Do Major Facilitator Superfamily Domain Containing Proteins Respond to Glucose Starvation?

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Abstract
The human brain weighs around 2% of the total body mass, nevertheless it consumes about 20% of the total glucose intake. Glucose, the main energy source of the brain, is important for many processes, for instance as energy for synthesis of neurotransmitters. Therefore a stable glucose concentration in the brain is crucial. Unlike other macronutrients, glucose is able to cross the BBB through facilitated transport by glucose transporters (GLUTs) that belong to the solute carrier (SLC) superfamily. There are currently 65 SLC families with over 400 members in total. Out of 65 families, many belong to the protein family (Pfam) class major facilitator superfamily (MFS). There were 28 putative SLC transporters, 18 of them were called major facilitator domain containing proteins (MFSDs). Recently MFSD2A, MFSD2B, MFSD4A, MFSD4B and MFSD5 were grouped into SLC families making the total amount of current putative SLCs 23 In this project the effects of glucose starvation on MFSD6, MFSD6L, MFSD8, MFSD9 and MFSD10 in primary mouse cortex cultures were studied on protein and gene level through immunocytochemistry (ICC) and quantitative polymerase chain reaction (qPCR). All proteins except for MFSD10 were detected in the ICC. All except MFSD8 displayed a change in fluorescent intensity. MFSD6, MFSD6L and MFSD9 were upregulated after 3 h of glucose starvation compared with control. Gene expression was detected for all targets except for Mfsd6l. Gene expression alterations were found for Mfsd8, Mfsd9 and Mfsd10. The 3 h glucose starvation resulted in an acute response in the gene expression for Mfsd9 and Mfsd10 but was back to control levels after 12 h, while Mfsd8 respond after 12 h of glucose starvation. All of them were back at similar levels as controls when re-fed with glucose. In conclusion, all five MFSDs responded to glucose starvation at some point. For instance MFSD6 responds to glucose starvation on a protein level, Mfsd6 was however also the only gene out of the four tested that did not respond to glucose starvation on a gene level.
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1.0 Introduction

The brain uses glucose as its main energy source and the human brain consumes about 20% of the glucose that is taken in, even though it only weighs 2% of the total body mass (Mergenthaler et al., 2013). The neurons in the human brain require a steady level of energy and thus need continuous energy supply (Mergenthaler et al., 2013). The Blood Brain Barrier (BBB) is, unlike other organs, not permeable to macronutrients due to tight junctions and the uptake of neuroactive substances to the brain is limited. However, some macronutrients such as glucose can cross the BBB through facilitated transport by Solute Carrier (SLC) proteins. Glucose is not only used as an energy source but also as a precursor to synthesize components within the brain, such as glutamate, that are needed for neuron activity but cannot cross the BBB (Mergenthaler et al., 2013). Glucose is metabolized to pyruvate through glycolysis which leads to the production of NADH, with the help of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Díaz-García et al., 2017). The pyruvate and NADH is then used to make ATP, energy. To initiate an action potential the neurons require a lot of energy, and most energy consumed by the brain is, according to Mergenthaler et al (2013), believed to go towards synaptic activity.

1.1 The importance of astrocytes

Astrocytes are a type of glia cell that do not send signals through action potentials as neurons do. Instead they can sense changes and communicate with surrounding cells through chemical signalling (Volterra and Meldolesi, 2005). Astrocytes modulate neurons and are, among other things, important for neuronal synaptic activity and they defend nearby neurons from oxidative stress. However, pathogenic conditions such as Alzheimer’s disease may affect astrocytes negatively, leaving neurons without astrocytic defense (Bélanger and Magistretti, 2009). In the adult brain astrocytes store glucose as glycogen that can easily be metabolized and used as energy during, for instance, fasting periods (Falkowska et al., 2015).

In 1994, Pellerin and Magistretti suggested that glutamate release from nearby cells leads to glucose metabolism in astrocytes and the release of lactate from astrocytes to surrounding
neurons. They believed that the lactate was used as fuel by neurons, which resulted in the astrocyte-neuron lactate shuttle (ANLS) hypothesis. The hypothesis indicates that neurons receive most energy from this shuttle rather than from direct glucose uptake (Mangia et al., 2009). However, Díaz-García et al (2017) suggest that neuronal metabolic responses are mostly due to direct glucose consumption by neurons themselves (Díaz-García et al., 2017).

1.2 Effects of disturbed glucose homeostasis

Lack of glucose can lead to negative effect on the brain function, such as loss of consciousness, cognitive function and/or other permanent brain damage. It can even in the worst case lead to death (Ritter, 2017). It is therefore very important that the glucose concentration in the brain is stable.

Diabetes mellitus (DM) type 2 is one example where the glucose homeostasis is negatively affected. Insulin, the hormone affected in DM, is important for lowering blood glucose levels when needed and regulates stored glycogen and fat during fasting periods. In DM patients, insulin deficiency or resistance (for instance deficient insulin receptor effect) occur, sometimes even both (Rang et al., 2016). Cognitive dysfunction linked to an unstable glucose homeostasis and memory impairment is something that has found in DM patients (Cai et al., 2012). Studies show that insulin is needed for memory processing and learning, and that insulin receptors are upregulated in the brain to compensate insulin dysfunction in Alzheimer’s disease (AD) patients (Cai et al., 2012). The Rotterdam study performed by Ott et al (1999) indicates that DM increases the risk of developing AD. New glucose transporters could therefore possibly work as drug targets to regulate the glucose metabolism for these two conditions, and also other conditions where metabolic dysfunction affects the glucose homeostasis, such as Huntington disease (HD) and Parkinson’s disease (PD) (Cai et al., 2012).
1.3 Classification

1.3.1 Solute carrier protein families

Solute carriers (SLCs) are membrane bound proteins that transport solutes such as ions and glucose across membranes (César-Razquin et al., 2015). The SLCs are divided into 65 families with more than 400 members (Perland and Fredriksson, 2017). The members are divided into families based on their structure, function and sequence (Schlessinger et al., 2014).

SLC transporters need to undergo a conformational change after binding to solutes to be able to release the solute on the opposite site of which it binds. It transforms from an inward open conformation to an outward open conformation after binding, or the opposite, depending on the direction of the transport (Quistgaard et al., 2016). SLCs are secondary active or facilitator transporter proteins, they are uniporters, symporters or antiporters (Schlessinger et al., 2014). Uniporters are facilitated transporter that use passive diffusion to transport solutes and is therefore dependent on the solute’s concentration gradient. Symporters and antiporters transport two solutes at the same time but only depend on the concentration gradient of one of the solutes. Symporters transports the two solutes in the same direction and are called cotransporters. Antiporters however transport the two solutes in different directions and are thereby called exchangers (Wolfersberger, 1994).

SLCs are needed for basic physiological mechanisms, many of them interact with drugs and some of them are involved in diseases. This makes them very interesting as possible drug targets (César-Razquin et al., 2015). SLC6A3 for instance is a dopamine transporter and is important for maintaining dopamine in cells such as neurons. Mutation in the gene coding for this transporter can lead to the development of Parkinson’s disease (Kurian et al., 2009). Another example is the SLC5 family that is involved in the basic physiological functions such as glucose reuptake in the kidneys (César-Razquin et al., 2015).
1.3.2 Protein family clans

Protein family (Pfam) is a database in which proteins can be divided into different protein families (Pfam) clans based on their functional domains. The two biggest Pfam clans for the SLCs are major facilitator superfamily (MFS) and amino acid/polyamine/organocation (APC). The MFS proteins have a structure that consists of 12, 14 or 24 transmembrane helices (Sreedharan et al., 2011). Out of the 65 SLC families, many belong to the MFS Pfam clan (SLC tables). The SLC2 transporter family, also known as glucose transporters (GLUT), is one example of an SLC family that belongs to the MFS Pfam clan. They transport glucose, among other sugars, across the BBB and membranes of both neurons and astrocytes. (Mueckler and Thorens, 2013).

1.4 Putative SLCs

In addition to the known SLCs there are some proteins of similar structure as the SLCs. These do not belong to an SLC family yet, hence they are called putative SLCs. Most of these (24) belong to the MFS Pfam clan and the remaining putative SLCs are TMEM104 and OCA2. TMEM104 belong to the APC Pfam clan and the OCA2 clusters with the ion transporters (IT) Pfam clan (Perland et al., 2017a). There were 30 putative SLCs with 18 called major facilitator superfamily domain containing (MFSD) proteins. However six putative SLCs were recently grouped into SLC families and five of those were MFSDs (MFSD2A, MFSD2B, MFSD4A, MFSD4B and MFSD5) (SLC tables). The current putative SLCs that belong to the MFD Pfam clan are therefore; MFSD1, MFSD3, MFSD6, MFSD6L, MFSD7-12, MFSD13a, MFSD14A and MFSD14B. The MFSD proteins studied in this experiment were MFSD6, MFSD6L, MFSD8, MFSD9 and MFSD10.

Little information was found about MFSD6 and MFSD6L. McCulloch et al (2017) speculate that MFSD6 may be involved in synaptic transmission since it is found in presynaptic vesicles, (McCulloch et al., 2017). According to The Human Protein Atlas (HPA), MFSD6 is both intracellular and membrane bound while MFSD6L only is predicted to bind to the membrane.
MFSD8, also called ceroid lipofuscinosis neuronal 7 (CLN7), is a lysosomal membrane bound protein that when mutated can result in CLN7 disorder (Brandenstein et al., 2016). This is a type of neurodegenerative disorder with symptoms of this disease, like seizures, first appear between the ages of 1.5 and 6 years and progress rapidly during teen years. However the function of MFSD8 and weather it is a solute carrier or not is still unknown (Brandenstein et al., 2016). According HPA, MFSD8 is mainly localized in nucleoplasm and in vesicles.

There is very limited information about MFSD9. A phylogenetically cluster of MFSDs show that MFSD9 is most closely related to MFSD10, MFSD14A and MFSD14B but also to the SLC46 family (Perland et al., 2017b). MFSD9 shares a sequence identity above 20% with SLC46A1, a folate transporter, and could therefore belong to the SLC46 family. However it also shares a sequence identity above 20% with MFSD14B (which unlike SLC46A1 is predicted to transport organic ions) and MFSD10 (Perland et al., 2017b). The substrate profile for MFSD14B remains unknown. The difference in solute profile for MFSD14B, MFSD10 and SLC46A1 makes it difficult to predict the substrate profile for MFSD9. On the other hand, Perland et al (2017) suggest that MFSD9 is involved in nutritional processes due to the fact that Mfsd9 was found to be highly expressed in kidneys and intestines (Perland et al., 2017c). MFSD9 is a predicted membrane protein and according to HPA mainly localized in nucleoli. Nevertheless Perland et al (2017) performed immunohistochemistry on MFSD9 with anti- MFSD9 (SC247973 Santa Cruz). No findings of MFSD9 staining in nucleoli was presented and therefore not discovered.

The amino acid sequence of MFSD10 is similar to a tetracycline transporter found in Escherichia coli and is therefore also called tetracycline transporter-like protein (TETRAN) (Ushijima et al., 2008). This protein transports organic anion found in the proximal tubules in the kidneys. Most of the non-steroidal anti-inflammatory drug (NSAID) are organic anions and are therefore suspected to be substrates of MFSD10 (Ushijima et al., 2008). It has been shown to transport the NSAID indomethacin and can therefore perhaps also transport other NSAIDs such as diclofenac (Ushijima et al., 2008).
1.5 Methods
In this project Immunocytochemistry (ICC) and quantitative polymerase chain reaction (qPCR) were used. ICC is used to investigate the proteins through protein staining. Staining proteins, neurons and nuclei enables detection of staining patterns, localizations and changes in protein expression. Unlike ICC, qPCR is a method used to look at gene expression. In these experiments five housekeeping genes were used for the qPCRs. Housekeeping genes are genes involved in basal and vital functions of a cell and are not affected by changes such as glucose intake. These are thereby used as reference genes to normalize the gene expression of the targets (Kozera and Rapacz, 2013).

2.0 Aim
The aim of this project is to see if and how some of the putative SLC proteins belonging to the MFS family and found in neurons in the brain, are affected by glucose starvation on a protein and on a gene level. It is also to investigate how the MFS react when glucose is added back to their diet after glucose starvation.

3.0 Materials and Methods

3.1 Locating the proteins
MFSD6, MFSD6L, MFSD8, MFSD9 and MFSD10 were located in 3 h and 12 h glucose starved mouse primary cortex cultures and qualitatively analyzed through Immunocytochemistry (ICC) on fixed cells. The cells were blocked using a milk blocking solution to minimize possible unspecific binding. Primary antibodies were then added to the cells and incubated with milk blocking solution on an orbital shaker over night at 4°C to bind to the proteins before adding fluorescent secondary antibodies the next day to enable visualization of the protein staining. Neurons were stained using a cocktail of primary antibodies called PAN (neurofilament antibody) (Purified anti-Neurofilament Marker, Bioledgend). All primary antibodies were diluted in 5% milk blocking solution (BioRad). Primary antibody for MFSD6L (HPA023164 Sigma) was diluted to 1:100, primary antibodies
for MFSD6 (SAB2502050 Sigma) and MFSD8 (HPA044802 Sigma) were diluted to 1:50 and primary antibodies for MFSD9 (SC247973 Santa Cruz Biotechnology) and MFSD10 (HPA037398 Sigma) were diluted to 1:25. 200 µl, 5 mg/ml DAPI (Thermo Fisher) diluted to 1:15000 in PBS was used to stain cell nucleus and 1 µl PAN diluted in 200 µl of the blocking grade was used to stain neurons. 1µl secondary fluorescent antibody (diluted 1:1 in glycerol) was diluted in 200µl milk blocking solution (Thermo scientific) and used in this experiment to stain the MFSD proteins and neurons. The secondary antibodies used were Alexa Fluor 488 Donkey anti-goat IgG (Thermo scientific) for MFSD6 and MFSD9, Alexa Fluor 488 Donkey anti-rabbit (Thermo scientific) for MFSD6L, MFSD8 and MFSD10 and IgG Alexa Fluor 594 Chicken anti-mouse IgG (Thermo scientific) for PAN. Images were required with an Olympus microscope BX55 with an Olympus DP73 camera. The cellSens Dimension v1.14 (Olympus) images used in the analysis were taken using a 40x objective and then handled using Image J.Fiji edition (Schindelin et al., 2012). Fluorescent intensities were measure in Image J as intensity/cell and statistically analyzed using Mann Whitney-U test. Approximately 10 sets of images were taken of each coverslip and used for the analysis. Co localization with PAN, staining pattern and fluorescent intensity of the targets were analyzed. However not all images were used in the intensity measurements due to unclear cell count. Four coverslips were used for each target in the ICC. Two were starved, one for 3h and one for 12h. The other two coverslips contained control cells for the 3h starved cells and the 12h starved cells. Paring of the mice were approved and treated as described by Perland et al (2016) (Perland et al., 2016).

3.2 RNA extraction and cDNA synthesis

Some primary cortex cultures from wild type mouse embryos were glucose starved for 3h and 12h and some were starved for 3h and 12h then refed with glucose before RNA was. The cells were homogenized and lysed according to the manufacturer’s recommendation for the AllPrep DNA/RNA Micro Kit (QIAGEN). The RNA was separated from the DNA in the homogenized lysate through a provided AllPrep DNA spin column. The RNA was then separated from proteins, washed and eluted. Concentrations of the RNA were measured using ND-1000 spectrophotometer (NanoDrop Technologies) before performing cDNA synthesis. High Capacity RNA-to-cDNA kit (Applied Biosystems AB) was used to perform cDNA synthesis. Method was performed according to manual provided from the manufacturer.
3.3 Analyzing the gene expression

Quantitative polymerase chain reaction (qPCR) was used to determine the mRNA expression changes of MFSDs in primary cortex cells subjected to glucose starvation for 3 h and 12 h as well as cells subjected to 3h and 12h glucose starvation and refed for 12h. Wells on qPCR plates were loaded with 3 µl of the synthesized cDNA together with a master mix with a volume of 17 µl making the total volume per reaction 20 µl. The master mix consisted of 11.52 µl nuclease free water, 1.0 µl DMSO (Sigma Aldrich), 3.60 µl 10x DreamTaq buffer (Thermo Scientific), 0.20 µl 25 mM dNTP (Thermo Scientific) prepared by mixing dATP, dCTP, dGTP and dTTP in a 1:1:1:1 relation, 0.05 µl 100 pmol/µl of the forward and reverse primers from Thermo Scientific (designed with Beacon Design 8, Premier Biosoft) presented in table I, 0.5 µl SYBR green diluted 1:1000 in 1xTE buffer pH 7.8 (Thermo Scientific) and 0.08 µl DreamTaq polymerase (5 U/µl, Thermo Scientific). The plate was placed in CFX96 connect machine (Bio-Rad) and the data was collected using CFX maestro (Bio-Rad). This method was repeated for five housekeeping genes (Beta actin, Beta tubulin, Cyclo, Rpl19 and H3a), see Table II for sequences. The following protocol was used; Denaturation at 95°C for 30 sec once, then denaturation at 95°C for 10 sec, annealing at the optimal temperature for the primes (see Table I and II) and elongation at 72°C for 30 sec for detection for 50 cycles then elongation for 3 min and last melting at 55°C for 10 sec for 81 cycles with 0.5°C increase for each cycle.

Table I. Forward and reverse target primers used in the qPCR and the optimal temperatures used in the annealing step for each set of primers.

<table>
<thead>
<tr>
<th>MFSD target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Optimal temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mfsd2b (Thermo Scientific)</td>
<td>cgtccagttggaacaaagtt</td>
<td>acaagggccaaacataggg</td>
<td>58.9</td>
</tr>
<tr>
<td>Mfsd6 (Thermo Scientific)</td>
<td>caactcaccagattcttca</td>
<td>cctacttactccttcacaa</td>
<td>54.8</td>
</tr>
<tr>
<td>Mfsd8 (Thermo Scientific)</td>
<td>attctaactgccccatte</td>
<td>ctatactcttacctcactt</td>
<td>56.0</td>
</tr>
<tr>
<td>Mfsd9 (Thermo Scientific)</td>
<td>tgtgtctgttctcagagt</td>
<td>tgtgaagcaaatctccta</td>
<td>57.0</td>
</tr>
<tr>
<td>Mfsd10 (Thermo Scientific)</td>
<td>cctgctcttggaatatggt</td>
<td>cagtgtcttggaacagaa</td>
<td>57.0</td>
</tr>
</tbody>
</table>
Table II. Forward and reverse primers for the housekeeping genes used in the qPCR. Also the optimal temperatures used in the annealing step for each set of primers.

<table>
<thead>
<tr>
<th>Housekeeping genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Optimal temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta actin (Thermo Scientific)</td>
<td>cctcttgggtatggaatctctgtg</td>
<td>cagcactgttgcttgagatagg</td>
<td>55.0</td>
</tr>
<tr>
<td>Beta tubulin (Thermo Scientific)</td>
<td>agtgctctctctacag</td>
<td>tattctcgtaatgcagtgc</td>
<td>55.0</td>
</tr>
<tr>
<td>Cyclo (Thermo Scientific)</td>
<td>tttgggaaggtgaagaggg</td>
<td>acagaaggaatggttgatggg</td>
<td>55.0</td>
</tr>
<tr>
<td>Rpl19 (Thermo Scientific)</td>
<td>aatgcaaatgccaactc</td>
<td>ggaatgacatgcaggg</td>
<td>55.0</td>
</tr>
<tr>
<td>H3a (Thermo Scientific)</td>
<td>ccttgtgggtctgtttga</td>
<td>cagttggtctgtctgg</td>
<td>55.0</td>
</tr>
</tbody>
</table>

Data was exported from the qPCR machine and used in LinRegPCR to obtain primer efficiency, which was later on used in GraphPad software to detect outliers through the extreme studentized deviate (ESD) method (GraphPad QuickCalculs: outlier calculator). The primer efficiency mean was used in excel, after excluding outliers, to calculate corrected Ct-values. Geomean values for the housekeeping genes were obtained by using the corrected Ct-values in the GeNorm protocol (Vandesompele, De Preter K Fau-Pattyn et al. 2002). The protocol revealed if the housekeeping genes were stable enough (value < 1.5) to use for normalization of the mRNA expression of the targets, making it possible to compare the relative mRNA expression of the targets in the starved and starved then refed samples with their controls but also with each other. The relative mRNA expression was statistically analyzed using Mann Whitney U-test.

4.0 Results

Fluorescent immunocytochemistry was performed on primary cortex cell cultures obtained from mice. The cells were glucose starved for 3 h and 12 h. Controls where included in the ICC to compare intensity changes and changes in location after starvation. Intensities were measured using Image J.Fiji edition and statistically analyzed with Mann Whitney U-test. This experiment was performed for MFSD6, MFSD6L, MFSD8, MFSD9 and MFSD10. Unfortunately ICC results on starved cells for MFSD10 could not be obtained due to the absence of a successfully optimized protocol for this specific protein.
4.1 ICC

4.1.1 MFSD6

Figure 1 illustrates the results obtained for MFSD6 after ICC on 3 h and 12 h starved cells. This protein was mostly expressed in PAN positive cells. However it was also expressed in some cells that were not PAN positive. Two staining patterns were found for MFSD6. One where the whole cell body was stained green and one where only the area close to the membrane and dendrites/axons were stained green. The different patterns also had different fluorescent intensities. The total fluorescent intensity for MFSD6 after 3 h glucose starvation was significantly higher than controls, Figure 2. However the fluorescent intensity in the 12 h starved cells was not significantly different when compared to control, Figure 3.

4.1.2 MFSD6L

ICC results for MFSD6L illustrate that it mostly co-localizes with PAN positive cells, however also co-localizes with some PAN negative cells, Figure 4. MFSD6L only has one staining pattern. It stains the cell body close to the membrane in a dotted way, also seen in Figure 4. Intensity measurements revealed a higher intensity for MFSD6 after a 3 h glucose starvation compared to control, Figure 5. No significant difference was however found for MFSD6 after a 12 h starvation when compared to control, Figure 6.

4.1.3 MFSD8

Figure 7 illustrates that MFSD8 does co-localize with some PAN positive cells and a few PAN negative cells. It has two staining patterns one where it is centered in the cell body and one where it stains the cell body close to the membrane as well as axons/dendrites in a dotted way. The centered pattern is nucleus like and has a higher intensity than the other cells. This nucleus like pattern is found in some PAN positive and negative cell while the other pattern is found only in PAN positive cells. There is no significant difference in fluorescent intensity between the starved cells and controls for 3 h starved, Figure 8, as well as for 12 h starved, Figure 9.
4.1.4 MFSD9

MFSD9 co-localizes with PAN positive cells, Figure 10. This protein has three staining patterns. Some cells were MFSD9 positive within the whole cell body some were stained in a PAN staining like patterns and some cells were stained solely in the center of the cell in a nucleus like pattern. The fluorescent intensity was overall higher for the 3 h starved cells compared to controls, Figure 11. The 12 h starved cells showed no significant difference in fluorescent intensity compared with control, Figure 12.
Figure 1. Fluorescent ICC illustrations of MFSD6 in 3 h controls and 3 h glucose starved cells as well as 12 h control and 12 h glucose starved cells. Images were taken with an Olympus DP73 camera with 100x objective. Separate images for a. MFSD6 (green) and b. neurons (red) were taken and merged together in c with nucleuses stained with DAPI (blue) Most cells are PAN positive however images 1.c and 3.c show MFSD6 expression in cells that are not PAN positive. Images 1.a, 2.a, 3.a and 4.a illustrate two staining patterns. Some cells were stained within the whole cell body while other cells were stained in areas close to the membrane as well as axons/dendrites.

Figure 2. Statistical analysis of MFSD6 fluorescent intensity in cell starved for 3 h. This illustrates a higher intensity for MFSD6 in 3 h glucose starved cells compared with 3 h control. *= p<0.05, **=p<0.01, ***=p<0.001.

Figure 3. Fluorescent intensity analysis of MFSD6 after 12 h starvation. No difference in fluorescent intensity was seen for MFSD6 after 12 h starvation.
1. MFSD6L, 3 hours starved control.

2. MFSD6L, 3 hours starved.

3. MFSD6L, 12 hours starved.

4. MFSD6L, 12 hours starved control.

**Figure 4.** ICC illustrations of MFSD6L in 3 h control (1), 3 h glucose starved (2), 12 h control (3) and 12 glucose starved cells (4). These images were also taken with Olympus DP73 camera with 100x objective. Images named a illustrates protein staining, b PAN positive cells and c illustrates a and b merged together with Dapi stained nucleuses. MFSD6L mostley colocalized with PAN positive cells. Images 1.c and 3.c show that MFSD6 also merge with PAN negative cells. One staining patterns was found. It stains the cell body in areas close to the membrane in a dotted way.

**Figure 5.** Mean fluorescent intensity of MFSD6L control and MFSD6L 3 h starved measured as fluorescence/cell. MFSD6L showed higher intensity in 3 h starved cells compared with controls. *= p<0.05, **=p<0.01, ***=p<0.001.

**Figure 6.** Statistical analysis of fluorescent intensity of MFSD6L in 12 h starved cells. No difference in fluorescent intensity of MFSD6L was found in 12 h starved cells when compared to control.
1. MFSD8, 3 hours starved control.

2. MFSD8, 3 hours starved.

3. MFSD8, 12 hours starved control.

4. MFSD8, 12 hours starved.

**Figure 7.** Protein expression of MFSD8 in 3 h starved, 3 h starved control, 12 h starved and 12 h starved control cells. Olympus DP73 camera with 100x objective was used to take these images. MFSD8 expression in PAN positive cells are presented in b. Images named c show a merged illustration of a and b with DAPI stained nucleuses. Cells in 1 are control cells for 3 h glucose starved cells which are presented in 2. Controls for 12 h starved are shown in 3 and 4 then presents the 12 h glucose starved cells. The illustrations used in the analysis revealed two staining patterns for MFSD8. It stains the cell body in areas close to the membrane as well as axons/dendrites in a dotted way. It also stains the center of the cell body in a nucleus type structure.

**Figure 8.** Fluorescent intensity results of MFSD8 in controls and 3 h starved cells. No difference in fluorescent intensity was found for MFSD8 in 3 h starved cells compared to controls.

**Figure 9.** Fluorescent intensity results of MFSD8 in controls and 12 h starved cells. No difference was found for MFSD8 in 12 h starved cells when compared to controls.
Figure 10. ICC illustrations of MFSD9 in 3 h control (1), 3 h starved (2), 12 h control (3) and 12 h starved (4) cells taken with Olympus DP73 camera with 100x objective. Images were taken on the protein staining 1.a, 2.a, 3.a and 4.a as well as on the PAN positive cells 1.b, 2.b, 3.b, and 4.b. Images in 1 were merged together with DAPI stained nucleuses and are presented in 1.c. Same process was repeated for 2, 3 and 4. MFSD9 had three staining patterns. It stains the cell body in areas close to the membrane as well as axons/dendrites in some cells. In other cells it stains the whole cell body or solely the cell body or the cell body in a nucleuse like pattern.

Figure 11. Fluorescent intensity analysis of MFSD9. The intensity was higher in the 3 h starved cells compared with control cells. *=p<0.05, **=p<0.01, ***=p<0.001.

Figure 12. Fluorescent intensity of MFSD9 in 12 h starved cells. No significance was found for the fluorescent intensity of MFSD9 in
4.2 qPCR

qPCR was used to analyze the gene expression of Mfsd6, Mfsd8, Mfsd9 and Mfsd10. The gene expression for Mfsd6L could not be analyzed due to the absence of working primers. Results from the qPCRs were used to analyze changes on gene level by measuring the relative mRNA expression in 3 h control, 3 h starved, 12 h control and 12 h starved cells as well as in cells that had been refed after a 3 h and 12 h glucose starvation and their controls. The data received from the qPCRs were normalized using housekeeping genes, then plotted and statistically analyzed in GraphPad using Mann Whitney U-test.

4.2.1 qPCR of Mfsd6

The relative mRNA expression for Mfsd6 was at the same level in 3 h starved cells as its control. The same was seen for the 3 h starved and refed, 12 h starved and 12 h starved and refed samples. In other words, no change in relative mRNA expression for Mfsd6 was found in any of the samples, Figure 13. The figure also show significantly higher expression for the refed samples (as well as their controls) compared with the starved samples (and their controls).

4.2.2 qPCR of Mfsd8

Statistical analysis revealed a significantly lower relative mRNA expression for Mfsd8 in 12 h glucose starved cells compared to control samples. The gene expression of Mfsd8 in 3 h starved, 3 h starved then refed and 12 h starved the refed did however not show any changes, Figure 14.

4.2.3 qPCR of Mfsd9

The relative mRNA expression of Mfsd9 was significant for the 3 h starved cells. The 3 h starved cells had a lower gene expression of Mfsd9 compared with the control. The gene expression was however not significantly different compared to controls in the rest of the samples, Figure 15.
4.2.4 qPCR of Mfsd10

Significant difference was also found for Mfsd10 in the 3 h glucose starved sample. The rest of the Mfsd10 samples showed no significance. The gene expression was lower in the 3 h starved sample compared to its control.

![Mfsd6](image)

**Figure 13.** Relative mRNA expression of Mfsd6 in 3 h starved, 3 h starved and refed, 12 h starved and refed samples as well as their controls. The expression was analyzed statistically using Mann Whitney U-test. No significance was seen for Mfsd6 in any of the cells.
Figure 14. Summarized qPCR results for Mfsd8. Cells were either starved for 3 h, starved for 3 h and refed, starved for 12 h or starved for 12 h and refed. The gene expressions were statistically analyzed resulting in a significantly lower relative mRNA expression of Mfsd8 in 12 h starved cells compared with controls. *= p<0.05, **=p<0.01, ***=p<0.001.

Figure 15. Relative mRNA expression of Mfsd9 measured in cells that had been either starved for 3 h, starved for 3 h then refed with glucose, starved for 12 h or starved for 12 h and refed with glucose. Samples were statistically compared to controls. The Relative gene expression of Mfsd9 was only significant in the 3 h glucose starved sample. *= p<0.05, **=p<0.01, ***=p<0.001.
Figure 16. The relative mRNA expression of *Mfsd10* measured in 3 h starved cells, 3 h starved and refed cells, 12 h starved cells as well as in 12 h starved and refed cells. The expressions were statistically analyzed through Mann-Whitney U-test in GraphPad. The relative mRNA expression of *Mfsd10* was significant in the 3 h starved cells. *= p<0.05, **=p<0.01, ***=p<0.001.
5.0 Discussion

In this project changes in gene and protein levels for the putative SLCs MFSD6, MFSD6L, MFSD8, MFSD9 and MFSD10 in response to glucose starvation were studied. It helps determine if these proteins can be possible glucose transporters or not. If future experiments reveal that these proteins or other putative SLC are glucose transporters than these can be used as targets for new active substances. This opens up new possibilities for new treatments in diseases where the glucose homeostasis is affected.

5.1 Protein expression

5.1.1 Localization and pattern

ICCs in this project revealed that all targets co-localized with PAN positive cell concluding that they are in fact found in neurons. Apart from neurons MFSD6, MFSD6L and MFSD8 were all also found in PAN negative cells. To be sure that these PAN negative cells are not neurons, further experiment needs to be done. The staining pattern differed between the proteins and could indicate that the proteins are found in different areas in the cell. For instance MFSD8 and MFSD9 were both localized in the centre of the cell in a nucleus type structure and merged with DAPI stained nuclei. Therefore it could be assumed that they are nucleus bound proteins. The most transport like proteins located in nucleus are proteins that make up pore complexes to enable transportation of other proteins and are called Nuclear Pore Complex (NPC) (Wente and Rout, 2010). According to HPA MFSD9 is mainly localized in nucleus however Perland et al (2017) did not present any results supporting that prediction after performing Immunohistochemistry on MFSD9. This could be due to the fact that HPA used HPA055293 antibody while Perland et al (2017) used SC247973 antibody. In other words the nucleus staining pattern could possibly but not necessarily be an artefact. MFSD6L and MFSD8 both stained the cell in areas close to the membrane as MFSD6 and MFSD9 however in a dotted way (unlike MFSD6 and MFSD9). The reason for the dotted pattern is unclear. Further ICCs need to be done.
Lekholm et al (2017) used Giantin (Golgi marker) and KDEL (endoplasmic reticulum marker) to determine if two other putative SLCs, MFSD14A and MFSD14B, are found in those organelles (Lekholm et al., 2017). The same markers could be used for MFSD6L and MFSD8 to find out if that is the reason for the dotted staining patterns. Other staining markers for other organelles like, lysosomes, mitochondria or vesicles are also an option.

5.1.2 Fluorescent intensity

Lekholm et al (2017) performed ICC on eight putative SLCs. These were MFSD1, MFSD2A, MFSD2B, MFSD11, MFSD14A, MFSD14B and UNC93A. The proteins were deprived of sugar from 4.5g/L to 1.0g/L for 3 h and 12 h and then later on compared to controls. According to the results, all showed an increase in protein expression after 3 h. Only MFSD14A had a significantly lower protein expression after 12 h (Lekholm et al., 2017).

In this project MFSD6, MFSD6L and MFSD9 had a higher fluorescent intensity after 3 h glucose starvation which indicates an upregulation of these proteins, as was seen for some of the putative SLCs (UNC93A, MFSD11 and MFSD14B) studied by Lekholm et al (2017). The upregulation of MFSD6, MFSD6L and MFSD9 was however not seen after 12 h of starvation and not after being refed after 3 h and 12 h starvation. The results could indicate an acute response to the glucose starvation as seen for SLC2A1 (GLUT) and SLC2A4 (GLUT4) during hypoxia (Zhang et al., 1999). SLC2A1 and SLC2A4 have been proven to be translocated to the membrane when acutely stimulated by hypoxia (Zhang et al., 1999). If these are transporters the upregulation could be a stress reaction from the cell so the transporters can transport in glucose. It would also explain why the transporters are not affected after being refed with glucose. However it does not explain why they are not upregulated after 12 h starvation. Could this possibly be due to a cell adaption or due to compensation from nearby astrocytes? The results obtained from the ICC may not be 100% true due to quick florescence fading. However multiple sets of images were used for each protein and a mean value was obtained therefore the fading affects should not have a major impact.
5.2 Relative gene expression

*Mfsd6, Mfsd8, Mfsd9 and Mfsd10* have previously been studied in cells that have been deprived of glucose for 3 h and 12 h receiving only 1g/L glucose unlike the cells used in this project which did not receive any glucose at all for the same periods of time (Lekholm et al., 2017). The results from the experiment presented a downregulation of *Mfsd10* after 3 h of glucose deprivation but no difference for *Mfsd6, Mfsd8* or *Mfsd9*. After 12 h of glucose deprivation they found that *Mfsd9* and *Mfsd10* were upregulated and that *Mfsd6* and *Mfsd8* were not changed when compared to the control (Lekholm et al., 2017). The result for *Mfsd6* presented by Lekholm et al (2017) support the results obtained for *Mfsd6* in this project as well as the results obtained for *Mfsd8* and *Mfsd10* after 3 h starvation. However their results for *Mfsd9* after 3 h and 12 h starvation and *Mfsd8* and *Mfsd10* after 12 h starvation do not support the findings in this project. Instead results presented in this report indicate that *Mfsd8* was downregulated after 12 h of glucose starvation whereas no response as seen after glucose deprivation and that *Mfsd9* and *Mfsd10* are downregulated after 3 h of glucose starvation and not upregulated as they were after 12 h of glucose deprivation. Since *Mfsd6* did not respond to glucose deprivation or glucose starvation it is thought to not be involved in the transport of glucose.

Microarray study of different gene categories during amino acid starvation for 1, 2, 3, 5 and 16 h, performed by Hellsten et al (2017), revealed that many genes products for metabolic processes were downregulated after 1 and 3 h amino acid starvation and then upregulated after 5 and 16 h of starvation. The expression of *Mfsd8* after glucose deprivation and starvation both go against these findings. It could thereby mean that it may be an important protein for processes other than glucose transport, since it is not downregulated until the cell is in the most critical stage of the tested (12 h glucose starvation). *Mfsd9* could possibly be a gene coding for a glucose transporter. *Mfsd10* is an organic anion transporter and could be important for other metabolic processes in the cell. These two genes almost follow the expression pattern for metabolic process genes after amino acid starvation (Hellsten et al., 2017). They were either down regulated or not affected by glucose deprivation and glucose starvation after 3 h and were either upregulated or not affected by glucose deprivation and glucose starvation after 12 h. It is therefore more likely that these two are glucose transporters.
than Mfsd6 or Mfsd8. Another reason for them to not be affected could be that they have different affinities to glucose. For instance the SLC2A1 (GLUT1) and SLC2A3 (GLUT3) are two glucose transporters with different affinities to glucose that have been proven to have different gene expressions under certain conditions. For instance Slc2a3 could be upregulated while Slc2a1 did not change at all (Desideri et al., 2014).

The relative mRNA expression of Mfsd6 and its control when refed were high compared to the starved samples and starved sample controls. This could be because the refed cells were treated better and given glucose under a longer period of time.

5.3 Protein expression compared to gene expression

The results from the ICC do not support the results obtained from the qPCR runs. Three proteins were tested on both levels (MFSD6, MFSD8 and MFSD9). Only MFSD9 showed alterations on both protein and gene level. It was upregulated on a protein level after 3 h starvation and down regulated on a gene level after 3 h starvation and could be due to negative feedback on the gene expression from an upregulation of the protein. To be sure that a change in protein expression is or is not directly linked to alterations on a gene level, further tests needs to be done. Tests where cells, are subjected to glucose starvation about every hour for 12h instead of just at two time points. This because different proteins have different turnovers, balance between protein synthesis and protein degradation (Poortmans et al., 2012). This is preferable but may not be practical.

5.4 Mouse models for human proteins

The function of the proteins may be the same in mouse as in humans even though the expression in mouse did not turn out to be the same as the HPA predictions for all the targets. The expression of some of the MFSDs were alike the HPA predictions and some were not. According to HPA predictions MFSD6 is both intracellular and membrane bound, MFSD6L is only predicted to bind to the membrane, MFSD8 is mainly localized in nucleoplasm and in
vesicles and MFSD9 is mainly localized in nucleolus. These predictions are made for human transporters but are similar to the patterns found in this project. The experiments performed in this project were executed in mouse cells. When comparing the protein expression of MFSD6 and MFSD8 in mice with the protein localization prediction by HPA, it looks like they are relatively similar. However MFSD6L is predicted to bind to the membrane but showed a dotted pattern which could indicate that it binds to intracellular organelles. MFSD9 is predicted to mainly be localized in nucleoli which were not the results in this project. Instead MFSD9 had three staining pattern and was mainly stained within the cell in areas close to the membrane. This could however be due to the fact that the antibody used by HPA was not the same as the antibody used in this project.

5.5 Future experiments

More ICCs and qPCRs can be done at more time points, to find out if changes in protein expression are directly linked to changes on the gene expression and why the protein staining pattern differs in different cells. Can the difference detected be because the cells are different types of neurons? Are these proteins expressed in cells other than neurons or are the PAN negative cells with positive protein expression just other types of neurons that do not bind to PAN as well as the rest? Also ICCs need to be performed for MFSD10 and qPCR need to be performed for Mfsd6L. ICC need to be optimized for MFSD10 perhaps by performing the experiment with new primary antibodies. For Mfsd6L primers need to be optimized by designing new primers and finding their optimal temperature for annealing. A microarray could also be done to follow changes in gene expression of different gene categories during glucose deprivation and starvation.

5.6 Conclusion

In conclusion, all MFSD proteins studied in this project responded to glucose starvation at some point. Three of the MFSDs tested through ICC (MFSD6, MFSD6L and MFSD9) were all upregulated after a 3 h starvation. None of the proteins responded to glucose starvation after 12 h glucose starvation on a protein level and only MFSD8 did not respond to glucose
starvation on a protein level at all. Mfsd6 was the only gene that did not respond to glucose starvation on a gene level out of the tested. Mfsd8 had a delayed response to glucose starvation that was compensated when refed with glucose. Mfsd9 and Mfsd10 both had an acute response to the glucose starvation that was back at normal levels after a 12 h starvation as well as compensated when refed with glucose after a 3 h starvation. The expression of Mfsd6, Mfsd8, Mfsd9 and Mfsd10 in refed cells were also tested in this project. Not a single gene showed a difference in expression when compared to controls. This indicates that the genes that rebounded may be involved in glucose transport, metabolism or processes affected by glucose since they were compensated by being refed.

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7.0 Sammanfattning
Glukos är hjärnans främsta energikälla och behövs bland annat som energi för att viktiga processer ska kunna genomföras i kroppen. Substansen är dock för stor för att på egen hand kunna passera blod-hjärnbarriären. Glukos behöver därför transporteras över barriären. Detta görs med hjälp av glukostransportörer så kallade solute carriers (SLC). Det finns över 400 SLC medlemmar och även några proteiner som troligen tillhör SLC familjen men som inte riktigt bevisats ännu. I detta projekt har fokus varit på fem stycken sådana proteiner. Dessa har samlingsnamnet Major facilitor superfamily domain containing proteins (MFSD). I detta projekt har följande proteiner studerats: MFSD6, MFSD6L, MFSD8, MFSD9 och MFSD10. Syftet var att ta reda på om och hur dessa proteiner reagerar på glukossvält på proteinnivå och gennivå. De två metoderna som användes för detta var Immunocytokemi för att studera proteinuttrycket och Kvantitativ polymeraskedjereaktion för att mäta och jämföra genuuttrycket av de olika MFSDarna. För Immunocytokemien användes celler som svälts på glukos i 3 h respektive 12 h och kontroller för dessa som fått glukos. För den kvantitativa polymeraskedjereaktionen användes även här celler som svälts i 3 h respektive 12 h, samt kontroller, men även celler som svält i 3 h respektive 12 h men sedan fått glukos igen, samt kontroller för dessa. Statistiska analyser utfördes på dessa experiment och resulterade i att MFSD6, MFSD6L och MFSD9 hade en högre intensitet i jämförelse med kontroller efter 3 h glukossvält. På gennivå var Mfsd8, Mfsd9 och Mfsd10 signifikanta. Mfsd8 hade ett lägre relativ mRNA uttryck i cellerna som hade svält i 12 h medan Mfsd9 och Mfsd10 hade ett lägre mRNA uttryck i cellerna som hade svält i 3 h. Slutsatsen av detta blir då att alla någon gång påverkades av glukossvält. MFSD9 var den enda som påverkades på protein- samt gennivå av de som testades på båda. MFSD6, MFSD6L och MFSD9 påverkades på proteinnivå, de var alla uppreglade efter 3 h glukossvält i jämförelse med kontrollerna. På gennivå var Mfsd8, Mfsd9 och Mfsd10 påverkade. Mfsd8 vad nedreglerad efter 12 h medan Mfsd9 och Mfsd10 var nedreglerade efter 3 h. Detta innebär alltså att en förändring i protein nivå inte nödvändigtvis behöver vara kopplat till förändringar i gennivå. För att säker ställa detta krävs dock fler försök vid fler tidpunkter än två.
8.0 References


