Characterization of Amino Acid Transporters in the Brain

Molecular and Functional Studies of Members within the Solute Carrier Families SLC38 and SLC6

MARIA HÄGGLUND
Abstract


Solute carriers (SLCs) comprise the largest group of transporters in humans and there are currently 52 SLC families. They are embedded in cellular membranes and transport numerous molecules; defects in many of the genes encoding SLCs have been connected to pathological conditions, and several SLCs are potential drug targets.

The SLC38 family has in total eleven members in humans and they encode transporters called SNATs. In paper I and paper II, we reported molecular and functional characterization of Slc38a7 and Slc38a8, two of the previous orphan members in the family which we suggested to be named SNAT7 and SNAT8, respectively. Using in situ hybridization and immunohistochemistry, these transporters showed similar expression pattern and localized to neurons in the brain. For functional characterization proteins were overexpressed in X. laevis oocytes and an Uptake Assay and electrophysiological recordings showed preferred transport of L-glutamine, L-histidine, L-alanine, L-asparagine, L-aspartate and L-arginine for SNAT7. A similar pattern was seen for SNAT8 in a slightly different order of affinities. We classified SNAT7 as a system N transporter and SNAT8 as belonging to system A, and suggests that SNAT7 and SNAT8 could play a role in the glutamine/glutamate(GABA) cycle (GGC) in the brain.

Furthermore, we studied the vesicular B^0AT3 (Slc6a17) transporter in paper III, and the sodium-coupled amino acid transporter B^0AT2 (Slc6a15) in paper IV. Tissue expression studies showed similar localization of Slc6a17 and Slc6a15 mRNA using in situ hybridization and real-time PCR. In paper III, vesicular localization of B^0AT2 was shown in both excitatory and inhibitory neurons. When challenging the monoaminergic system with drugs both Slc6a17 and Slc6a15 were upregulated. Suggested roles for the transporters are thereby in synaptic remodeling by regulating the availability of free amino acids used as precursors needed in neurotransmitter synthesis. Moreover, in paper IV, immunohistochemistry showed B^0AT3 localization to neurons, astrocytes and epithelial cells of the choroid plexus. Leucine injections caused a smaller reduction of food intake as well as higher neuronal activation in the paraventricular hypothalamic nucleus in Slc6a15 KO mice, compared with wild type mice. This suggests B^0AT2 involvement in the anorexigenic effects of leucine.

Keywords: Amino acid transporter, Solute Carrier, Glutamine, Leucine, SNAT, B0AT

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ISSN 1651-6206
ISBN 978-91-554-8832-1
urn:nbn:se:uu:diva-212610 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-212610)
If the human brain was simple enough for us to understand, we would still be so stupid that we couldn’t understand it.

Jostein Gaarder
Cover: Immunohistochemistry labeling of primary cell culture with membrane marker WGA (red), nucleus marker DAPI (blue) and the vesicular transporter B₀AT3 (green).
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Arc</td>
<td>arcuate hypothalamic nucleus</td>
</tr>
<tr>
<td>APC</td>
<td>amino acid/polyamine/organocation</td>
</tr>
<tr>
<td>B&lt;sup&gt;0&lt;/sup&gt;AT</td>
<td>system B&lt;sup&gt;0&lt;/sup&gt; amino acid transporter</td>
</tr>
<tr>
<td>CPA/AT</td>
<td>cation:proton antiporter/anion transporter</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DBI</td>
<td>diazepam binding inhibitor</td>
</tr>
<tr>
<td>Gad67</td>
<td>glutamic acid decarboxylase, 67 kDa</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>Gln</td>
<td>glutamine (Q)</td>
</tr>
<tr>
<td>GGC</td>
<td>glutamine/glutamate (GABA) cycle</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamate (E)</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneally injection</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridization</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>KO</td>
<td>knockout mice</td>
</tr>
<tr>
<td>Leu</td>
<td>leucine (L)</td>
</tr>
<tr>
<td>MeAIB</td>
<td>α-(methyl)amino isobutyric acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclei (antibody marker)</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>SLCs</td>
<td>solute carriers (human gene name)</td>
</tr>
<tr>
<td>SLC38A7</td>
<td>solute carrier family 38, member 7</td>
</tr>
<tr>
<td>SLC38A8</td>
<td>solute carrier family 38, member 8</td>
</tr>
<tr>
<td>SLC6A15</td>
<td>solute carrier family 6, member 15</td>
</tr>
<tr>
<td>SLC6A17</td>
<td>solute carrier family 6, member 17</td>
</tr>
<tr>
<td>SNAT</td>
<td>sodium coupled neutral amino acid transporter</td>
</tr>
<tr>
<td>SNRI</td>
<td>serotonin–norepinephrine reuptake inhibitor</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin re-uptake inhibitor</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>WT</td>
<td>wild type mice</td>
</tr>
</tbody>
</table>
Introduction

Transporters in the central nervous system (CNS)

Transport of substrates such as amino acids, neurotransmitters, nucleotides, sugars, purines, ions and drug molecules, occur by ATP-dependent (primary active transporters) and ATP-independent (passive and secondary active transporters) mechanisms in the CNS (Figure 1).

The ABC transporter and other ATP driven pumps are either open or closed and use energy released by ATP hydrolysis to move substrates across membranes against their electrochemical gradient. By contrast, ion/water channels exist in the closed state in most cases and substrates are transferred down their electrochemical gradient (Figure 1A).

The cellular localization of members within the solute carrier (SLC) superfamily are in mitochondrial (1), vesicular (2), peroxisomal (3), lysosomal (4) and cellular membranes (not found in nuclear membrane) and they include exchangers, coupled transporters and passive transporters (5). Exchangers, also termed antiporters, move two or more molecules (ions or substrates) in opposite direction across the membrane. Coupled transporters (symporters or co-transporters) move two molecules in the same direction across the membrane, while passive transporters, also known as uniporters or facilitative transporters, allow diffusion of substrates across membranes down their electrochemical gradient (Figure 1B). What is common for the SLC superfamily are that the proteins have multiple transmembrane (TM) domains embedded within the membrane. SLCs transport a specific substrate considerably slower than channel proteins and it is generally believed that a conformational change of the protein is important in the transfer process (5).

Understanding how membrane bound amino acid transporters are part of the flow of substrates in the CNS is a challenging task for modern neuroscience. Functional characterization of transporters and understanding the mechanisms they are part of is relevant to many fields, such as medicine, genetics, developmental biology, pharmacology and cancer chemotherapy (5). Amino acid transporters in the brain are important as transporters of neurotransmitters and neurotransmitter precursors, but also for the role they play as nutrient sensors and as transporters for metabolic substrates into all cells (5,6). Amino acid transporters are especially important in neurons for neurotransmitter signaling, for several aspects of food intake regulation and general metabolism. Neurons have a lower capacity for de novo synthesis of
amino acids than other cells and are therefore more dependent on amino acids from external sources. Among the known amino acid transporters some have a promiscuous substrate profiles while others are very specific. Also, the specific repertoire of transporters expressed by a certain cell type varies. One approach to get a deeper knowledge about novel transporters to further identify their functional role is to localize the type of cells and tissue expressing the transporter and to identify their preferred substrate profile.

**Figure 1.** Localization and mechanism of transporters in a cell. **A.** Membrane bound ATP-dependent and ATP-independent transporters with proteins belonging to SLC transporters (red) or non-SLC transporters (grey) localized in the plasma membrane or in intracellular compartments. **B.** The transport of solutes by SLCs can either be coupled, exchanged or passive. This figure has been adapted from Heidiger *et al.* 2013 (6).

**Solute carriers (SLCs)**

**Evolution of SLCs**

The SLC superfamily is the largest group of transporters in the human genome (5) and they have been categorized into at least 52 different families with varied biochemical properties (6). In order to be classified into a partic-
ular family, the encoded protein has to have at least 20-25% amino acid sequence identity to the other members (5). The transporters within the superfamily are ancient with members present in most prokaryotes, as well as all eukaryotes (7). One of the first overall phylogenetic classification of all SLC members revealed that 15 of the SLC families form four major phylogenetic groups, termed α-, β-, γ- and δ-groups, with proteins in each group having a common evolutionary origin in mammals (8). Subsequently, it was shown through sequence profile analysis that 24 of the SLC families belong to three PFAM clans (9), termed major facilitator superfamily (MFS), amino acid/polyamine/organocation (APC) and monovalent cation:proton antiporter/anion transporter (CPA/AT) (7). This suggests that there exist a higher order of relationship between the SLC families.

The phylogenetic information provides foundation for overall classification of SLC genes and is valuable for annotation and prediction of substrates for the many SLCs within the superfamily that have not yet been characterized and tested in experimental transport assays. Most of the SLC families are not studied in detail and it is possible that the number of SLC transporters and families will continue to expand through the discovery of novel genes.

SLCs are with its 395 members the largest group of ATP-independent transporters in the human genome (6). This large number indicates the importance of having regulated membrane transport over the cell membrane as well as intracellular membranes. However, the complex genomic structure with a high number of introns, the heterogeneity among their functions and the difficulty to clone and express these very large proteins, has delayed their functional characterization. There are currently close to 120 orphan SLCs, of these 51 are most closely related to known amino acid transporters and are hence possible novel amino acid transporters (8).

**SLC38 family**

**Naming, expression pattern, classification and function**

The solute carrier family 38 (SLC38) is the system A and system N sodium-coupled neutral amino acid transporter family. The family belongs, together with the SLC32 and SLC36 families, to the β-group (8) and is classified into the APC superfamily of PFAM clans (7).

Within the superfamily is SLC32A1, vesicular inhibitory amino acid transporter (VIAAT, also termed VGAT), the only member of the SLC32 family. The VIAAT transporter mediates H+ driven uptake of GABA and glycine into synaptic vesicles in CNS (10). The members of the SLC36 family, also known as PATs, consist of the four members SLC36A1-A4 (PAT1-
4). The functionally characterized PATs are all proton-coupled amino acid transporters with preferences for L-proline, with additional preference for glycine and alanine (PAT1 and PAT2), and L-tryptophan (PAT4). PAT3 is still orphan with unknown substrate profile. The PATs are expressed ubiquitously, with the exceptions of PAT2 which not is found in brain and PAT3 which is exclusively expressed in testis (11). PAT1 (12) and PAT2 (13) are found on the cellular membrane of neurons, and PAT1 is also found on the lysosomal membranes (14).

The largest family within the superfamily is the SLC38 family, which is known as system A and system N sodium-coupled neutral amino acid transporters and consists of 11 members (SLC38A1-A11) (Table 1). SLC38A1-A5 are the most studied (11), while SLC38A6-A11 transporters were more recently discovered (15). The SLC38 family is old in evolutionary terms with at least two SLC38 members, SLC38A10 and SLC38A7/8 (SLC38A7 and SLC38A8 originate from the ancestral SLC38A7/8) present before the split of plants and animals, while SLC38A1-A5 are the most recently developed members (11).

Today, all except SLC38A6 and SLC38A9-A11 have been characterized regarding their expression, cellular localization, preferred substrates and transport properties. The encoded proteins of the characterized SLC38 members are known as sodium coupled neutral amino acid transporters (SNATs). The transporters within the family have wide expression patterns with all members expressed in the brain, and they are also found in many peripheral tissues (11,16). The SLC38A1 (SNAT1) (17-20) and SLC38A2 (SNAT2) (21,22) are localized both in astrocytes and neurons, SLC38A3 (SNAT3) (23-25) and SLC38A5 (SNAT5) (26) only in glial cells, SLC38A7 (SNAT7) (paper I) (27,28) and SLC38A8 (SNAT8) (paper II) (29,30) are found in all types of neurons, while the type of cells expressing SNAT4 in the brain (31) has not yet been characterized.

In general, all characterized SNATs are transporters of L-glutamine, L-asparagine, L-histidine and L-alanine. They have also been classified into the N/A systems, with SNAT1, SNAT2, SNAT4 and SNAT8 belonging to system A and SNAT3, SNAT5 and SNAT7 associated with system N (11,29). The SNATs are co-transporters for sodium ions with the substrate uptake driven by the inward directed ion gradient across the plasma membrane. The System N transporters SNAT3 (25,32) and SNAT5 (33) also use hydrogen ion exchange to drive the transport, while the ability of hydrogen ions to drive transport through SNAT7 and SNAT8 has not been studied. The classification according to the N/A systems depends among other things on patterns of expression and substrate recognition (Table 2), and the more recently discovered members of the family have not yet been classified according these systems.
Table 1. SLC38 family of system A and system N sodium-coupled neutral amino acid transporters as well as not yet classified SLCs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Alias</th>
<th>Substrates</th>
<th>Tissues</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>System A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC38A1</td>
<td>SNAT1</td>
<td>ATA1, GlnT, NAT2, SA2, SAT1</td>
<td>Gln, Asn, His, Ala, Met, Ser, Cys, MeAIB, Gly, Pro, Thr</td>
<td>Brain, retina, heart, placenta, adrenal gland</td>
<td>(17,19,34,35)</td>
</tr>
<tr>
<td>SLC38A2</td>
<td>SNAT2</td>
<td>AT2, KIAA1382, SA1, SAT2</td>
<td>Ala, Ser, Pro, Cys, Met, Asn, Gln, Gly, His, Phe, Thr, MeAIB</td>
<td>Ubiquitous</td>
<td>(22,36,37)</td>
</tr>
<tr>
<td>SLC38A4</td>
<td>SNAT4</td>
<td>FLJ10191, NAT3, PAAT, SAT3</td>
<td>His, Arg, Lys, Asn, Ala, Ser, Gly, Gln, Cys, Thr, Pro, Met, (Asp), MeAIB</td>
<td>Brain, retina, liver, kidney, adipose tissue, placenta</td>
<td>(31,38-40)</td>
</tr>
<tr>
<td>SLC38A8</td>
<td>SNAT8</td>
<td>Gln, Arg, Ala, His, Asp, Leu, Pro, Glu, Asn, MeAIB</td>
<td>Brain, pituitary, eye, thymus, stomach, adrenal gland</td>
<td>(15,29,30)</td>
<td></td>
</tr>
<tr>
<td><strong>System N</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC38A3</td>
<td>SNAT3</td>
<td>g17, NAT, SN1</td>
<td>Gln, His, Ala, Asn</td>
<td>Liver, skeletal muscle, kidney, pancreas</td>
<td>(25,34)</td>
</tr>
<tr>
<td>SLC38A5</td>
<td>SNAT5</td>
<td>JM24, SN2</td>
<td>Gln, Asn, His, Ser, Gly, Ala, Met</td>
<td>Stomach, brain, liver, lung, small intestine, spleen, colon, kidney, blood</td>
<td>(26,33)</td>
</tr>
<tr>
<td>SLC38A7</td>
<td>SNAT7</td>
<td>Gln, His, Ser, Ala, Asn, Asp, Glu, Met, Leu, Gly, Arg, Lys</td>
<td>Brain, liver, skeletal muscle, uterus, pituitary</td>
<td>(15,27,28)</td>
<td></td>
</tr>
<tr>
<td><strong>System ?</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC38A6</td>
<td>n/a</td>
<td>NAT-1</td>
<td>Brain, eye, heart, liver, kidney</td>
<td>(15)</td>
<td></td>
</tr>
<tr>
<td>SLC38A9</td>
<td>n/a</td>
<td></td>
<td>Ubiquitous</td>
<td>(15)</td>
<td></td>
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<tr>
<td>SLC38A10</td>
<td>n/a</td>
<td></td>
<td>Brain, pituitary, lung, kidney, liver</td>
<td>(15)</td>
<td></td>
</tr>
<tr>
<td>SLC38A11</td>
<td>n/a</td>
<td></td>
<td>Testis, stomach, skeletal muscle, pituitary, adrenal gland, brain</td>
<td>(15)</td>
<td></td>
</tr>
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</table>
Table 2. System A vs. System N

<table>
<thead>
<tr>
<th>System A</th>
<th>System N</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeAIB selectivity</td>
<td>No MeAIB selectivity</td>
</tr>
<tr>
<td>(hallmark)</td>
<td></td>
</tr>
<tr>
<td>Broad substrate profile</td>
<td>Narrow substrate profile</td>
</tr>
<tr>
<td>(L-Gly, L-Ala, L-Cys, L-Gln and the hallmark L-Thr)</td>
<td>(L-Gln, L-His and L-Asn)</td>
</tr>
<tr>
<td>Wide expression pattern</td>
<td>Tissue specific expression</td>
</tr>
<tr>
<td>(often in liver)</td>
<td></td>
</tr>
<tr>
<td>Na⁺-dependent</td>
<td>Na⁺-dependent and H⁺ exchanger</td>
</tr>
<tr>
<td>Not/partly tolerate Li⁺ substitution to Na⁺</td>
<td>Tolerate Li⁺ substitution to Na⁺</td>
</tr>
<tr>
<td>pH-dependent</td>
<td>pH-dependent</td>
</tr>
</tbody>
</table>

Classical system A transporters show transport of the amino acid analog (methylamino)isobutyric acid (MeAIB) across cellular membranes (41), as well as a broad range of amino acids such as L-glycine, L-alanine, L-cysteine, and L-glutamine, L-proline, whereas system N has a more narrow profile with L-glutamine, L-histidine, and L-asparagine as primary substrates. System A members are known to be ubiquitously expressed, while the system N transporters have more restricted expression, often high in liver. The system N transporters co-transport amino acids and sodium ions and exchange hydrogen ions and can also use lithium ions as a substitute to sodium ions, whereas sodium dependent system A members are suggested to not (or only partly) tolerate this substitution. Investigation of pH-dependency is commonly used to separate the classical system N/A from the classical ASC system (42–44).

The classification into the systems is not always straightforward and some of the characterized transporters have less clear cut distinction between the systems. Many of the known SNATs belonging to system A have also shown to partly tolerate lithium substitution (45,46), making sensitivity to lithium substitution a diagnostic criteria for systems that should be taken with caution. The SLC38A7 is classified into system N but shares some of the physiological features of both the A and N systems (paper I) (27,28). It is also unclear whether the classification into the systems could possibly be different dependent on what species the study is performed in i.e. the human SLC38A4 have also been reported to belong to the system N transporters (46).

In this thesis, we determined the brain expression and functionally characterized the previous orphan SLC38A7 (paper I) (27,28) and SLC38A8 (paper II) (29,30) transporters and further classified them into the A/N systems.
Physiological function

Glutamate is the most important excitatory transmitter in normal brain function and elevated concentrations of extracellular glutamate are toxic to neurons. Glutamate does not cross the blood-brain barrier and therefore must be synthesized de novo in neurons. The neurotoxic properties of glutamate are the reason why controlled circulation and reuptake of glutamate are important to prevent and avoid excitotoxicity. Tight control of glutamate is also an essential process in the complex balance of neuronal amino acid metabolism. Glutamine, on the other hand, is the most abundant non-toxic circulating amino acid in the body and functions as a precursor for nucleic acids and nucleotides and is used in protein synthesis and also as a precursor for the neurotransmitter glutamate by being an intermediate in the glutamine/glutamate(GABA) cycle (GGC) (47).

The amino acid L-glutamine is the favored substrate for most of the members in the SLC38 family. The SNAT3 (23,25) and SNAT5 (26) transporters are found on astrocytes, while SNAT1 (19,35), SNAT2 (22,37), SNAT7 (paper I) (27,28) and SNAT8 (paper II) (29,30) are neuronal. Neurons and astrocytes are cells known to be involved in the GGC, and SNATs with their high expression in the brain were therefore assigned a role in the cycle (11,48) (Figure 2). The exact involvement in GGC of the recently characterized SNAT7 and SNAT8, with high expression in the neuronal axons and in the soma, and the other orphan SNATs is not yet elucidated. Also, the exact contribution of the SNAT proteins in the GGC is not clear. For example, the lack of expression of SNAT1 and SNAT2 in the nerve terminals, where most of the glutamine transport of the GGC into neurons is thought to occur, indicates that SNAT1 and SNAT2 are actually not involved in the cycle (49). Rae et al. (2003) suggested the neuronal system A members SNAT1 and SNAT2 to not play a prominent role in neurotransmitter cycling, instead being responsible for glutamine transport mainly into the soma and thereby supplying the cells with glutamine used for general biosynthesis (50).

The GGC also has an important role in liver, where it facilitates ammonia detoxification from portal blood. In the liver, SNAT3 is known to play an important role in ammonia detoxification (25), while SNAT2 is upregulated by glucagon to mediate uptake of amino acids as precursors for gluconeogenesis (51). Both SNAT4 (52) and SNAT5 (53) allow net import of amino acids required for protein biosynthesis and metabolic purposes in liver. In addition, it was recently shown that SLC38A8 gene variants are associated with increasing adiposity and insulin resistance related nonalcoholic fatty liver disease (NAFLD) (54). Also, another study showed that an allelic variant of the SLC38A8 gene is connected to foveal hypoplasia, a condition known to be involved in a spectrum of ocular disorders (55).
Figure 2. Overview of glutamine/glutamate(GABA) cycle (GGC) in rodent CNS. The cycle starts with release of the neurotransmitter glutamate by the presynaptic glutamatergic neuron into the synaptic cleft. Glutamate can act on post-synaptic neurons by activating the ionotropic glutamate receptors NMDA, AMPA or the metabotropic mGluRs, while the excitatory action of glutamate is terminated by rapid removal of the transmitter from the cleft. Two systems remove glutamate, one is through reuptake mediated by EAAC1 (Slc1a1) (known as EAAT3 in human) back into the presynaptic neuron where it is repacked into vesicles through vesicular glutamate transporters VGLUT1, 2 or 3 (Slc17a7, a6 or a8). The other is the mechanism generally believed to be the most important and it occurs through membrane bound GLAST (Slc1a3) or GLT-1 (Slc1a2) (known as EAAT1 and EAAT2, respectively in human) into surrounding glia cells lining the synapse. After uptake into the glia cell and amidation of glutamate by glutamine synthetase (GS), glutamine is transported out of the glia cell into the extracellular space via System N transporters, which is mediated via SNAT3 or SNAT5 (Slc38a3 or a5), but most likely other amino acid transporters are also involved. Glutamine is taken up from the extracellular space into the presynaptic neuron by SNATs, originally thought to be mediated by SNAT1 (Slc38a1) or SNAT2 (Slc38a2) as well as one or several unknown transporters that could be SNAT7 or SNAT8 (Slc38a7 or a8). In the presynaptic neuron glutamine is converted into glutamate by phosphate-activated glutaminase (GLS) (also known as PAG) and packed into vesicles by VGLUTs. Neuronally located SNATs and PATs also transport a number of various amino acids into neurons used for general protein synthesis. Glutamate taken into postsynaptic neurons is used in the synthesis of GABA, catalyzed by the GAD67 and GAD65 enzymes, and GABA together with glycine are transported into synaptic vesicles by VIAAT (Slc32a1). Not all mechanisms are represented, for reviews see (47,56,57). Figure adapted from Schiöth et al. 2013 (11).

There are also other proposed functions of the members of the SLC38 family. Amino acid transport systems are in general upregulated in different types of cancer and transporters of glutamine play a certain role in this context as glutamine is known to be necessary for fast growing cells (58). SNAT1 (59), SNAT2 (60) and SNAT3 (61) have all been shown to be activated during different cancer states. It is also known that SNAT2 can acts as a transceptor (62) by modulate intracellular amino acid pools (63) and by sensing and signaling amino acid availability, and have thus been suggested to indirectly activate the mammalian target of rapamycin (mTOR) pathway (64,65) which is important for cell growth and proliferation. In addition, the transport activity of the system A member SNAT2 increases after amino acid deprivation (66), results which together with the involvement in different cancer states suggests that expression levels of the SNATs are highly dynamic and easily regulated.

There are currently no drugs on the market or drugs in development that have members from any of the families within the β-group as their primary targets (67). Characterization regarding substrate profiles and other biochemical properties, tissue distribution as well as the cell type specificity and subcellular localization, are important for understanding the physiological role and possible pharmaceutical potential of novel system A and system N
transporters. Transgenic studies and pharmacological substances could also be crucial in understanding the physiological roles of the transporters. Difficulties arise with knocking out a specific transporter, since other transporters with overlapping substrate profiles may compensate for the effects, resulting in only small phenotypic changes. Multiple knockouts or conditional transgenes may be necessary for studying the SNATs.

SLC6 family

Naming, expression pattern, classification and function

The SLC6 family of transporters includes 20 members and two pseudogenes in the human genome and comprises mostly of sodium (and some hydrogen or chloride) dependent transporters for uptake of solutes against its concentration gradient (68). Phylogenetic analysis of the members in the SLC6 family has further classified these transporters into the four subgroups; (I) GABA, osmolyte, neurotransmitter and creatine transporters (GAT1-3, CT1-2, TAUT and BGT1), (II) monoamine, amino acid, and neurotransmitter transporters (DAT, NET and SERT), (III) amino acid and neurotransmitter transporters I (GLYT1-2, PROT and ATB0), and (IV) the previously orphans now renamed amino acid and nutrient transporters II (B0AT2, NTT5, B0AT3, XT2, B0AT1 and IMINO) (68-71).

The SLC6A15 and SLC6A17 belongs to the nutrient amino acid transporter II subgroup (Table 3), which is known to contain transporters with preference for neutral amino acids and branched chained (BCAA) amino acids, with exception of the proline and betaine transporting SLC6A20 (IMINO) (68) and the orphan SLC6A16 (NTT5). The SLC6A15 gene codes for the B0AT2 protein and the protein product of the SLC6A17 gene is, after name suggestion of Zaia et al. 2009 (72), termed B0AT3. Unfortunately, an additional group published another study in the same number of the journal and they suggested the same protein name for the gene product of SLC6A18 (73), a name that now has been more accepted. In this thesis, the protein name for SLC6A17 is B0AT3 (as in paper III) (74,75), but it is termed NTT4 or XT1 in other places. In addition, here the protein name for SLC6A18 is termed XT2.
Table 3. SLC6 members within the amino acid and nutrient transporters II sub-group. BCAA; branched chain amino acids (Leu, Ile, Val) and neutral amino acids (Ser, Thr, Cys, Asn, Gln, Met, Trp). See explanation for B₀AT3* in the main text.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Alias</th>
<th>Substrates</th>
<th>Tissues</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC6A15</td>
<td>B₀AT2,</td>
<td>SBAT1,</td>
<td>BCAA, neutral amino acids, Pro, Ala</td>
<td>brain, eye, muscle, placenta</td>
<td>(71,76-79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V7-3, NTT7-3, FLJ10316</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC6A16</td>
<td>NTT5</td>
<td></td>
<td>Unknown</td>
<td>testis, liver, lung, pancreas</td>
<td>(77)</td>
</tr>
<tr>
<td>SLC6A17</td>
<td>B₀AT3</td>
<td>NTT4, XT1</td>
<td>Neutral amino acids, Leu, Pro, Ala, Gly</td>
<td>brain, eye, skeletal muscle, intestine, epididymis</td>
<td>(72,74,75,80-82)</td>
</tr>
<tr>
<td>SLC6A18</td>
<td>XT2</td>
<td>B₀AT3*</td>
<td>BCAA, neutral amino acids, Ala, Gly</td>
<td>kidney, adrenal gland, uterus, brain</td>
<td>(70,73,83)</td>
</tr>
<tr>
<td>SLC6A19</td>
<td>B₀AT1</td>
<td>HND</td>
<td>BCAA, neutral amino acids, Pro, Ala, His, Phe, Gly</td>
<td>kidney, intestine, lung, uterus, brain, heart</td>
<td>(70,84-87)</td>
</tr>
<tr>
<td>SLC6A20</td>
<td>IMINO</td>
<td>SIT1</td>
<td>Pro,OH-Pro, betaine</td>
<td>intestine, eye, lung, brain, kidney</td>
<td>(70,71,83,88)</td>
</tr>
</tbody>
</table>

The B₀AT2 (Slc6a15) transporter is almost exclusively expressed in the CNS, with high expression in caudate putamen, cerebral cortex, amygdala, hippocampus and in hypothalamus (paper IV) (70,71,76-79,89,90). Comparably expression studies on the related SLC6 transporters revealed that Slc6a17 mRNA, as well as the B₀AT3 protein, is widely distributed throughout the CNS (paper III) (74,75,81,91), while the orphan Slc6a16 member is found mostly in peripheral tissues (77). The Slc6a17 is found in the same areas as Slc6a15 with addition of thalamus expression, and another difference is that Slc6a17 is generally more abundant than Slc6a15 (90,92). The highly overlapping expression patterns for the two transporters could indicate of similar functions (90). However, cellular localization shows cell surface location of the B₀AT2 protein in neurons, a limited number of astrocytes close to the third ventricle (3V) and in epithelial cells of the choroid plexus (paper IV) (78,79), while B₀AT3 (Slc6a17) is known to be expressed on vesicles in axon terminals of glutamatergic and GABAergic neurons (paper III) (72,74,75,82,92-95).

The secondary active transporters in the SLC6 family are dependent on electrochemical sodium, chloride or hydrogen ion gradients to drive the transport. Sodium ion gradients are maintained over the plasma membrane of all cells, while hydrogen ion gradients are maintained over internal membranes such as synaptic vesicles. In general, B₀AT2 prefer electrogenic transport of large neutral essential amino acids with hydrophobic side chains,
with sodium ions as co-transport (71,76), while \( \text{B}^0\text{AT3} \) acts as a transporter of a broad range of neutral amino acids, with additional affinity for proline, leucine, glycine and alanine (72,82). Conflicting data show sodium ion gradient (72) as well as hydrogen ion gradient (82) dependent transport by \( \text{B}^0\text{AT3} \). The vesicular localization support the hydrogen ion driven transport, while recently histological staining with additional expression of \( \text{B}^0\text{AT3} \) in the soma of neurons, most likely in the plasma membrane, support the sodium ion dependency (paper III) (74,75). The \( \text{B}^0\text{AT2} \) (\( \text{Slc6a15} \)) transporter show most similarities with \( \text{B}^0\text{AT3} \) (\( \text{Slc6a17} \)) regarding sequence similarities, tissue expression, as well as substrate profiles compare to the other members within the family.

Studies with the \( \text{Slc6a15} \) gene knockout (KO) mice, originally termed v7-3 (\( \text{Slc6a15} \)) KO mice (96), have further characterized the transporter. The \( \text{Slc6a15} \) KO mice have a 40% reduced sodium ion dependent uptake of leucine, and a 15% reduced uptake of proline compared with wild type mice (96).

In this thesis, we characterized the brain expression and specify the cellular localization of \( \text{B}^0\text{AT3} \) (\( \text{Slc6a17} \)) (paper III) (27,75) and \( \text{B}^0\text{AT2} \) (\( \text{Slc6a15} \)) (paper IV) (78,79). In addition, we challenged the monoaminergic system with drugs and used models of acute food deprivation and chronic food restriction to study effects on mRNA expression in various brain regions (paper III). We also used \( \text{Slc6a15} \) KO mice and investigated whether \( \text{B}^0\text{AT2} \) is important for mediating the anorexigenic effect of leucine (paper IV).

### Physiological function

Only twelve of all the SLC members are currently utilized as therapeutic drug targets (97). Of these twelve, four are members of the SLC6 family and used for treatment of depression, epilepsy and addiction (67). For example, the main GABA transporter GAT1 (encoded by \( \text{SLC6A1} \)) plays an important role in the brain and is strongly inhibited by anti-epileptic drugs such as tiagabine and nipecot acid (98). In addition, the monoamine transporters DAT (\( \text{SLC6A3} \)), NET (\( \text{SLC6A2} \)) and SERT (\( \text{SLC6A4} \)) are mainly located in CNS and specific inhibitors of these transporters include cocaine-analogs, the appetite suppressant mazindol and the anticholinergic drug benztropine (used in treatment of Parkinson's disease). Additionally, selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, and serotonin–norepinephrine reuptake inhibitors (SNRIs) antidepressants are targeting the monoaminergic transporters (99). A number of the other SLC6 family members are known to be important for pathological conditions and several are potential drug targets.

In synaptic transmission several SLC6s are crucial for termination of neurotransmission, through uptake of neurotransmitters from the synaptic cleft into presynaptic terminals or glial cells, as well as for maintaining low tonic
neurotransmitter concentrations outside synapses. Different types of neurons containing either glycine-reuptake transporters (GLYT1 (SLC6A9), GLYT2 (SLC6A5)), GABA-reuptake transporters (GAT1, BGT1 (SLC6A12), GAT2 (SLC6A13), GAT3 (SLC6A11)) or monoamine-reuptake transporters (DAT, NET, SERT) are all part of ending the signaling through neurotransmitter activated receptors by neurotransmitter reuptake from the synapse (reviewed by Pramod et al. 2013) (68) (Figure 3). The three monoamines are also associated with regulation of appetite and energy homeostasis (100), and SNRIs (sibutramine) has been one of the most widely used drugs against obesity (101).

The exact role of the vesicular B\textsuperscript{0}AT3 and the cell membrane bound B\textsuperscript{0}AT2 within synaptic transmission in the CNS is less clear, but changes in monoamine levels are known to regulate B\textsuperscript{0}AT3 and B\textsuperscript{0}AT2. Strong upregulation of the Slc6a17 (B\textsuperscript{0}AT3) transporter is seen in a model for the monoamine system in brain, using SSRI (fluoxetine) and SNRI (bupropion), and a similar but weaker effect is seen for the Slc6a15 (B\textsuperscript{0}AT2) transporter (paper III) (27,82). A suggested role for the transporters are thereby in regulating synaptic remodeling, a process important in the long term action of antidepressant drugs (102), by regulate the availability of free amino acids used as pools for precursors needed in the synthesis of neurotransmitters. The B\textsuperscript{0}AT2 results are also supported by several studies where the transporter is associated with depression (103-106).

Moreover, the B\textsuperscript{0}AT3 preference for glycine and the upregulation of the transporter by activating drugs suggest the transporter to be involved in glutamatergic signaling and NMDA receptor function (72,74,75,82). This theory is strengthened by results showing high upregulation of the transporter in response to starvation (paper III) (74,75). Starvation increases levels of circulating ghrelin that can activate neurons in hypothalamus to release glutamate, which in turn can activate NMDA receptors and hence stimulate feeding (107).

Many of the amino acid transporters in the SLC6 family are known to play important roles in several pathological conditions including obesity e.g. the ATB\textsuperscript{0+} (SLC6A14) transporter is associated with this condition (108-110). Recent studies with Slc6a15 KO mice show transporter mediated activation of the mTOR pathway and anorexigenic response to rapid administration of leucine, suggesting B\textsuperscript{0}AT2 to be part of leucine activated signaling in hypothalamic neurons related to food intake (paper IV) (78,79). Also, B\textsuperscript{0}AT2 is involved in leucine's ability to reduce intake of normal chow and weight gain when having access to a high fat diet in gender selective perspective (111). The essential amino acids leucine and valine cannot
Figure 3. Role of several SLC6 family members in synaptic transmission in the CNS. Schematic representation of transporters in synaptic terminals of monoaminergic, GABAergic and glycineergic neurons. Glycine-reuptake transporters (GLYT1, GLYT2), GABA-reuptake transporters (GAT1, BGT1, GAT2, GAT3) and monoamine-reuptake transporters (DAT, NET, SERT) are all part of ending the signaling of neurotransmitter activated receptors by neurotransmitter reuptake from the synaptic cleft. The vesicular glycine and neutral amino acid transporter B0AT3 is located in excitatory as well as inhibitory neurons, while the cell membrane bound BCAA and neural amino acid transporter B0AT2 is found on most neurons and on a limited number of astrocytes. The vesicular VIAAT protein, a member of the SLC32 family, transport GABA and glycine into synaptic vesicles in GABAergic and glycineergic neurons, while the vesicular SLC18 family members VMATs transport dopamine, norepinephrine and serotonin into synaptic vesicles in terminals of monoaminergic neurons. Figure adapted from Pramod et al. 2013 (68).
be synthesized *de novo* and must therefore be taken up from the diet. Central icv administration of leucine has been shown to decrease food intake and body weight through activation of the mTOR signaling in the hypothalamus (112,113). Leucine injection induce activation of the mTOR downstream factors S6 and S6K1, proteins in a pathway known to be involved in cellular processes including protein synthesis, cell growth and metabolism (Figure 4A) (114,115).

The B0AT2 transporter, and to some extent also B0AT3, prefer transport of the BCAA which is known to play an important role in providing brain neurons with a constant supply of glutamate by function as a nitrogen donor for glutamate synthesis (Figure 4B) (116-118).

**Figure 4.** The mTOR and BCAA pathways. Schematic diagram showing processes contributing to enhanced mRNA translation and synthesis of glutamate through the mTOR pathway (A) and glutamate synthesis within the BCAA pathway (B). (A) Amino acid transporters are the initial contact between the cell and the environment and play a central role in nutrient sensing and signaling. The mTOR complex respond to increases in amino acids, particularly leucine, and signals to translational effectors such as the ribosomal S6 kinases (S6Ks) and eIF4E-binding protein (4E-BP) to enhance mRNA translation. (B) The BCAA provide amino groups from α-ketoglutarate and function thereby as nitrogen donors for the synthesis of glutamate. The branched-chain α-keto acid dehydrogenase complex (BCKDH) converts BCAA into Acyl-CoA derivatives that are converted either into acetyl-CoA acetoacetate or succinyl-CoA ending up in the TCA cycle (reviewed in Tokunaga *et al.* 2004 (119).
Aims

The overall aim of this study was to understand how novel amino acid transporters function regarding substrate specificity, cell type specificity and physiology. This will allow us to dissect the functional characteristics of the amino acid sensing systems in the brain at the single gene level and identify the transporter responsible for a given cell-physiological function. Eventually this will allow us to elucidate the physiological role of the different amino acid transporters.

For this purpose, I used wild type mice, a transgene mouse line and X. laevis oocytes as research tools to study the transporters on a histological, behavioral and functional level. The specific aims for each paper were:

Paper I
This study aimed to screen the expression of Slc38a7 (SNAT7) and to investigate the cellular and subcellular localization in the CNS. Moreover, we wanted to provide a functional characterization to find the substrate profile and ion coupling properties.

Paper II
As a continuation of paper I we wanted to examine the expression Slc38a8 (SNAT8) in the CNS, and study similarities and interaction between SNAT7 and SNAT8. In addition, we investigated the substrate preference and uptake properties.

Paper III
The aim with this study was to study the CNS expression of the vesicular Slc6a17 (B0AT3) transporter in detail. We also investigated if the transporter plays a role in regulation of monoaminergic and glutamatergic synapses.
Paper IV

Herein we sought to test the hypothesis that the \textit{Slc6a15} (\textit{B}{^0}\textit{AT2}) transporter plays a role in mediating the effect on reduction of food intake and activation of the mTOR pathway after leucine injection. We also aimed to study the expression profile in the CNS.
Material and methods

More detailed descriptions of the experimental procedures used in this thesis work are provided in the individual papers. In the following section, I discuss the methods I have used the most.

Gene expression analysis

PCR and quantitative real-time PCR

The polymerase chain reaction (PCR) was developed in the early 1980s by the American biochemist Kary Mullis (120). Today, the method is one of the most powerful tools in cloning, genetic engineering and sequencing and Mullis was awarded the Nobel Prize in Chemistry in 1993 for his achievement. The main principle of PCR is multiple repeating of a three-step reaction in which the amount of a specific DNA product is amplified. A limitation with regular PCR is the ability of using the reaction as an analytic technique and for quantification. However, Higuchi et al. 1993 invented real-time PCR (RT-PCR), a kinetic PCR based analytic method used for detection and quantification of RNA (121). The method is capable of measuring the product in each cycle, in real time, if a dye is added during the reaction and the cycle at which the signal reaches a certain threshold value may then be used to calculate the original concentration.

In this thesis we used the PCR method for genotyping of transgenic mice (paper IV). The tip of the tail from the pups was cut for collecting DNA and the PCR method was used together with a set of three designed primers for sensitive detection of small amounts of the targeted DNA sequence. The PCR product was run on an electrophoresis gel for visualization (Figure 5).
Figure 5. PCR genotyping of *Slc6a15* KO mice. A set of three primers were used for genotyping of pups by PCR for simultaneous amplification of wild type (WT) and knockout (KO) alleles. Figure redrawn from Drgonova *et al.* 2007 (96).

Additionally, we used the quantitative RT-PCR method to determine gene expression levels for genes of interests in relevant tissue samples collected from wild type mice and rats and from transgenic mice (paper III and IV). RNA was extracted and cDNA synthesized by using reverse transcriptase. A number of house-keeping genes were tested and the most stable set of genes were used for internal references to correct for variations in dissection, RNA extraction and reverse transcriptase efficiency (122). Primers for house-keeping genes and for genes of interest were designed to specific amplify only segments of these genes.

**In situ** hybridization

**In situ** hybridization (ISH) is a sensitive histological coloring technique for expression studies in morphologically preserved cells in fixed tissue sections. The main principle, first described by Gall and Pardue in 1969 (123), is sequence specific hybridization of a labeled nucleotide sequence, an *in vitro* transcribed RNA probe, to complementary target mRNA expressed inside cells. The probe is visualized and detected *in situ* in the cell body, and since the mRNA is located in the cell soma, this area becomes visible. Non-radioactive labeled probes are visualized using enzyme based detection with enzyme-coupled antibodies (124). The cells with the transcription of the gene of interest are visualized by addition of a substrate that becomes colored (or fluorescent) by the enzyme-coupled antibody.

The semi quantitative method is used for detection of gene expression within one single cell. Identification of mRNA expression through the process of ISH is used as a tool in clinical diagnosis and in basic scientific research (125). The method can be used as one of the first steps to elucidate the function of newly discovered genes which can lead to identification of in what kind of tissues and in what kind of cell populations the gene of interest
is expressed, which is required for understanding the physiological role of the gene (126).

In all papers (paper I-IV) the single probe ISH method was used with RNA probes synthesized and used on coronal and sagittal tissue sections for localization of genes of interest in specific cells within mouse CNS. Both chromogenic and fluorescent substrates have been used interchangeably without any noticeably difference in sensitivity. Additionally, in paper III double ISH was used for detection of two probes at the same time for visualization and co-localization of the gene of interest together with a marker. In the double ISH method is the first probe detected in one color and the enzyme needed for detection is then inactivated prior the detection of the second probe in another color.

Protein expression studies

Immunohistochemistry

The method immunohistochemistry (IHC) was described already in the early 1940s by Coons et al. (127). The method is used for histological staining and a powerful tool for localizing proteins in cell cultures or tissue sections. The principle of the method is un-conjugated primary antibody binding to a specific antigen binding characteristic, epitope, of an expressed protein. The localized protein can thereby be visualized by using a fluorochrome conjugated secondary antibody for amplification of the signal and ultraviolet light (128). An alternative staining procedure is using diaminobenzidine (DAB) precipitation. If using two, or even three, different primary antibodies simultaneously (termed double/ triple IHC) it is possible to detect several antigens at the same time, in the same cell or tissue section. This allows comparative studies such as co-localization of a protein without known function and proteins with known and specific cellular functions.

In this thesis, the IHC method was used with custom made antibodies in all papers (paper I-IV) for localization and co-localization of novel proteins in brain and spinal cord tissue. A disadvantage of using antibodies is background and unspecific staining (129). Optimization of the IHC protocol was made for each antibody for improvement of the signal by using different blocking reagents and antigen retrieval to reduce background signal and unmasking the protein epitope. Precaution has been made to make sure the staining is representative by evaluating the specificity of the custom made antibodies and comparing the expression with matching ISH results. An advantage with the IHC is the possibility to combine it with ISH for detection of both gene and protein at the same time, and the combined method was used in paper I-III.
Functional characterization

*X. laevis* oocyte system

The South African Clawed frog *Xenopus laevis* (*X. laevis*) is a non-mammalian animal model used in most of disciplines of modern biology including biochemistry, embryology, toxicology and biomedical research. In 2012 John Gurdon received the Nobel Prize in medicine for his work with nuclear transplantation experiments on egg cells from *X. laevis*. The unfertilized egg cell, the oocyte, is a resilient cell and the high translational capacity was first described in 1971 (130). Oocytes can be used for transient expression of heterologous proteins by microinjection of either exogenous RNA and/or DNA. The RNA can either be total mRNA or poly(A) mRNA extracted from native tissue samples or *in vitro* transcribed cDNA-derived mRNA (cRNA). This powerful system is commonly applied for studying receptors, channels and transport proteins with several techniques (131). An advantage with the oocyte system is that *X. laevis* is easily kept and bred in captivity and therefore commercially available at low costs (132).

Ovaries from *X. laevis* females contain a mixture of six different stages of mature oocytes (I-VI) and only the latest stage is used in overexpression studies. A schematic drawing of the oocyte is presented in Figure 6. The human oocyte has a size of 100 µm in diameter while the *X. laevis* oocyte has a diameter of about 1.3 mm (133). An advantage with the *X. laevis* oocyte is thereby the large cell size allowing for high overexpression of the heterologous protein of interest on the large cell surface. The high number of overexpressed proteins on the cell surface gives strong signals when performing experiments, something that is important for example when studying novel low affinity transporters. The system has also generally low background of endogenous transport activity compared with other whole-cell heterologous expression systems. Endogenously expressed transporters and ion channels in *X. laevis* oocytes are at a low number as the oocyte does not rely on uptake of nutrients or ions from extracellular sources for their development (134). Disadvantages with heterologous protein overexpression in oocytes could form heteromeric complexes, interact with endogenous proteins, upregulate endogenous transport systems and altered protein properties could occur due to differences in the ambient temperature or altered cellular environment (131).
Figure 6. Schematic drawing of the oocyte. Injection of cDNA is performed in the huge oocyte nucleus, also called a germinal vesicle, which is located in the darker animal pole, while cRNA injections should be performed in the cytosol in the light vegetal pole. Figure modified from Carolina Åstrand.

In this thesis, small batches of oocytes were obtained by surgical removal of single ovarian lobes. Liberase enzyme treatment of the ovary tissue released the individual oocytes, detached the surrounding layer of follicle cells and removed the vitelline membrane prior the experiments. Ion-dependent substrate preference was investigated on stage V-VI oocytes after cytoplasmic cRNA microinjections for overexpression of the novel proteins of interest (paper I and paper II). Only cRNA was used for microinjection in oocytes for studying membrane bound proteins. Using a cRNA vector allows for manipulations of the genetic sequence, which in this case is 5’ and 3’ untranslated regions (UTRs) from a X. laevis globin ribosome binding gene used for enhancing the translation efficiency of the heterologous cRNA transcripts in oocytes (135). A disadvantage with using cRNA is that only homomultimeric protein complexes will be formed even though most membrane-bound proteins form complexes with other membrane bound and cytoplasmic proteins and also with cytoskeleton elements (136). Overexpression of proteins of interest and subcellular distribution of the protein was also verified with immunostaining on sectioned cRNA injected oocytes. The oocyte system was then used for studying plasma membrane transport proteins by either radioisotope flux measurements or electrophysiological recordings and a flow scheme of the experimental setups is shown in Figure 7.
**Figure 7.** Flow scheme of experimental setups with radioisotope flux measurements and electrophysiological recordings. Day 1; Surgery performed on *X. laevis* frog under anesthesia for removal of ovarian lobes, and for isolation and collection of oocytes. Day 2; Microinjection of exogenous cDNA/ cRNA construct into oocytes. Day 3-4; Incubation for overexpression of protein of interest. Day 5; Uptake Assay by incubation of oocytes in radiolabeled substrate, washes and measuring of radioactive decay (in cpm) in cell lysate. Electrophysiology by two-microelectrode voltage-clamp for measuring the co-transport of sodium ions (in nA) pass the plasma membrane in oocyte superfused with substrate solution. Day 6; Data analysis for evaluation of results.

**Uptake Assay**

In short, radioisotope uptake measurements are initiated by adding medium containing the appropriate radiolabeled substrate to groups of cRNA-injected oocytes and control oocytes (n = 8-12/group). After incubation, the excess of labeled substrate is removed by rapid ice-cold washes with isotope-free transport buffer. The individual cells are then lysed and dissolved in detergent for quantification of oocyte-associated radioactivity by liquid scintillation counting (in cpm), corresponding to substrate uptake of transporter (134).

The large size of the oocyte makes the initial rates of uptake sustained for longer periods, typically 1 min to 1 h, compared with what can be achieved with vesicles, bacteria, yeast or cultured cells, and the incubation time is experimentally determined for each studied transporter. A number of functional studies can be measured with the Uptake Assay, including substrate specificity, competition sensitivity, pH- and ion-dependence and efflux.

In this thesis, novel amino acid transporters were functionally characterized using Uptake Assay on overexpressing *X. laevis* oocytes (paper I and paper II). Additionally, recombinant protein expression in *X. laevis* oocytes was confirmed with IHC on sections from overexpressing and control oocytes.
Two-electrode voltage-clamp

With two-electrode voltage-clamp, transport which is electrogenic can be studied. A wide range of transporters from different protein families can be studied with two-electrode voltage-clamp on overexpressing *X. laevis* oocytes because the uptake of substrates across the cellular membrane is ion-dependent. The transport capacity of the substrate together with the co-transported cation e.g. \( \text{Na}^+ \), \( \text{H}^+ \) or \( \text{K}^+ \) or anion e.g. \( \text{Cl}^- \) is slow for transporters \( (10^{-1}-10^{-4} / \text{sec}) \) compared with ion-channels \( (\geq 10^6 / \text{sec}) \) (134).

In two-electrode voltage-clamp (TEVC) is a bath-grounding electrode together with two intracellular glass microelectrodes (impaled into the oocyte) used for current recordings during superfusion with substrate solution (131). One intracellular electrode records and clamps the oocyte membrane to a predetermined holding potential (minus 50-70 mV), while the other delivers a current (in nA) to maintain that potential. The recording membrane potential electrode is connected to a feedback amplifier where the signal is compared to the voltage clamp command given by a generator. The highly amplified difference of these signals is applied as a current through the current-delivering electrode and to the grounding electrode. The current needed to hold the desired membrane potential can be used to show the measured ion flow over the cellular membrane through overexpressed electrogenic transporters, ion channels or pumps, representing the substrate transport or flow over the membrane (Figure 8).

The two-electrode voltage-clamp method can be used as an alternative to the Uptake Assay for investigating properties such as ion dependence and substrate specificity for novel transporters. As we are working with transporters of the SLC class, which are driven by ion gradients, uptake of amino acids (or other substrates) induces a change in intracellular ion concentrations and hence a change in membrane potential. By monitoring changes in current to keep the membrane potential constant using clamp recordings, it would be possible to assess amino acid uptake without the need for radiolabeled substrates. An advantage with this method is that substrates not available in radioactive labeled form can easily be tested, but to correlate transport induced currents (ion movement) and transport (substrate movement) uptake of radiolabeled substrates may be measured simultaneously in oocytes under identical conditions.

For the experiments in this thesis, we employed two-electrode voltage-clamp in paper II for functional characterization of the novel transporter.
Figure 8. Two-electrode voltage-clamp setup on *X. laevis* oocyte. By attaching electrodes to an oocyte, the substrate transport into the cell can be shown by measuring the flow of sodium-ions across the cellular membrane that are co-transported with the substrate. The oocyte chamber contains a protein overexpressing oocyte superfused with Na⁺-containing substrate (in red) controlled by a perfusion system and a vacuum pump. Channel 1 (CH1) is the voltage electrode that clamps the membrane at desired potential (E), while channel 2 (CH2) is the electrode that delivers a current (I) to maintain the membrane potential. The system contains a current ground and a voltage ground, one grounding electrode placed in the chamber and one in KCl solution on the other side of an agar bridge. FBA is the feedback amplifier measuring current and voltage and delivering the current. Figure redrawn from (131).

**Behavioral analysis**

**Transgenic mice**

Transgenic mice are important animal models for studying genes whose functions have not been determined. Mario Capecchi, Martin Evans and Oliver Smithies won the Nobel Prize in 2007 for developing the KO mouse by gene targeting (137-139). Conventional (full) KO mice have a null mutation introduced into a target allele by homologous recombination, while conditional KO mice allows for conditional compensation depending on the promoter in the transgene. The Cre-LoxP (140) and flipase recombination...
target (FRT) (141) systems were developed to circumvent hindrances of full KO mice, such as embryonically lethal strains that cannot undergo further analysis of the gene at different developmental stages.

The generation of the full Slc6a15 KO mouse, originally termed v7-3 (Slc6a15) C57BL/6J KO mouse, is described in detail elsewhere (96). In short, for inactivation of the Slc6a15 gene product exon 3, known to contain the initiation codon and the TM domain involved in substrate binding (142), was disrupted in embryonic stem (ES) cells using homologous recombination (Figure 9). Correctly recombinant ES cells, selected by antibiotic (neomycin) and antiviral drug (ganciclovir), were identified by PCR and confirmed by Southern blotting. ES cells were injected into blastocysts and inserted into pseudopregnant females resulting in chimeric transgenic mice. Mating of the heterozygous progeny of the chimeric mice produced wild type, heterozygous and KO mice, and RT-PCR further confirmed the disruption of the locus in KO mice. In this thesis the Slc6a15 KO mouse was used in paper IV.

![Figure 9](image.png)

**Figure 9.** Disruption of exon 3 in the Slc6a15 gene in ES cells using homologous recombination. Red boxes represent exons, arrows the EcoRI (RI), BglII (BII) and SacI (SI) restriction sites, neomycin antibiotic resistance gene (Neo) and viral enzyme tyrosine kinase (TK). Figure redrawn from Drgonova et al. 2007 (96).

Study of Slc6a15 KO mice

Administration of leucine is known to decrease food intake and body weight through activation of the mTOR signaling and mTOR downstream factors S6 and S6K1 in the hypothalamus (113). In this thesis (paper IV), leucine was intraperitoneally (ip) injected in Slc6a15 KO mice (96). We then measured the food intake and body weight of the mice, and c-Fos activated neurons, by counting cells in food related areas in the brain, to assess the importance of the B0AT2 in mediating the effect on reduction of food intake from leucine.
Results and discussion

Paper I & II

The SLC38 family of transporters has in total eleven members in humans and they encode amino acid transporters called sodium-coupled amino acid transporters (SNATs) (15). To date, five SNATs have been characterized and functionally subdivided into System A (SNAT1, SNAT2 and SNAT4) and System N (SNAT3 and SNAT5) showing the highest transport for L-glutamine (11). In paper I and II we present functional characterization of the novel glutamine transporters encoded by the \textit{Slc38a7} and the \textit{Slc38a8} genes and we suggest the encoded proteins should be named SNAT7 and SNAT8, respectively, to adhere with the SNAT nomenclature.

In paper I, the \textit{Slc38a7} mRNA was found to be abundantly expressed in brain and spinal cord when using \textit{in situ} hybridization. Strong expression of \textit{Slc38a7} was identified in ventromedial hypothalamus (VMH), basolateral amygdala (BLA), ventral tegmental area (VTA) and locus coeruleus (LC). These areas are known to be involved in many different physiological functions including feeding (143,144). Immunohistological double-labeling with a custom made antibody, for detection of the SNAT8 protein, and neuronal markers showed localization in GABAergic and other neurons in the CNS. The cellular distribution of SNAT7 indicated expression in the soma, the cell membrane and on the cellular axon.

Functional characterization was performed with $[^3]$H-labeled L-amino acids added to SNAT7 overexpressing oocytes. The Uptake Assay revealed L-glutamine as the preferred substrate of transport and a time course showed increasing amounts of uptake with time up to 60 minutes, indicating that SNAT7 is a relatively low capacity transporter. Further characterization of the coupled transport properties of SNAT7 showed sodium-dependent amino acid transport. A competition analysis was performed with amino acids, neurotransmitters and MeAIB, to examine the substrate profile for SNAT7. The substrate profile of SNAT7 was ranked: L-glutamine $>$ L-histidine $>$ L-serine $>$ L-alanine $>$ L-asparagine $>$ L-aspartic acid $>$ L-glutamic acid $>$ L-methionine $>$ L-leucine $>$ L-glycine. SNAT7 showed an unusually broad substrate profile. The amino acid analogue MeAIB, a system A substrate, did not inhibit the uptake of L-glutamine. The preferred substrate profile, tissue expression pattern and sodium-dependent transport, indicated that SNAT8 is
a novel member of the system N amino acid transporter system. *Slc38a7* (SNAT7) is unique in being the first system N transporter expressed in GABAergic and also other neurons. The preferred substrate and axonal localization close to the synaptic cleft indicates that SNAT7 could have an important function for the reuptake and recycling of glutamate.

In paper II, we provide a comprehensive CNS expression profile in mouse brain for the *Slc38a8* gene and the SNAT8 protein. The *Slc38a8* mRNA was expressed in restricted areas such as paraventricular hypothalamic nucleus (Pa), VMH and arcuate hypothalamic nucleus (Arc) in hypothalamus, BLA in amygdala, VTA and LC in pons. In addition, SNAT8 was shown to be expressed in all neurons, both excitatory and inhibitory, but not in astrocytes or vesicles in mouse brain using immunohistochemistry.

Functional characterization was performed by overexpressing SNAT8 in *X. laevis* oocytes to identify coupled transport properties and the preferable substrates by applying both a radiotracer technique (Uptake Assay) and two-electrode voltage-clamp. The substrate investigation showed SNAT8 preference for uptake of L-glutamine, L-arginine, L-histidine, L-alanine L-glutamate, L-aspartate, L-leucine, and L-proline. The transport was sodium-dependent and could not be substituted with lithium, and the amino acid analogue MeAIB was also a preferred substrate. We suggest SNAT8 to belong to the system A transporters based on the tissue expression and functional characteristics of SNAT8.

*Slc38a7* (SNAT7) and the closely related *Slc38a8* (SNAT8) originate from a common ancestor present already in placozoans (11). In this paper we showed that both *Slc38a7* (SNAT7) and *Slc38a8* (SNAT8) are found in amphibians. We also visualized protein-protein interactions between SNAT8 and SNAT7. The SNAT8 expression on axons of neurons together with the substrate profile suggests that SNAT8, similar as SNAT7, could play a role in sustaining the glutamate neurotransmitter pool through the GGC in the brain.

**Paper III**

The vesicular B^0^AT3 transporter (*Slc6a17*), one of the members of the SLC6 family, is a transporter for neutral amino acids and is exclusively expressed in brain (72,82). High *Slc6a17* gene expression was seen exclusively in rat CNS using real-time PCR on a panel of central and peripheral tissues. We provide a comprehensive expression profile of *Slc6a17* (B^0^AT3) in mouse brain using *in situ* hybridization and immunohistochemistry. Abundant mRNA expression of *Slc6a17* was seen in mouse brain and spinal cord and *Slc6a17* mRNA, expression also found in POMC and NPY neurons and in both excitatory and inhibitory neurons. A custom made B^0^AT3 antibody was
used to obtain detailed co-labelling with several cell type specific markers. Once again the neuronal expression was confirmed and the protein was found in the cell body, axon and synapses of inhibitory and excitatory neurons. The B⁰AT3 expression was highly overlapping with vesicular glutamate transporter 2 (VGLUT2) and vesicular glutamate transporter 1 (VGLUT1).

We also showed that Slc6a17 mRNA is up-regulated in animals subjected to short term food deprivation as well as in animals treated with the serotonin reuptake inhibitor fluoxetine and the dopamine/noradrenaline reuptake inhibitor bupropion. This suggests that the B⁰AT3 transporter have a role in regulation of monoaminergic as well as glutamatergic synapses.

Paper IV

In paper IV we studied the B⁰AT2 protein, a product of the Slc6a15 gene and a member of the SLC6 subfamily that has been shown to be a transporter of essential branched-chain amino acids (71,76).

A qPCR expression analysis of Slc6a15 showed widespread expression in brain and spinal cord, and low or no expression in peripheral tissues. A screen of mouse brain and spinal cord showed high expression of Slc6a15 mRNA in striatum, cortex, hippocampus, amygdala, hypothalamus, pons, and in spinal cord using in situ hybridization. The Slc6a15 transporter was found in areas known to be part of the regulation of food intake such as supraoptic nucleus (SON), VMH, anterior hypothalamic area (AH), BLA, Arc, and LC. We generated a polyclonal B⁰AT2 antibody and used immunohistochemistry to identify the cell-types expressing the transporter. We showed that B⁰AT2 immunoreactivity is mainly neuronal, including localization in many GABAergic neurons and spinal cord motor neurons. The B⁰AT2 protein was also found in astrocytes close to ventricles, and co-localized with cytokeratin and diazepam binding inhibitor (DBI) in epithelial cells of the choroid plexus. The expression suggests B⁰AT2 to partly function as a transporter contributing to the uptake of circulating amino acids from the bloodstream into the brain.

B⁰AT2 function as an amino acid transporter with high affinity for leucine (71,76). We aimed to further characterize the B⁰AT2 transporter in CNS, and used Slc6a15 KO mice to investigate whether B⁰AT2 is important for mediating the anorexigenic effect of leucine. Leucine was ip injected and we measured food intake and activation of neurons in food related areas in the brain in wild type mice compared with Slc6a15 KO mice. The Slc6a15 KO mice showed lower reduction of food intake as well as lower neuronal activation in VMH in response to leucine injections, suggesting B⁰AT2 to be
involved in hypothalamus senses variations in leucine availability to regulate energy balance.

In this study we also discuss if B⁰AT2 has a physiological function as a transporter contributing to the uptake of essential BCAA from the bloodstream into the brain or as a provider of neurotransmitter precursors, or both.
Conclusions and perspectives

The focus of the thesis was to characterize amino acid transporters expressed in the CNS. Characterization regarding substrate profiles and other biochemical properties, tissue distribution as well as the cell type specificity and subcellular localization, and behavioral investigations of transgenic models are all important features for understanding the physiological role and possible pharmaceutical potential of transporters.

The SNAT transporters with the amino acid L-glutamine as favored substrate (for most of the members) and with their high expression in the brain have been suggested to be part of the GGC and for supplying cells with glutamine as substrate for general biosynthesis in the brain. The two SNAT7 (Slc38a7) and SNAT8 (Slc38a8) transporters share almost identical substrate specificities and are co-expressed in several brain tissues. The extent of functional redundancy between these two transporters is unknown and the exact involvement of the recently characterized SNAT7 and SNAT8 and the other novel SLC38 members in GGC is not yet elucidated.

Our aim is to continue the characterization of the recently discovered members in the SLC38 family. We will perform in situ hybridization and immunohistochemistry to map the tissue and cellular expression and use Uptake Assay and electrophysiology to evaluate the substrate profile and transport properties for the novel SLC38 members. A full molecular and functional characterization of the orphan Slc38a10 is in the pipeline. In addition, custom made antibodies for the SNAT7 (SLC38A7), SNAT8 (SLC38A8) and SLC38A10 will be used identify the exact cellular localization using electron microscopy. This will ultimately show if these transporters are localized to synapses and hence sort out if the novel transporters are important for the uptake of amino acids in the GGC. In addition, cellular compartmental localization can be used for evaluating membrane type expressing the transporters using western blot.

It would also be of interest to further investigate SNAT7 and SNAT8 and see if one of the two transporters becomes up-regulated during starvation of amino acids, while the other one is unaffected and function more as a housekeeping gene required for the maintenance of basic cellular function that are expressed in all cells of an organism under normal condition. Finally, we are planning to generate conditional knockout mice for SLC38A7 and SLC38A8, and the other novel members within the family, by utilizing the
Cre-LopP system, which will allow us to study the physiological function at whole body level.

We have access to the *Slc6a15* KO mouse through collaboration with a research group in Baltimore, USA. Studies with *Slc6a15* KO mice show transporter mediated activation of the mTOR pathway and anorexigenic response to rapid administration of leucine, suggesting B⁰AT2 to be part of leucine activated signaling in hypothalamic neurons related to food intake.

Central intracerebroventricular (icv) administration of leucine into the 3V has shown an even stronger decrease of food intake and body weight through activation of the mTOR signaling in the hypothalamus. During icv administration leucine can directly affect hypothalamic signaling and does not have to be transported with the blood from the abdominal to the brain and does not have to pass the blood-brain-barrier. It would therefore be of interest to further investigate how B⁰AT2 (SLC6A15) is involved in the anorexigenic effects of leucine by performing icv administration of leucine directly into the 3V in *Slc6a15* KO and wild type mice.

Changes in monoamine levels are known to regulate the vesicular B⁰AT3 (*Slc6a17*) and cell membrane bound B⁰AT2 (*Slc6a15*), but the role of the two transporters within synaptic transmission in the CNS is less clear. A suggested role for the transporters are within synaptic remodeling by regulate the availability of free amino acids used as pools for precursors needed in the synthesis of neurotransmitters. In addition, the B⁰AT3 and B⁰AT2 preference for BCAA suggests a role in glutamate synthesis through the BCAA pathway. Moreover, upregulation of B⁰AT3 (*Slc6a17*) is also seen as a response to starvation, suggesting the transporter to be involved in glutamatergic signaling and NMDA receptor function.

SLC6A15 is associated with depression, and there are commonly used methods for measuring depression-like behavior in mice. It would therefore be of interest to further evaluate the *Slc6a15* KO mice and the role of the transporter in depression with methods such as the Porsolt forced swim test, also in combination with SSRIs, and the tail suspension test to further evaluate the role of B⁰AT2 in depression.
Acknowledgements

It is with deep sincerity that I want to thank my colleagues at the Department of Neuroscience and elsewhere. The five years I have spent in HelgiLab have been great and I'm glad I had such nice colleagues to share them with.

My greatest gratitude goes to my main supervisor Robert Fredriksson who has been an excellent mentor and friend throughout these years. I have learned a lot about being a scientist from you and I would like to thank you for always having time for questions, encourage me and for great ideas. Thanks to my co-supervisor Helgi Schiöth for creating such a great environment to do research in. You have taken my projects in a new direction when being stuck and functioned as stand-in for Robert during his parental leave. Special thanks for successful collaboration to my unofficial co-supervisor Örjan Wrange at KI. I have learned a lot about frogs from you and you have inspired me to not give up in hard times.

I would also like to thank my co-authors and project students, who all have been important and necessary for the work presented in this thesis. Especially thanks to Smitha, Sofie, Sahar, and Sonchita for all the fun and good scientific discussions. I am very grateful to Erik, Victor, Sofie B, Edvin, Gaëtan, Aniruddha, Olga S, Ali, Yinan, Ingrid, Atieh, Luca, Galina and Victoria. It has been educational to supervise you all and thanks for the hard work in the lab, the genuine interest in the projects and help pushing my projects forward.

Anna and Karin, you are the best! Without you my time as a PhD student would have been half as fun as it was. Thanks for fantastic experiences on and outside the SfN conferences in Chicago, San Diego and Washington. Thanks for all the small and big talks about everything and nothing and the time we have spent together in the gym.

Thanks Pleunie and Lill-Anders for being great office mates. Thank you all past (Marcus, Josefin, Kalle, Johan and Chris) and present (Mathias, Emilia, Emilie, Vanni, Mike, Anica, Philip, Jonathan, Christian, Madeleine, Emil and Olga) members of HelgiLab for all the great times and for creating such a warm atmosphere.
If knowledge (and friendship) was for any reason not found in HelgiLab I have been “using” Dan or Klas and their lab crews Christina, Helena, Görel, Daniel, Bo, Ingrid, Anders, Emma, Martin, Nadine, Nicole, Kali, Christiane, Lina, Kia, Malin and Kasia, which I would like to sincerely thank.

To all people who daily have been eating lunch with me (you know who you are), thanks for waiting for me to finish my meal!

Thanks to Birgitta (autoklavtanten) for all the things you do and to Emma, Mariana, Lena, Ulla, Berit and Cecilia for handling administration.

Writing papers is not all. Thanks to all my friends and my family that have contributed on my path towards a PhD and this thesis. To my grandma, grandpa, Lars, “min allra allra bästa” brother, Lisa, mum and dad, and finally my biggest love Fredrik and Elsa, I would like to say that I love you all and that you are the most important in my life.
References


104. Li, M., Ge, T., Feng, J., and Su, B. (2013) SLC6A15 rs1545843 and depression: implications from brain imaging data. The American journal of psychiatry 170, 805


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