics of 29 novel atypical SLCs of MFS type: evolutionary conservation, predicted structure and neuronal co-expression. *Open Biology* 7(8), DOI: 10.1098/rsob.170142

* Equal contribution

Additional publications

V **Perland**, **E**., and Fredriksson, R. (2017). Classification systems of secondary active transporters. *Trends Pharmacol Sci.* 38(3), 305-315. DOI: 10.1016/j.tips.2016.11.008.

VI Lekholm, E., **Perland**, **E**., Eriksson, M., Hellsten, S., Lindberg, F., Rostami, J., et al. (2017). Putative membrane-bound transporters MFSD14A and MFSD14B are neuronal and affected by nutrient availability. *Frontiers in Molecular Neuroscience* 10(11). DOI: 10.3389/fnmol.2017.00011.

VII Hellsten, S.V., Eriksson, M.M., Lekholm, E., Arapi, V., **Perland**, **E**., and Fredriksson, R. (2017). The gene expression of the neuronal protein, SLC38A9, changes in mouse brain after *in vivo* starvation and high-fat diet. *PLoS One* 12(2), e0172917. DOI: 10.1371/journal.pone.0172917.

VIII Wiemerslage, L., Islam, R., van der Kamp, C., Cao, H., Olivo, G., Ence-Eriksson, F., Castillo, S., Larsen, A. L., Bandstein, M., Dahlberg, L. S., **Perland**, **E**., et al. (2017). A DNA methylation site within the KLF13 gene is associated with orexigenic processes based on neural responses and ghrelin levels. *Int J Obes* (Lond). 41(6), 990-994 DOI: 10.1038/ijo.2017.43.

IX Williams, M.J., **Perland**, **E**., Eriksson, M.M., Carlsson, J., Erlandsson, D., Laan, L., et al. (2016). Recurrent sleep fragmentation induces insulin and neuroprotective mechanisms in middle-aged flies. *Frontiers in Aging Neuroscience* 8(180). DOI: 10.3389/fnagi.2016.00180.

X Roshanbin, S., Lindberg, F.A., Lekholm, E., Eriksson, M.M., **Perland**, **E**., Ahlund, J., et al. (2016). Histological characterization of orphan transporter MCT14 (SLC16A14) shows abundant expression in mouse CNS and kidney. *BMC Neurosci* 17(1), 43. DOI: 10.1186/s12868-016-0274-7.

XI Williams, M.J., Goergen, P., Rajendran, J., Zheleznyakova, G., Hagglund, M.G., **Perland**, **E**., et al. (2014). Obesity-linked homologues TfAP-2 and Twz establish meal frequency in Drosophila melanogaster. *PLoS Genet* 10(9), e1004499. DOI: 10.1371/journal.pgen.1004499.

XII Bagchi, S., **Perland**, **E**., Al-Walai, N., Kheder, S., Lundgren, J., and Fredriksson, R. (2017) Probable role for Major facilita-

tor superfamily domain containing 6 (MFSD6) during variable energy consumption. *Submitted manuscript*

XIII Eriksson, M.M., Lekholm, E., **Perland**, **E**., Hellsten, S.V., and Fredriksson, R. (2017) The putative membrane-bound transporter protein UNC93A response highly to starvation in mice. *Manuscript*

XIV Eriksson, M.M., Aggarwal, T., **Perland**, **E**., Fasching, A., Palm, F., Williams, M.J., and Fredriksson, F. Identification of a putative osmoregulatory membrane transporter protein in *Drosophila melanogaster*. *Manuscript*

XV Lekholm, E.*, Eriksson, M.M.*, Klaesson, A., **Perland**, **E**., Schweizer, N., Söderberg, O., and Fredriksson, R. (2017) Sugar deprived mouse embryonal cortex cultures respond by alerting MFS transporter expression and localization. *Manuscript*

* Equal contribution

Contents

Abbreviations

AMTF	Atypical MFS transporter family
APC	Amino acid/polyamine/organocation
BBB	Blood-brain barrier
CLN	Ceroid-lipofuscinosis, neuronal
CPA/AT	Cation:proton antiporter/anion transporter
DAB	3,3'-Diaminobenzidine
DAT	Dopamine transporter
DMT	Drug/metabolite transporter superfamily
EAAT	Excitatory amino acid transporter
HFD	High-fat diet
HGNC	Human genome nomenclature committee
HMM	Hidden Markov model
GABA	Gamma-aminobutyric acid
GMQE	Global model quality estimation
IT	Ion transporter clan
L-Dopa	Levodopa
MFS	Major facilitator superfamily
MFSD	Major facilitator superfamily domain containing
NET	Norepinephrine transporter
Pfam	Protein family database
SERT	Serotonin transporter
SD	Standard deviation
siRNA	Small interfering ribonucleic acid
SLC	Solute carrier
SNAT	Sodium-coupled neutral amino acid transporter
SPNS	Sphingolipid transporter
SSRI	Selective serotonin reuptake inhibitor
SV2	Synaptic vesicles glycoprotein 2
SVOP/SVOPL	SV2 related proteins
PLA	Proximity ligation assay
qPCR/qRT-PCR	Quantitative real-time PCR
TCDB	Transporter classification database
TMS	Transmembrane segment
UNC93	Uncoordinated-93 protein
VGLUT	Vesicular glutamate transporter
VIAAT	Vesicular inhibitory amino acid transporter

Introduction

Solute transport between cellular compartments is vital, as it allows interaction between different cellular environments. As lipid bilayers surround the cell and its organelles, specific membrane-bound transporters are needed to convey influx and efflux of solutes. There are three major functional groups of proteins responsible for this cross-membrane transport (Almen et al., 2009): channels, primary active transporters and secondary active transporters. Channels translocate molecules down their electrochemical gradient while active transporters require ion/solute gradients to constitute movement (Hediger et al., 2004). Active transporters are thus energy dependent and can move molecules in both directions over the membrane (Wilkens, 2015). The primary active transporters independently generate and maintain electrochemical ion gradients for uphill transport (Hediger et al., 2004), while the secondary active transporters exploit already existing gradients to mediate their molecular transport (Hediger et al., 2004; Wilkens, 2015). In humans, secondary active transporters constitute the most common type of transporters (Almen et al., 2009), and they are called solute carriers (SLC).

Solute Carriers

An SLC is a transmembrane protein, moving substances across membranes. At present, there are 430 known SLC proteins (Perland and Fredriksson, 2017), of which 30 % are still considered orphans regarding expression profile and/or function. The SLCs are divided into families based on function, homology or phenotype (Gray et al., 2016), and currently there are 52 SLC families (Hediger et al., 2013). As there is relatively high similarity in structure and function among the SLCs, a protein must share more than 20 % sequence identity with other members to be placed in a family (Hediger et al., 2004). Each SLC family has specific substrate profiles (listed in Table 1), but combined, they have a broad spectrum of substrates, and shuffle essential molecules such as amino acids, ions, sugar, neurotransmitters, xenobiotic compound and therapeutic drugs (Hediger et al., 2004).

Table 1. *SLC families, number of family members, substrates and Pfam clan*

SLC family	Members	Substrate	Pfam Clan
SLC1	7	Glutamate and neutral amino acids	No clan
SLC2	14	Sugar	MFS (CL0015)
SLC3	2	Amino acids	Tim barrel (CL0058)
SLC4	10	Bicarbonate	APC (CL0062)
SLC5	12	Sugar	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)
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	Sugar	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	Ion/Sugar/Phospholipids	MFS (CL0015)
SV2	3	Sugar	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

Substrates for the SLC families and the atypical SLC proteins were obtained from the SLC tables (http://slc.bioparadigms.org/), and MFSD2A (Esnault et al., 2008), MFSD4B (Horiba et al., 2003b), MFSD5 (Tejada-Jimenez et al., 2011), MFSD10 (Ushijima et al., 2008), MFSD14A (Matsuo et al., 1997) and SV2A (Madeo et al., 2014).

SLCs are evolutionary well-conserved proteins (Hoglund et al., 2011; Sreedharan et al., 2011; Schioth et al., 2013; Perland et al., Aug. 2017), expressed in most cellular membranes: the plasmalemma (Hediger et al., 2004; Nishimura et al., 2014; Perland et al., 2016; Wang et al., 2016; Perland et al., 2017), the membranes of synaptic vesicles (Hediger et al., 2004; Takamori, 2006; Gronborg et al., 2010; Mendoza-Torreblanca et al., 2013; Loscher et al., 2016), endosomes/lysosomes (Siintola et al., 2007; Storch et al., 2007; Palmieri et al., 2011; Chapel et al., 2013; Montalbetti et al., 2013; Ratajczak et al., 2014) mitochondria (Yanagisawa et al., 2003; Hediger et al., 2004; Haitina et al., 2006), Golgi (Ishida et al., 2005) and the endoplasmic reticulum (Tabeta et al., 2006; Angers et al., 2008) (Fig. 1).

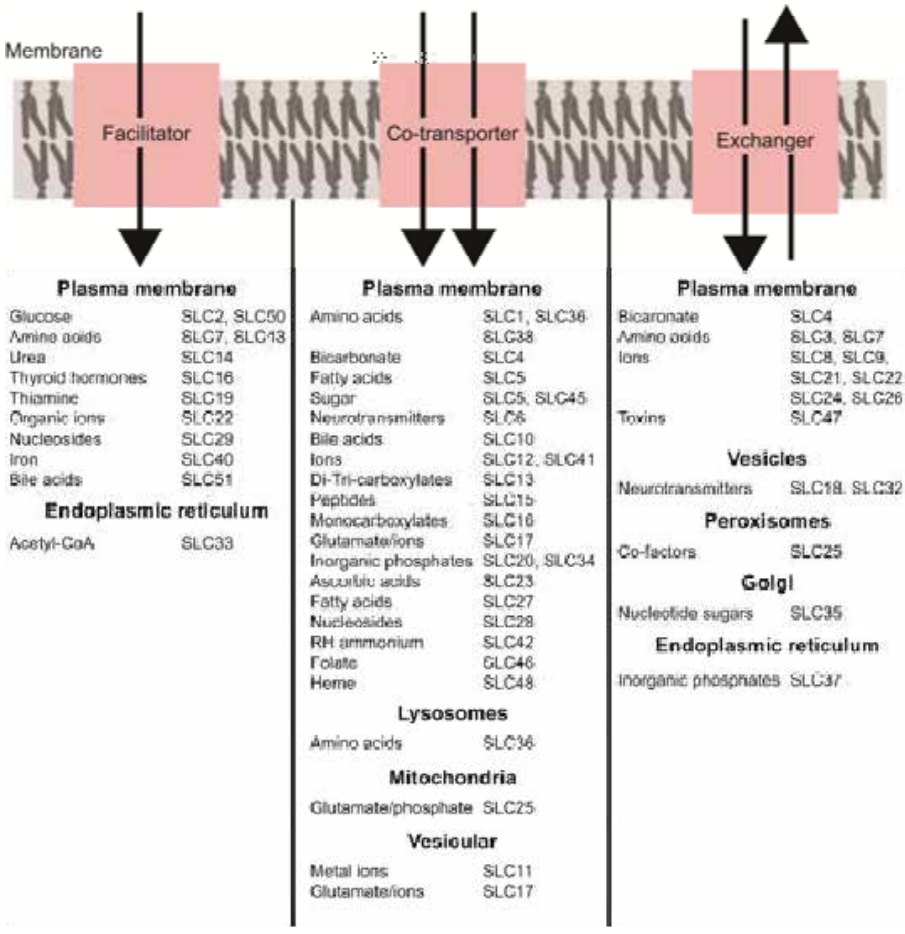

Figure 1. General or predicted location, mechanism and substrate of known SLC families. Information obtained from the SLC tables (http://slc.bioparadigms.org/). Cell membrane was retrieved from Somersault18:24 (www.somersault1824.com), shared under a creative commons license (CC-BY-NC-SA 4.0).

General functions of known SLC families

SLCs have three general transport mechanisms, facilitative diffusion (uniport), coupled-(co-) transport (symport) or exchange (antiport) (Fig. 1) (Hediger et al., 2004) Facilitators are passive transporters mediating passage over membranes along an electrochemical gradient (Hediger et al., 2004), and the facilitative SLCs are generally located to the plasma membrane (Fig. 1). Substances such as glucose (SLC2 (Mueckler and Thorens, 2013), SLC50 (Wright, 2013)) and amino acids (SLC7 (Fotiadis et al., 2013), SLC43 (Bodoy et al., 2013)) can be moved via facilitative diffusion. Co-

transporters and exchangers, on the other hand, use energy from coupled ions and already existing ion gradients (Hediger et al., 2004) to enable bi-directional transport. The transporters couple the substrate to an ion, usually sodium (Na^+) or hydrogen (H^+), to allow passage along the ion's gradient, either in a unified way (co-transport) or in opposite directions (exchange).

Co-transport is the most common mechanism, and about half of the SLC families are constituted by proteins having this type of function. They are mostly found in the plasma membrane, but also in intracellular organelles like the lysosomes (SLC36 (Sagne et al., 2001; Roshanbin et al., 2014)) and mitochondrial (SLC25 (Palmieri, 2013)) membranes. Transport of iron (SLC11 (Montalbetti et al., 2013)), amino acids (SLC1 (Kanai and Hediger, 2004), SLC36 and SLC38 (Schioth et al., 2013)) and peptides (SLC15 (Smith et al., 2013)) are mediated by co-transporters (Fig. 1). Exchangers are also expressed in multiple cellular lipid bilayers, such as the plasma membrane (SLC3 and SLC7 (Fotiadis et al., 2013)), vesicular membrane (SLC18 (Lawal and Krantz, 2013), SLC32 (Gasnier, 2004)) and the Golgi membrane (SLC35 (Song, 2013)), and they have diverse substrate profiles. The exchanger families SLC18 and SLC32 are, for example, responsible for the filling of neurotransmitters into synaptic vesicles (Gasnier, 2004; Lawal and Krantz, 2013), while SLC3 and SLC7 regulate intracellular amino acid levels by mediating transport over the plasma membrane (Fotiadis et al., 2013).

SLCs in the central nervous system

Membrane transporters are essential for all cells, and they play important roles in the central nervous system. According to the SLC tables (http://slc.bioparadigms.org/), approximately half of all the SLCs have confirmed brain expression. As the brain is an enclosed organ with regards to the blood-brain barrier (BBB), substances cannot diffuse freely from the periphery to the central parts. Transporters are therefore needed to mediate substrate distribution (Nalecz, 2017). Transporters are expressed both on neurons and glial cells, and together, SLCs aid in providing cells with nutrition, controlling neurotransmission, and in preventing neurotoxicity by removing signalling substances from the extracellular milieu. As the following sections will evidence, the brain would not function properly without its SLCs.

SLCs gate nutrition over the blood-brain barrier

The blood-brain barrier (BBB) acts as a wall separating the brain from the rest of the body. This tight-junction structure prevents diffusion of compounds in and out of the brain, and therefore SLCs, among other transporters, are required to mediate molecular movement (Nalecz, 2017). There are

several SLCs expressed in the BBB, like members of SLC1, SLC2, SLC3, SLC5, SLC6, SLC7, SLC16, SLC21, SLC22, SLC38, SLC44 and SLC47 (Nalecz, 2017), where for example SLC2A1 provides energy to the brain in the form of different sugars through its extensive expression in cerebral endothelial cells (Mueckler and Thorens, 2013). Amino acids and ions are also transported over the BBB through various SLCs (Nalecz, 2017), such as SLC7 (Fotiadis et al., 2013) and SLC22 (Hosoya and Tachikawa, 2011) family members, respectively. Neurotransmitters are mainly synthesized in the brain, but their precursors can be distributed from the periphery and must then be gated over the BBB via transporter proteins to enter the brain. SLC6 and SLC38 members, for example, transport precursors for glutamate, GABA and catechol amines to the brain (Nalecz, 2017). Levodopa (L-Dopa), an intermediate in the biosynthesis of dopamine which is used as a prodrug, is also transported through an SLC (SLC7A5) (del Amo et al., 2008), enabling the addition of dopamine as treatment for patients suffering from Parkinson's disease. This particular example with L-Dopa is an excellent illustration of how pharmacology utilises endogenous systems for therapeutics, where the body's own transport system and synthesis machinery is utilised to create an active drug inside the brain.

The SLC machinery also exerts the opposite function, and removes molecules from the brain, where, for example, SLC6 transporters are responsible for the GABA efflux from the brain. The SLCs are needed to mediate the influx and efflux of solutes to and from the brain.

Glutamate-glutamine-cycle

Inside the brain, SLCs allow neurons to take up necessary precursors for neurotransmitters. A refined example of this is the glutamate-glutamine-cycle. Upon glutamate release, SLC1 members (SLC1A2 (EAAT2) and SLC1A3 (EAAT1)) located on the plasma membrane on astrocytes lining the synapse, move glutamate from the cleft and in to the glial cell (Shigeri et al., 2004; Bak et al., 2006). This prevents glutamate neurotoxicity and enables the glutamate recycling. In the glial cell, glutamate is transformed into glutamine (Albrecht et al., 2010), which is released from the astrocyte through SLC38A3 (SNAT3) and SLC38A5 (SNAT5), and reabsorbed by the neuron through SLC38A1 (SNAT1), SLC38A2 (SNAT2), SLC38A7 (SNAT7) and SLC38A8 (SNAT8) (Bak et al., 2006; Albrecht et al., 2010). Once inside the neuron, glutamine is again converted into glutamate, before being packed into synaptic vesicles to enable another excitatory release.

Vesicle filling

For neurotransmitters to be released into the synaptic cleft, they must first be packed into vesicles. There are several SLCs responsible for vesicle filling;

SLC17A6, SLC17A7 and SLC17A8 (the VGLUTs) pack glutamate via co-transport of chloride ions (Reimer, 2013), and SLC32A1 (VIAAT) is responsible for the filling of GABA and glycine in an exchange of hydrogen (Gasnier, 2004). Monoamines are also packed into vesicles by specific SLCs (the SLC18 family) (Lawal and Krantz, 2013), and this particular system is hijacked by drugs like amphetamine (Lawal and Krantz, 2013), and utilised as therapeutic targets for movement disorders (Rask-Andersen et al., 2013).

Termination of neuronal signalling

A synaptic signal is active as long as there are neurotransmitters in the synaptic cleft. The duration of the signal must be strictly regulated to control neural plasticity and to prevent neurotoxicity. The brain therefore has several ways to remove extracellular neurotransmitters; they can be degraded by specific enzymes in the cleft, bind to receptors/channels on the pre- and post-synaptic membranes, or be taken up through SLCs back to the pre-synapse and surrounding astrocytes for recycling. Glutamate can be transported back into both neurons and astrocytes through SLC1 transporters (Kanai and Hediger, 2004), while the reuptake of monoamines is mediated by the SLC6 family through transporters commonly known as DAT (SLC6A3), SERT (SLC6A4) and NET (SLC6A2) (Pramod et al., 2013), all located on pre-synaptic membranes. The reuptake systems for the monoamines are the most frequently exploited SLCs as pharmacological targets (Rask-Andersen et al., 2013), and they are targeted by the monoamine-reuptake inhibitors (SSRI) when treating depression (Lin et al., 2015).

Evolution of SLCs

Membrane-bound proteins are essential for maintaining the integrity of cells, and to maintain an appropriate milieu to thrive in, which is why approximately 27 % of the human proteome is constituted of membrane proteins (Almen et al., 2009). To survive, all living cells are in need of nutritional supplements like ions, sugar and amino acids, and the SLCs control an extensive part of the transport of these types of solutes. Based on what is known as of today, one might assume that this necessary transport is the reason why SLC-related proteins are identified in both the Bacteria and Eukaryta domains (Hoglund et al., 2011) (Fig. 2). Some families appear to be evolutionary old, whereas others developed more recently. Examples of this are the SLC2 sugar family having homologs all down to bacteria (Hediger et al., 2013) and the amino acid transporter families SLC32, SLC34 SLC38 which are found as far back as in green algae (Schioth et al., 2013), while the neurotransmitter transporters in the SLC18 family are mainly present in animals (Hoglund et al., 2011). Note that Figure 2 displays a schematic repre-

sentation of species evolution, including some of the phyla with confirmed SLC expression. There are disagreements in the field as to the origin of the roots, but regardless of the exact appearance of the tree of life, SLCs have been found in all three domains (Hediger et al., 2004; Hoglund et al., 2011).

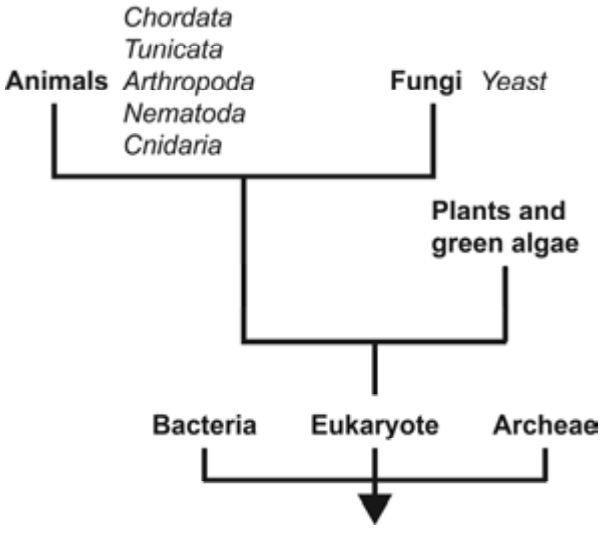

Figure 2. Schematic representation of species evolution, adapted from (Cavalier-Smith, 2010; Hoglund et al., 2011). All domains include SLC proteins, and listed are some of the eukaryotic phyla having confirmed SLC expression. Branch length does not correspond to actual evolutionary distance.

Transporter proteins having similar substrates usually have related sequences (Schlessinger et al., 2010) and share ancestral lineage. However, some families, like the amino acid transporters, do not cluster phylogenetically (Perland and Fredriksson, 2017). This suggests that different proteins have evolved independently with the aim to exert similar functions (Schlessinger et al., 2010), further emphasising the transporters importance.

The largest group of phylogenetically related SLCs are those which have the major facilitator superfamily (MFS) fold (Hoglund et al., 2011; Perland and Fredriksson, 2017). These proteins were originally identified in bacteria (Pao et al., 1998; Saier et al., 1999), but cover eukaryotic genomes as well (Hoglund et al., 2011). Among the human SLCs of MFS type, some families are well conserved, like SLC2 and SLC22, while others first seem to have occurred in animalia (e.g. SLC18, SLC19) (Hoglund et al., 2011). The sequence identity among the SLCs of MFS type is generally low, but their tertiary folding is conserved (Reddy et al., 2012), which allows similar mechanisms.

SLCs in association with diseases and therapies

To maintain a healthy milieu *in vivo*, solute transport must occur accurately. SLCs are normally co-expressed in specific patterns to coordinate regulatory circuit to control normal tissue functions, and disruption in the transport systems is associated with several Mendelian diseases. The organic anion transporter family SLC17 (Reimer, 2013) is, for example, linked to neuronal diseases such as schizophrenia, amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (Hediger et al., 2013), while polymorphisms in *SLC2A9* are associated with the metabolic syndrome, risk factors increasing the probability of developing cardiovascular disease (Kaur, 2014), due to its urea transport (Mueckler and Thorens, 2013; Lin et al., 2015).

Transporter proteins are also highly interesting for the pharmaceutical industry, due to their potential of being direct drug targets (Lin et al., 2015; Rives et al., 2017), as in SSRI drugs where members from the neurotransmitter SLC6 family are targeted (Rask-Andersen et al., 2013). Despite the evident potential of SLCs as therapeutic targets, only 12 individual SLCs have drugs exerting their primary action on the SLC itself (Cesar-Razquin et al., 2015). However, SLCs can also be targeted indirectly (Rask-Andersen et al., 2013) since they can influence the pharmacokinetics by mediating drug disposition (Cesar-Razquin et al., 2015). This is utilised in cancer treatment, for example, where SLCs can transport the drug into the cancer cell where it can exert its function (Nyquist et al., 2017). As cancer cells have increased demand for nutrients, they usually have increased expression of transporters as well (Nyquist et al., 2017), making them highly susceptible to this type of drug. At present, few SLC transporters are used in pharmaceuticals, but those which are used are the target of multiple drugs. For example, the SLC6 family is utilised in at least 42 US Food and Drug Administration (FDA) approved drugs (Rask-Andersen et al., 2013).

Nomenclature and classification

There have been several attempts to group SLCs into various clusters, and it has been summarised in the review *Classification systems of secondary active transporters* (Perland and Fredriksson, 2017). But in general, there are three major classification systems in use: the Human Genome Nomenclature Committee (HGNC), the Transport Classification Database (TCDB) and the Protein Family Database (Pfam). HGNC includes human genes and aims at giving each entry individual symbols according to a root system (Gray et al., 2016), the SLC nomenclature. HGNC families are built on homology, function and structure (Gray et al., 2016). TCDB aims at providing all transporter proteins identity numbers according to a superfamily-family system. TCDB is a representative database that includes functional and phylogenetic data

when creating families (Saier et al., 2006). Most secondary active transporters belong to the TCDB 2.A. family (Perland and Fredriksson, 2017), where the "2" represents secondary active transporters and the "A" indicates transport via facilitative diffusion, co-transport or exchange (Perland and Fredriksson, 2017). Pfam is also a multi-organism database, clustering proteins into families and clans based on sequence similarities (Finn et al., 2016), using Hidden Markov models (HMM). There are four Pfam clans including more than one SLC family: the major facilitator superfamily (MFS) clan contains SLC2, 15, 16, 17, 18, 19, SLCO, 22, 29, 33, 37, 40, 43, 45, 46, and 49; the amino acid/polyamine/organocation (APC) clan includes SLC4, 5, 6, 7, 11, 12, 23, 26, 32, 36, and 38; the cation:proton antiporter/anion transporter (CPA/AT) clan encompasses SLC9 and 10; and the drug/metabolite transporter superfamily (DMT) clan contains SLC35 and 39. In broad terms, the protein clustering in all three database systems is identical, differing largely only in how much information can be deduced from each name/entry.

In addition to the SLC family clustering, several of the human SLCs are grouped into four major phylogenetic clusters, denoted α (13 SLC families), β (5 SLC families), δ (2 SLC families) and γ (2 SLC families) (Fredriksson et al., 2008; Hoglund et al., 2011; Perland and Fredriksson, 2017). These analyses produced similar family clustering as HGNC, but they also allowed families to group together, visualising evolutionary relations. Since these studies were completed, approximately 50 new SLCs have been identified, and it is therefore likely that more proteins will fall into the four groups. Other attempts have been made to group and name proteins according to function (Schlessinger et al., 2010) or substrate (Broer and Palacin, 2011), but no matter how the classifications are performed, the results are relatively similar. Proteins clustering together based on sequence similarities usually have similar substrates and evolutionary ancestors, as it all comes down to protein topology. It is important to identify sequence relationship, as this increases the possibility finding a protein's character, including evolutionary conservation, mechanism and substrate specificity.

In the works presented in this thesis, the HGNC nomenclature was utilised in text, and the Pfam database was searched when identifying novel proteins.

Atypical SLCs

Through extensive genome annotation projects (Fredriksson et al., 2008) and proteome scanning using HMMs built on transporters from specific Pfam clans (Perland and Fredriksson, 2017), several atypical SLCs have been identified. These proteins are evolutionary related to SLCs (Fredriksson et al., 2008; Sreedharan et al., 2011; Perland et al., 2016; Lekholm et al., 2017;

Perland et al., 2017; Perland et al., Aug. 2017), but not named according to the SLC nomenclature (Perland and Fredriksson, 2017). Instead, most atypical SLCs are called according to the major facilitator superfamily domain containing (MFSD) terminology. The novel atypical SLCs are the 17 MFSD proteins (MFSD1, 2A, 2B, 3, 4A, 4B, 5, 6, 6L, 8, 9, 10, 11, 12, 13A, 14A and 14B), three synaptic vesicles glycoprotein 2 proteins (SV2A, SV2B and SV2C), two SV2-related proteins (SVOP and SVOPL), three sphingolipid transporters (SPNS1, SPNS2 and SPNS3), two Unc-93 proteins (UNC93A and UNC93B1), ceroid-lipofuscinosis, neuronal 3 (CLN3), transmembrane protein 104 (TMEM104) and the oculocutaneous albinism-related protein (OCA3). Those with known subcellular locations are included in Figure 3.

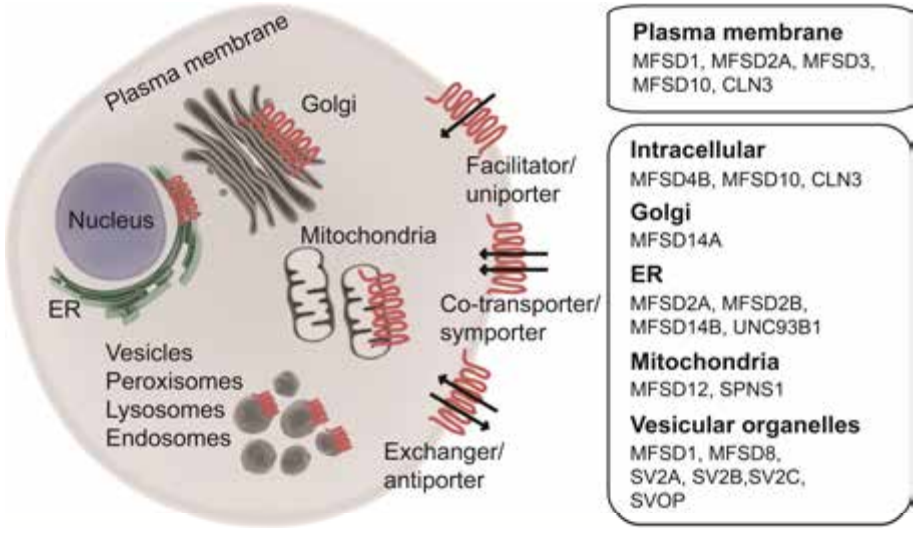

Figure 3. Schematic representation of the general locations of the SLC and atypical SLC proteins. Locations of atypical SLCs are listed in the boxes. Cell components were collected from Somersault18:24 (www.somersault1824.com), shared under a creative commons license (CC-BY-NC-SA 4.0).

HGNC ascertains that a given gene will only have one approved symbol (http://www.genenames.org) (Gray et al., 2016), meaning that the atypical SLCs will never be designated a name by the SLC root system, even though they fulfil the SLC definition. The atypical gene *MFSD7*, for example, is clustered into the SLC49 family (Khan and Quigley, 2013) and called *SLC49A3* in the SLC tables (http://slc.bioparadigms.org/), but its given name is still *MFSD7*.

All atypical SLCs, except CLN3, cluster into Pfam clans, where the MFSDs, SV2s, SVOP and SVOPL, SPNSs and UNC-93 proteins belong to

the MFS clan; TMEM104 is found in the APC clan and OCA2 cluster with the ion transporter (IT) clan.

Major facilitator superfamily

The term major facilitator superfamily (MFS) was coined in an article in 1993 (Marger and Saier, 1993), where the authors used statistical methods to cluster five bacterial facilitator families into one superfamily, the MFS. Since then, the number of families in the MFS has increased significantly (Pao et al., 1998; Saier et al., 1999; Perland and Fredriksson, 2017), and according to TCBD there are presently 85 subfamilies in the MFS transporter family, which includes about 25 % of all transporter proteins in prokaryotes (Law et al., 2008). The MFS was first considered as a bacterial protein family, but members were later found in several other classes as well, such as plants, insects and mammals (Pao et al., 1998; Sreedharan et al., 2011; Reddy et al., 2012). The human MFS proteins also share ancestral lineage with orthologues from several different species (Jacobsson et al., 2007; Hoglund et al., 2011; Sreedharan et al., 2011), as showed also in papers I-IV, suggesting that they are, or have been, essential for survival.

MFS proteins were primarily identified as membrane-bound transporters based on sequential analyses (Marger and Saier, 1993), but were later to include proteins whose transporter functions are still questioned, like the SV2 proteins. It is possible that MFS proteins have other functions in addition to transport, such as being receptors, enzymes or tranceptors. However, based on sequential analyses, all MFS proteins expressed in humans are considered as putative transporters until the contrary is proved.

28 of the atypical SLCs and all the proteins in the SLC2, 15, 16, 17, 18, 19, SLCO, 22, 29, 33, 37, 40, 43, 45, 46, and 49 families are MFS proteins belonging to the MFS Pfam clan (Hoglund et al., 2011; Perland and Fredriksson, 2017). CLN3 is not found within any Pfam clan, but it is listed as member of the Equilibrative nucleoside transporter family, which belongs to the MFS superfamily, according to TCDB.

The MFS is a very large and diverse superfamily (Pao et al., 1998; Reddy et al., 2012), exerting functions like nutrient uptake and ion transport (Table 1), and their transcription levels are affected by diet and changed nutritional status (Angers et al., 2008; Berger et al., 2012; Perland et al., 2016; Hellsten et al., 2017; Lekholm et al., 2017; Perland et al., 2017; Perland et al., Aug. 2017), suggesting involvement in cellular homeostasis.

Structure and mechanism of MFS proteins

MFS proteins are believed to originate from a common ancestor and share structural phenotype and mechanism (Reddy et al., 2012). MFS proteins are single polypeptides, constituted by transmembrane segments (TMS) combined by hydrophilic loops, with cytoplasmic N and C terminals (Reddy et al., 2012)(Fig. 4). They are generally composed of 12 TMS (Keller et al., 2014; Yan, 2015), which probably arose by gene duplication of a 6 TMS peptide or fusion of two 6 TMS peptides (Maiden et al., 1987; Reddy et al., 2012; Keller et al., 2014). However, there are disagreements in how the 6 TMS protein evolved; either it emerged through duplication of a 3 TMS peptide (Keller et al., 2014), or by triplication of a 2 TMS peptide (Reddy et al., 2012). There are functional similarities between every 3 TMS (Keller et al., 2014), where, for example, the first TMS in each triplet (TMS 1, 4, 7 and 10) constitutes the substrate binding pore (Yan, 2015) in the folded protein, providing emphasis to the duplication theory. However, sequence comparisons have stronger scores for 2 TMS similarities than 3 TMS (Reddy et al., 2012), and together with bioinformatic analyses focusing on conserved motifs and HMM results, the 3 TMS model is suggestively rejected (Vastermark and Saier, 2014). The controversy over the origin of the MFS peptide still continues.

Nevertheless, MFS proteins are composed of two 6 TMS peptides – the N and C domain – which are connected by a hydrophilic loop between TMS 6-7. The two domains are related by a 180º rotation (Yan, 2015), establishing the characteristic MFS-fold found in all MFS proteins. The cytoplasmic loop between the two domains is possibly required for proper protein function. In the GlpT protein (glycerol-3-phosphate transporter from *E.coli*), this loop connects to the N and C domains, and constrains their movement (Huang et al., 2003; Law et al., 2008), enabling transport via the rocker-switch mechanism while the protein sustains proper structure. Most MFS proteins mediate transport via the rocker-switch alternate access mechanism (Law et al., 2008), or via the refined clamp-and-switch model, which also include occluded proteins (Quistgaard et al., 2016), meaning that the N and C domains are rocking to unravel various conformational states, providing alternate access to both sides of the membrane (Law et al., 2008; Quistgaard et al., 2016). By changing its conformational state, the proteins allow transport of endogenous and essential compounds, xenobiotics and pharmaceuticals between cellular compartments.

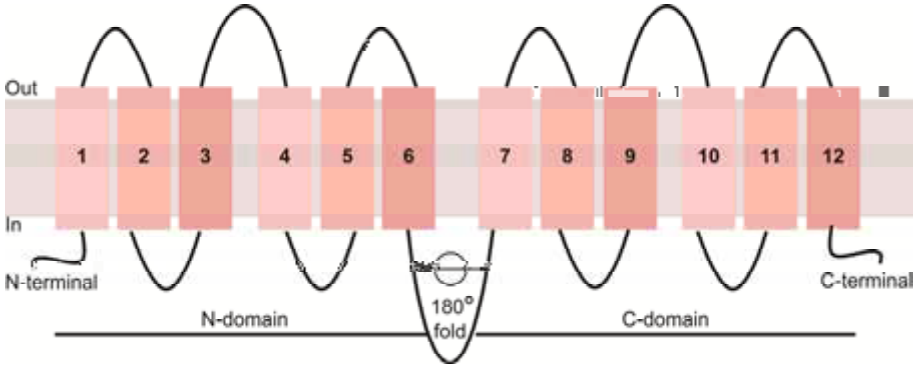

Figure 4. Illustration of how the general MFS secondary structure appears. TMS 1-6 constitutes the N-domain, while TMS 7-12 is within the C-domain. The domains are connected by a hydrophilic loop folding the peptide 180º, causing the MFS fold. The N and C terminals are located to the cytoplasm.

Aims

It is evident that SLC proteins are involved in several processes for maintaining physical well-being, and that they are important in pharmacokinetics. A systematic approach has been requested to elucidate characteristics of all plausible SLCs (Cesar-Razquin et al., 2015). The aims of the presented studies were therefore to investigate the atypical SLCs originating from the MFS Pfam clan. In Papers I-III, specific proteins were analysed, whereas in Paper IV, a cohesive understanding of 29 SLCs of MFS type was compiled. The overall aim was to create a solid foundation on which further work can be based; the individual aims for the papers were as follows below.

Paper I

The aims were to analyse the atypical SLCs MFSD5 and MFSD11, and their relationship with SLC families of MFS type. One aim was also to clarify the global mRNA and protein distribution in mouse tissues, with a focus on the brain, and their subcellular location in embryonic primary cortex cells. As SLCs are presumably involved in maintaining the homeostasis, transcription levels of *Mfsd5* and *Mfsd11* were analysed in mouse tissues after subjecting mice to different diets.

Paper II

One aim was to elucidate the neuroanatomical distribution of MFSD1 and MFSD3, and their subcellular locations. Other aims were to establish their resemblance with known SLCs by phylogenetic analyses, and to perform structural predictions to strengthen the hypothesis that they are transporters.

Paper III

MFSD4A and MFSD9 were studied with the aims to visualise their neuronal staining pattern in mouse brain, and to get a deeper understanding of their structural topologies. Proteomes were searched to identify orthologues to the human proteins, using HMM.

Paper IV

The aim was to obtain a cohesive understanding of 29 atypical SLCs of MFS type and to divide them into possible families to facilitate functional characterisation studies. Furthermore, understanding ancestral development by identifying orthologues in different species, using HMM, enhances the probability of finding the proteins' functions. As all of the studied proteins were relatively similar in structure and expression, another aim was to study their co-expression using single-cell RNA sequencing data, supplemented with epitope proximity studies using *in situ* proximity ligation assay. This provided understanding in how transporters co-work in single cells. Since previous data showed changed expression levels of atypical SLCs due to altered nutritional status *in vivo*, the final aim was to investigate if and how the atypical SLCs of MFS type reacted to reduced amino acid availability *in vitro*.

Methodological considerations

The experimental procedures are described in detail in the papers. Brief theoretical descriptions of the methods I have used in the papers are provided below.

Hidden Markov models

To identify proteins related to the human MFSDs, HMM were used. A model is built on two information paths, the underlying *state path* (the Markov) where the probability calculations occur in a hidden state, and the *observed sequence* which is the visible sequences (Eddy, 2004). In the state path, various probable outcomes (paths) are calculated, and algorithms identify the most likely result before calculating the confidence of the model (Eddy, 2004). For the identification of novel proteins, the HMM calculate the probability of how amino acids change over time. The *state path* are the proteins not yet found, whereas the *observed sequence* are the proteins included in the alignment used to build the HMM. The program thus calculates the probability of how the protein sequence would be in various species.

The atypical SLCs of MFS type were identified by HMM searches. A HMM was built on an alignment including all known human SLCs belonging to the MFS Pfam clan. The human proteome was then scanned, using the model, in search for related proteins (Perland and Fredriksson, 2017). The identified hits were the proteins targeted in the articles included in this thesis. In Papers I, III and IV, separate HMMs were built for each atypical SLC protein to enable identification of related proteins in other species.

Phylogenetic trees

With phylogenetic trees, the aim was to reconstruct evolutionary relationships. For all but two phylogenetic tree, MrBayes was used, and the program is based on Bayesian interference of phylogeny (Huelsenbeck and Ronquist, 2001). This is a statistical method where the probability of the model is updated as soon as more information becomes available. The program calculates possible trees and uses this information to calculate new trees, to finally predict a reliable tree (Huelsenbeck and Ronquist, 2001). With its stringent

computational models, MrBayes is a useful program concerning small to medium alignments. It allows fewer total numbers of built possible trees compared to likelihood analyses, making it a relatively fast calculation. However, for larger data sets MrBayes will not converge in reasonable time and a likelihood approach is more appropriate. Two of the trees (in Papers I and IV) were therefore built using RAxML (Stamatakis, 2014), which calculates likelihood through bootstrapping.

Structural predictions

There are several online tools available for predicting a peptide's topology (Hirokawa et al., 1998; Krogh et al., 2001; Kall et al., 2004; Dobson et al., 2015), where the predictions are based on parameters such as hydrophobicity, charge bias, helix lengths and signal peptides (Krogh et al., 2001; Kall et al., 2004). The available tools seem to evolve from each other, where each new program incorporates the good qualities of the already existing programs. When CCtop predicts structures, it anticipates topology according to 10 other programs (including TMHMM and phobius), and combines the results with experimental and computational data (Dobson et al., 2015), providing a final structure. CCtop also provides a probability calculation stating how accurate the prediction is.

There are also automated online tools that build homology models. The target sequence is searched against a database of structurally known sequences, from which potential templates are listed. At present, only bacterial MFS proteins are structurally determined and listed as possible templates, and these proteins share relatively low sequence similarities with human MFS protein sequences. However, as most MFS proteins share topology (Reddy et al., 2012; Yan, 2015), the MFS templates were still considered as a good option when building the tertiary models.

Quantitative real-time PCR

To quantify transcription levels of a given gene, qPCR/qRT-PCR was used. RNA was transcribed into cDNA and amplified in a cyclic process of denaturation, annealing and elongation (Kubista et al., 2006). The GeNorm procedure (Vandesompele et al., 2002) was used to normalise the obtained cycle's threshold values against the geometric mean of a set of reference genes. For all experiments, six different reference genes were run to identify the most stable genes. For the wild type panels, all reference genes were considered stable and used in the analyses. Reference genes can however be affected by altered food intake; only the three least affected genes were therefore used as references for the food paradigm experiments.

In Paper I, differences in expression levels were calculated using one-way ANOVA, with Bonferroni post-hoc test, as all three different experimental groups were included in a combined analysis, whereas in Papers II and III unpaired T-tests were used as analysis method. The reason for the changed analysis procedure was that no comparison was done between the starved and high-fat diet groups. Only two groups were compared per analysis: starved versus control (given normal chow) and high-fat diet versus control. Since the experimental diet groups were compared with the same control, significance levels were adjusted using Bonferroni correction for multiple testing.

There were 4–6 mice in each experimental group for the qPCR trials, and since the focus was not on biological difference, the RNA samples were pooled before the qPCRs were run. The difference between the triplicate runs, and the standard deviations, represents technical errors rather than biodiversity.

Immunostaining

To visualise proteins in tissue and cell samples, immunostaining techniques were utilised. Antibody-antigen interactions allow colorimetric or fluorescent detection of the proteins of interests.

Antibodies can be verified by several methods, using blocking peptides, siRNA knockdown, knockout mice or cells. However, as the MFSD proteins are novel, there were, at present, not many antibodies available, nor were there any blocking peptides or knockout mice that could be used for verification. Western blot was therefore used, which allows antibody-antigen interaction in a protein sample, producing stained bands at different sizes, in relation to a size-controller. Western blot was used in Papers I-III, where the antibodies bound proteins at correct size in a protein sample collected from mouse brain. In Paper I, co-staining and proximity ligation assays were performed with antibodies targeting different epitopes on the same protein, to prove that two different antibodies bound epitopes in proximity to each other. However, this could not be performed for Papers II or III due to the absence of antibody alternatives. Generally, all present antibodies were produced in the same species preventing co-staining, and only the utilised antibodies produced bands at correct sizes in western blot.

To display the global neuroanatomical distribution of MFSD proteins in larger tissue samples, enzymatic DAB staining was used. In this method, biotinylated secondary antibodies are allowed to form a complex with avidin. Avidin has multiple biotin binding sites, and a non-saturated solution of avidin/biotin is mixed. The biotins are conjugated to horseradish peroxidase enzymes to enable higher enzyme concentration at the antibody binding site. Upon adding the avidin/biotin-enzyme mixture to the tissue sample, the avi-

din-complex binds the biotinylated secondary antibodies (according to the manual from the kit used). This increased enzyme availability enables amplified colorimetric staining, as the DAB chromogen is oxidised by the enzymes into a visible insoluble precipitate (Ramos-Vara, 2005).

To determine which cells express MFSD proteins, fluorescent staining was implemented on tissues, as it allows co-staining between different cellular markers, by utilising various fluorophores.

Finally, subcellular location was studied in embryonic primary cortex cells, using fluorescent immunocytochemistry. Confocal microscopy (Paper I) and super-resolution microscopy (Paper II) provide the possibility to image individual cells at different focus levels, which enable visual separation of plasma membrane and organelle staining. It is more challenging to work with primary cultures than with immortalised cell lines, but they have the benefit of being better representatives of a healthy system as they include several different cell types and are not derived from cancer cells. Primary cultures also include developing cells, and the expression might not correspond to what is visualised in adult animals.

Results and discussion

The first three papers focused on outlaying the histological distribution of mRNA and protein level for six novel MFS transporters in mouse tissues, together with phylogenetic clustering and structural protein predictions of the human sequences. The fourth paper summarised what is known about atypical SLCs of MFS type, and extended this information with evolutionary development and protein sequence interrelations on phylogenetic, transcriptional and protein levels.

All results are described and discussed in detail in each paper. A more general discussion, focusing on specific issues is presented below.

Paper I

MFSD5 and MFSD11 were the first targets, and the material collected for the manuscript was later used in Papers II and III. From micro array data previously collected (Hellsten et al., 2017), it was known that transcription of *Mfsd11* was affected in mouse hypothalamic N25/2 cells upon complete amino acid starvation (Hellsten et al., 2017), leading us to consider if and how the genes were affected by changed diet *in vivo*. With brain sections and specific brain areas, transcriptional alterations upon food deprivation and high-fat diet (HFD) were monitored in mice. *Mfsd5* was generally reduced by both food paradigms in all but the most posterior brain section, whereas *Mfsd11* remained stable except in midbrain where upregulation was detected after both food deprivation and high-fat diet. The two genes responded differently with changed diet, even though they shared subcellular localisation and were found in both excitatory and inhibitory neurons. The difference in transcription regulation probably lies in their functions. Because they are evolutionary separated, it is likely they possess different transport mechanisms and substrate profiles. In the presented likelihood tree, MFSD5 clustered closest to the facilitative amino acid SLC43 family, whereas MFSD11 branched off together with the SLC46 family, a known co-transporter family that gate folates over membranes. As evolutionary-related proteins usually share sequence properties, they generally also share function and substrate profiles (Schlessinger et al., 2010). It is therefore plausible to consider that MFSD5 and MFSD11 have different functions and transport mechanisms.

Immunostaining was utilised to study protein localisation, where the global expression of MFSD11 was analysed by DAB staining, showing abundant staining. This colorimetric method could not be used with the MFSD5 antibody as it produced unsatisfactory staining, even though it stained well when using the fluorescent labelling protocol. In theory, DAB is more sensitive than fluorescent staining, as it amplifies the signal through the avidin-biotin complex. However, in the fluorescent staining protocol there was an antigen retrieval step, boiling the section in citric acid, to enable binding. This was not done for the floating sections used in the DAB staining. It is therefore possible that the antibodies could not reach the epitopes in the 70μm thick slides used for the DAB staining. In later manuscripts (Papers II and III), the protocol was developed to enable antigen retrieval for DAB staining as well, but this had not yet been done when this article was published. The MFSD5 results originated from fluorescent staining only.

MFSD5 and MFSD11 were abundantly expressed in both embryonic and adult brains, suggesting they could be needed to maintain nutrient homeostasis. That their transcription levels were adjusted by the nutritional availability strengthens the hypothesis that they are involved in the cellular response to changed energy availability. Their plasma membrane expression facilitates the movement of molecules in and out of cells, and enables the maintenance of an appropriate intra-cellular milieu.

Paper II

MFSD1 and MFSD3 were studied in a similar manner as the proteins in Paper I, but with more focus on the proteomic analyses, where topology and homology models were used to strengthen the hypothesis that the atypical SLCs are transporters.

Phylogenetically, both MFSD1 and MFSD3 clustered with the facilitative SLC transporter families of MFS type, closest to SLC29 and SLC33 respectively. However, they cannot be grouped into any of these families since they were less than 20 % identical to present family members, and their substrate profiles cannot be inferred. For Paper I, it was known beforehand that *Mfsd11* was altered after complete amino acid starvation, but nothing was known about *Mfsd1* or *Mfsd3*. It was found that the expression of *Mfsd1* was upregulated by the amino acid starvation in primary cortex cells, whereas *Mfsd3* was unaffected. *In vivo*, the two genes were altered in different brain areas, where *Mfsd1* was increased in brainstem and reduced in cortex and striatum, while *Mfsd3* was increased in both brainstem and cerebellum, but down-regulated in hypothalamus after starvation. Consequently, they appeared to possess different functions in different parts of the brain, even though both proteins were detected along neuronal plasma membranes.

Structurally, both proteins were predicted to contain 12 TMS divided into the N and C domains, and the terminals were on the intracellular side of the membrane. The proteins were also predicted to fold according to the common MFS structure, revealing a potential substrate pore. The hypothesis that MFSD1 and MFSD3 are novel membrane-bound transporters is supported by the fact that the proteins share phylogenetic clustering with known SLC transporters and that they are predicted to exhibit the common MFS structure.

Paper III

Here, the focus was on outlaying the basic properties of the atypical SLCs MFSD4A and MFSD9. They developed late in evolution, and no related proteins could be detected in *S. cerevisiae*, *C.elegans* or *D.melanogaster*. The mammalian orthologues, on the other hand, had high sequence identity, with more than 50 % identical amino acids between tested species, suggesting conserved substrate and mechanism. When building the homology models, several of the TMS were disrupted helixes, even though they probably possess the standard helical configuration. This could depend on the modelling, that the proteins were not thoroughly aligned with the templates, providing breaks in the model, or that the incomplete helixes had a lower hydrophobicity index. An amphipathic TMS could, for examples have hydrophobic amino acids lining the plasma membrane and a more hydrophilic inner core to enable transport of charged or polar compounds. When looking at the specific amino acids in the MFSD4A and MFSD9 models, it seems that some of the fractionated TMS contained non-polar amino acids in the non-helical segment, supporting flaws in the prediction. However, other areas also included charged amino acids, enabling transport of polar substrates. MFSD4A phylogenetically clustered with the sodium-dependent sugar transporter MFSD4B (Horiba et al., 2003a; Horiba et al., 2003b; Perland and Fredriksson, 2017), suggesting that MFSD4A also could transport sugar, ions or both. MFSD9 phylogenetically grouped with the folic acid co-transporter SLC46A1 (Zhao and Goldman, 2013; Perland and Fredriksson, 2017), and the organic ion transporter MFSD10 (Ushijima et al., 2008; Perland and Fredriksson, 2017), making it a plausible transporter of charged substrates as well.

Protein expression was studied on cell type level, showing co-localisation with neuronal markers. With the fluorescent labelling, the signal was seemingly condensed around the nuclei, but from DAB staining antibody signal was detected along both soma and projections. MFSD4A labelled both soma and projections in, for example, hippocampus and cerebellum, while MFSD9 displayed projections in areas such as cortex and hippocampus layer CA2. Both proteins were found in several brain areas, but with different staining

patterns; e.g. MFSD4A staining was constrained to layer 5 in cortex, whereas MFSD9 was found through all cortex layers. The correct protein sizes detected with western blot and the specific binding patterns found in brain sections indicated that the MFSD4A and MFSD9 stainings were correct.

Mfsd4a and *Mfsd9* were also affected by food deprivation and an HFD. Transcription changes were analysed in specific brain areas. In general, the response was similar for the two transporters, except in hypothalamus where *Mfsd4a* was unchanged upon food deprivation while *Mfsd9* was increased, and in striatum where an HFD reduced *Mfsd4a* and *Mfsd9* remained stable. The expression of both genes was influenced by the nutritional availability. This, combined with the predicted transporter structures, suggested that MFSD4A and MFSD9 also were plausible transporters.

Paper IV

When writing Papers I-III, it became clear that a general study representing the characteristics of all atypical SLCs of MFS type was lacking. Therefore, Paper IV summarises all that is presently known about the human atypical SLCs of MFS type. This knowledge was extended to include proteomic, genomic and phylogenetic data. The work originated from the 29 human proteins, MFSD1, MFSD2A, MFSD2B, MFSD3, MFSD4A, MFSD4B, MFSD5, MFSD6, MFSD6L, MFSD7, MFSD8, MFSD9, MFSD10, MFSD11, MFSD12, MFSD13A, MFSD14A, MFSD14B, SV2A, SV2B, SV2C, SVOP, SVOPL, SPNS1, SPNS2, SPNS3, UNC93A, UNC93B and CLN3, and the proteins were divided into 15 families based on phytogenic clustering using MrBayes and sequence resemblance where more than 20 % sequence identity was required to be grouped into the same family. Proteins clustering together are usually sequentially similar, and it is likely they share substrate profile and/or mechanism. Dividing the proteins into families thus provides a shortcut to revealing a protein's function.

A phylogenetic tree including all known human SLCs of MFS type showed that the atypical proteins listed above clustered among the SLC proteins, and not together in a separate group. That the atypical SLCs phylogenetically merge with known SLCs strengthens the probability that they are transporters. This hypothesis was reinforced as 28 of the studied protein (all except CLN3) were predicted to have the common MFS transporter structure, with 12 TMS divided into the N and C domain.

MFS proteins are evolutionary old, as were several of the atypical SLCs. All proteins had orthologues in mice, four of the proteins were lost in chicken, and only MFSD5 was lost in zebrafish. Furthermore, about half of the atypical SLCs were conserved in fruit fly and roundworms, whereas only CLN3 could be found in yeast. It is possible that the conserved proteins are

needed for vital functions, possibly as housekeeping genes, whereas proteins found only in higher species possibly evolved to perform specific tasks.

Single-cell RNA sequencing data was analysed to investigate if transcripts from two atypical SLC genes were expressed in the same cell. 21 of the 29 studied genes were found to be co-expressed. However, due to cut-off values in the analysis and the relatively shallow sequence depth, it is possible that some transcripts were lost. This means that the remaining eight studied atypical SLCs still could be co-expressed. This was confirmed at protein level, where interaction between antibodies could be measured even though the transcripts were not found together in the RNA sequencing. Some genes were rarely expressed, such as *Mfsd14b* which was found together with only three other genes, whereas *Mfsd11* was more frequent and co-found with 18 other genes. Co-expression was detected in about 9,700 cells (out of 10,289), with some genes co-expressed in several of these, while others only in one or two cells. There seems to be a difference in transcription levels and distribution of the atypical SLCs.

Conclusions and perspectives

Several orphan membrane-bound proteins of MFS type were identified and studied. Due to their phylogenetic and sequential resemblance to human SLC proteins, they were considered as plausible transporters. The aim was to reveal their elementary characteristics: histology, evolution and their interrelations. Hopefully, these papers will be used as a base on which further functional studied can be founded.

Papers I-III

Atypical SLCs are relevant to study for their potentials as drug targets, but before their ability as pharmaceutical molecules can be determined, more work is needed. The purpose of Papers I-III was to study the location of six novel proteins belonging to the MFS transporter family, and with homology models visualise their possibility to fold into substrate binding pores. From the work presented here, it was established that MFSD1, MFSD3, MFSD4A, MFSD5, MFSD9 and MFSD11 were expressed in neuronal membranes, and it would be of interest to study their localisation in other areas as well. If they are expressed in the blood-brain barrier, they have potential as drug targets mediating drug distribution into the brain. High expression throughout the body, on the other hand, could cause side effect if targeted by medicines.

In the Papers included in this thesis, the focus was to understand the expression in healthy cells, and a next step could be to find out how they are expressed in disease models. Cancer tumours, for example, have an increased metabolic demand to enable continued growth, which is correlated to changed SLC expression (Nyquist et al., 2017). This entails the possibility to use these increased levels of transporters to guide drugs into the tumour.

By identifying the atypical SLCs phylogenetic clustering and their shared structure topology with SLCs enhances the hypothesis that they are transporter proteins. Detection of their cellular location in healthy brain cells is the first step toward elucidating their function. All these proteins should be included in the continued systematic research that is requested regarding SLC transporters (Cesar-Razquin et al., 2015).

Paper IV

Atypical SLCs share ancestral lineage with SLCs, but without being named according to the SLC root system or classified into any of the 52 existing SLC families (Gray et al., 2016; Perland and Fredriksson, 2017). However, when comparing the SLC families, there is high sequence variation among family members, and the atypical SLCs do not deviate from this variation. That the studied atypical SLCs clustered among the SLCs when compiling phylogenetic trees, strengthens the hypothesis that they are SLC proteins. It is therefore plausible to consider that the atypical SLCs, and the AMTFs, constitute novel SLC families. It is also possible that some of the 15 identified families could be merged into already existing SLC families. MFSD7 (AMTF5) is already in the SLC49 family. AMTF1 phylogenetically cluster with the SLC46 family, and their members shared at least 20 % identity with one other SLC46 member. The AMTF1 proteins fulfil all criteria of belonging to the SLC46 family. If the AMTFs were considered including regular SLC proteins and combined with the SLC families, it would result in 64 families enclosing SLC proteins, as in comparison to the 52 presently known SLC families.

With HMM, proteins related to the human atypical SLCs were found in several species. This enables the use of several animal models when elucidating the proteins function. Studying a protein's function in an animal model such as fruit fly, zebrafish or mouse may suggest how it works in humans.

The atypical SLCs are widely expressed in the mouse brain, and they are sometimes expressed together. By utilising the SLC co-expression network, drugs could be created to pass both into certain organs, and into specific cells. This would enable the pharmacological industry to better guide drugs to a certain location and to avoid adverse side effects. It would help in understanding how certain mutations give rise to severe diseases, while others do not. Understanding the combined molecular transport would make it possible to unravel some of the brain's many secrets.

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