Functional Characterization of Centrally Expressed Solute Carriers and G Protein-Coupled Receptors

SMITHA SREENDHARAN
Dissertation presented at Uppsala University to be publicly examined in B42, BMC, Husargatan 3, Uppsala. Thursday, September 22, 2011 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Transmembrane proteins are gatekeepers of the cells; controlling the transport of substrates as well as communicating signals among cells and between the organelles and cytosol. Solute carriers (SLC) and G protein-coupled receptors (GPCR) are the largest family of membrane transporters and membrane receptors respectively. The overall aim of this thesis was to provide a basic understanding of some of the novel SLCs and GPCRs with emphasis on expression, transport property, evolution and probable function.

The first part of the thesis directs towards the study of some novel solute carriers. In an initial study, we provided an overall picture of the sequence relationship and tissue expression of 14 diverse atypical SLCs confirming some of their evolutionary conservation and highly specific expression pattern. The focus then was on the SLC17 family (mainly vesicular proteins) and a novel member named Slc17a9. This study revealed that SLC17 family could be divided into four main phylogenetic clades which were all present before the divergence of the insect lineage with Slc17a9 having the most restricted evolutionary history. Detailed expression study of Slc17a9 in the mouse brain suggests that it is also expressed in some regions important for purinergic neurotransmission. Further, we deorphanised an aminoacid transporter Slc38a7 which was expressed in a majority of neurons in the CNS and showed that it preferably mediate transport of L-glutamine and L-histidine.

The second part of the thesis focuses on the study of two GPCRs belonging to the Rhodopsin superfamily, Gpr162 and Gpr153. A phylogenetic analysis revealed that both Gpr153 and Gpr162 originated from a common ancestor before the radiation of the mammalian lineage. Expression study revealed that Gpr162 had a predominant expression in the CNS and relatively lower expression in the other tissue tested whereas Gpr153 had a more widespread and similar expression pattern in both CNS and peripheral tissues. The functional studies of the two GPCRs were done using the antisense oligodeoxynucleotide knockdown rat model. These studies provided evidence linking the orphan Gpr162 gene with the regulation of food intake– related behaviour whereas Gpr153 gene caused only a slight reduction in food intake.

Keywords: GPCR, SLC, Gpr153, Gpr162, Slc17, Slc38

Smitha Sreedharan, Uppsala University, Department of Neuroscience, Functional Pharmacology, Box 593, SE-751 24 Uppsala, Sweden.

© Smitha Sreedharan 2011

ISSN 1651-6206 0346-5462
urn:nbn:se:uu:diva-156832 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-156832)
In a day, when you don’t come across any problem
– you can be sure that your travelling in the wrong path

Swami Vivekananda

To Amma and Achan
List of Papers

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


V Sreedharan S, Almen MS, Carlini VP, Haitina T, Stephansson O Sommer WH, Heilig M, de Barioglio SR, Fredriksson R, Schiöth HB. (2011) Gpr153 shares common origin with Gpr162 and is highly expressed in central regions including the thalamus, cerebellum and the arcuate nucleus. *Submitted Manuscript*

Reprints were made with permission from the respective publishers.
Contents

Introduction ................................................................................................... 11
Transmembrane proteins .......................................................................... 12
Solute carriers........................................................................................... 13
  Phylogeny of Solute carriers ................................................................. 13
  SLC17 family .......................................................................................... 14
  SLC38 family .......................................................................................... 15
G protein-coupled receptors .................................................................. 17
  Functional studies on GPCRs ................................................................. 18
  GPCR evolution and classification .......................................................... 19
Rhodopsin family ...................................................................................... 21
  Functions of Rhodopsins in the CNS ...................................................... 21
Aim ............................................................................................................... 23
Paper I ...................................................................................................... 23
Paper II .................................................................................................... 23
Paper III .................................................................................................. 23
Paper IV ................................................................................................... 24
Paper V ..................................................................................................... 24
Methodological review ................................................................................. 25
  Phylogenetic analysis ............................................................................. 25
  Gene expression studies ........................................................................ 26
    Quantitative real time PCR (qPCR) ...................................................... 26
    In situ hybridization (ISH) ................................................................... 27
    Immunohistochemistry ........................................................................ 27
Functional Studies .................................................................................... 28
  Transport assay in Xenopus leavis oocytes ............................................. 28
  Antisense oligodeoxynucleotide technology ......................................... 28
  Feeding study ......................................................................................... 29
  Behavioral studies ................................................................................ 29
Results and Discussion ................................................................................. 31
Paper I ...................................................................................................... 31
Paper II .................................................................................................... 32
Paper III .................................................................................................. 32
Paper IV ................................................................................................... 33
Paper V ..................................................................................................... 34
Work in progress................................................................. 36
  Generation and functional analysis of a novel transporter SLCZ1 mutant mice ............................................................................................................. 36
    Generation of SLCZ1 mutant mice ........................................ 36
    Analysis of SLCZ1 null mutant mice ...................................... 38

Conclusions and perspectives .................................................. 39

Acknowledgements......................................................................... 42

References...................................................................................... 46
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral amygdala</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPA_AT</td>
<td>Cation proton antiporter and Auto-transporter</td>
</tr>
<tr>
<td>DMT</td>
<td>Drug metabolite transporter</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocynate</td>
</tr>
<tr>
<td>FZD</td>
<td>Frizzled receptors</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma amino isobutyric acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>D-Glyceraldehyde 3 phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>GPS</td>
<td>GPCR proteolytic cleavage site</td>
</tr>
<tr>
<td>GrDG</td>
<td>Granular layer of dentate gyrus</td>
</tr>
<tr>
<td>HBD</td>
<td>Hormone binding domain</td>
</tr>
<tr>
<td>HGNC</td>
<td>Hugo gene nomenclature committee</td>
</tr>
<tr>
<td>HKG</td>
<td>Housekeeping gene</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridization</td>
</tr>
<tr>
<td>IT</td>
<td>Ion transporter</td>
</tr>
<tr>
<td>LC</td>
<td>Locus coeruleus</td>
</tr>
<tr>
<td>MeAIB</td>
<td>Methyl amino isobutyric acid</td>
</tr>
<tr>
<td>MFS</td>
<td>Major facilitator superfamily</td>
</tr>
<tr>
<td>OF</td>
<td>Open field</td>
</tr>
<tr>
<td>Py</td>
<td>Pyramidal cells of the hippocampus</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real time PCR</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier</td>
</tr>
<tr>
<td>SNAT</td>
<td>Sodium coupled neutral amino acid transporter</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SV2</td>
<td>Synaptic vesicle 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>T2R</td>
<td>Taste 2 receptor</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>VEAT</td>
<td>Vesicular excitatory amino acid transporter</td>
</tr>
<tr>
<td>VFTM</td>
<td>Venus fly trap module</td>
</tr>
<tr>
<td>VGLUT</td>
<td>Vesicular glutamate transporter</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
</tr>
<tr>
<td>VNUT</td>
<td>Vesicular nucleotide transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
</tbody>
</table>
Introduction

Cells and their organelles are aqueous compartments enclosed by a biological membrane. These membranes play a very important role in various cellular functions apart from serving as a boundary separating the cellular compartment and the cell interior from their exterior. This basic idea about the biological membrane led to several studies directed towards the in-depth understanding of the molecular organization of membranes. The most widely accepted generalization of the gross structure of functional biological membrane was provided by Singer and Nicolson in 1972 and was termed “The Fluid mosaic Model” [1]. According to this model the cell membrane was viewed as “a two-dimensionally oriented solution of integral proteins (or lipoproteins) in the viscous phospholipid bilayer solvent” [1]. New findings over four decades have further increased our understanding of biomembranes and have driven the revision of the fluid mosaic model in certain aspects (Figure 1). The membranes are now viewed as more mosaic than fluid, dynamic but structured and have proteins and lipids that act in concert forming functional molecular entities in the cell membrane [2-6].

![Image](image1.png)

**Figure 1.** A) Schematic view of the fluid mosaic model as proposed by Singer and Nicholson in 1972 illustrating the sparse distribution of monomeric protein dispersed in a sea of unperturbed lipid bilayer. B) An amended version of the above model is a modified view sketched by David Engelman exemplifying the nonrandomly protein (monomeric and multimeric) crowded membrane in a lipid bilayer of varied thickness. Figure adapted from [5].

The basic composition of biomembranes has not undergone any major changes through decades of studies. The membranes are composed of lipids, carbohydrate and proteins. However the diversity and complexities of the lipid and protein moiety and their complex interactions in the membrane
have been further established. The focus of this thesis is on membrane protein and hence limits the further discussion of lipid diversity and function in the membranes. Membrane proteins, an important functional unit has gained central role in membrane studies from the beginning [7]. They constitute a major fraction by weight in most functional biomembranes [7, 8]. Membrane proteins can be broadly divided into two categories termed peripheral and integral [9]. Peripheral membrane proteins are associated with the membrane but do not penetrate the hydrophobic core of the membrane. Most of them adhere weakly and temporarily to the membrane and are often attached to the integral membrane protein with an exception of glycoprophosphatidylinositol (GPI) anchored protein. Peripheral membrane proteins include peripheral enzymes such as D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aldolase and electron carriers such as cytochrome C. Integral membrane proteins are embedded into the hydrophobic core and are strongly bound to the membrane. Integral membrane proteins include a largely diverse group of transmembrane proteins which spans the entire length of the cell membrane.

Transmembrane proteins

Transmembrane (TM) proteins constitute an important class of membrane protein that plays several roles in the functioning of the cells. TM proteins can be classified based on their structure into alpha(α)-helical and beta(β)-barrel proteins [10]. β-barrel TM proteins are embedded into the membrane through multiple amphipathic β-sheets arranged in a cylindrical shape (Figure 2). These proteins are only found in the outer membrane of Gram-negative bacteria, mitochondria and chloroplasts [11]. α-helical proteins are a more diverse and a major class of TM proteins. These proteins consists of single or multiple α- helices spanning the membrane (Figure 2). They function as receptors, transporters, enzymes and cell adhesion molecules.

This diverse family of membrane proteins based on the presence of TM domains, is estimated to constitute between 15% to 39% of the human proteome [12]. A recent study has estimated that 27% of all human proteins to be alpha-helical membrane proteins [13]. The human membrane transporters mainly include ion channels, ATP binding cassette (ABC) transporters, pumps, water pumps and solute carriers. The receptors can be classified into metabotrophic receptors; G protein-coupled receptors (GPCR), ionotropic receptors, receptor tyrosine kinase etc. Among these GPCR is the largest with over 800 members in humans [14]. The focus of this thesis is on one class of membrane transporters called solute carriers and another class of membrane receptors known as GPCRs.
Figure 2. Schematic representation of different structural classes of transmembrane proteins a and b represent alpha helices and c represents beta barrels.

Solute carriers

Solute carrier (SLC) superfamily is the largest family of membrane transporters, which comprises of coupled transporters, exchangers driven by cellular sodium gradient and passive transporters. Their cellular localization is not limited to the cell membrane, but are also found in mitochondria [15], synaptic vesicles [16] and peroxiosomes [17] (Figure 3). Transported substrates include most endogenous substrates, drugs, metals and many environmental toxicants. This wide substrate specificity of SLCs infers diverse and important physiological functionality to these proteins. The availability of completely sequenced and annotated genomes, have led to the identification of several novel SLC’s which encompasses 384 genes [18]. The solute carrier were functionally divided into forty three subfamilies [19]; since then more families have been added including five new subfamilies, SLC44 - SLC48, according to the Hugo Gene Nomenclature Committee (HGNC). In addition to these families there are several proteins that are most closely related to the SLC’s based on sequence similarity measures and are called atypical SLCs [18].

Phylogeny of Solute carriers

Phylogenetic analysis allows proteins to be interrelated in an evolutionary perspective and thus displays their degree of relatedness. Such a comparative analysis also provides insight into the structure – function relationships [20]. Availability of sequence information from a large number of species provides a unique opportunity for phylogenetic analysis studies on large gene families like the SLCs. Such an analysis on entire human solute carriers explained the clustering of 15 SLCs subfamilies into four main groups, namely α-, β-, γ-, and δ-groups [18]. The α-group is the largest containing 7 families of SLCs (SLC2, 16, 17, 18, 22, 37 and 46) together with a cluster of proteins
known as synaptic vesicle 2 (SV2) proteins. The β-group includes three amino acid transporter families (SLC32, 36 and 38) whereas the γ-group (SLC7 and 12) and δ-groups (SLC8 and 24) contains two families each [18]. In this thesis, we study transporters belonging to SLC17 and SLC38 family.

**Figure 3.** A view of the both SLC and non-SLC membrane transporters. The upper panel shows that SLCs includes a variety of transporters such as coupled transporters, exchangers and passive transporters expressed in different intercellular compartment apart from the plasma membrane. This figure has been adapted from [19].

**SLC17 family**

The SLC17 family was initially reported to be Na+ dependent phosphate transporters. However, recent studies determined that all the members of this family are involved in the transport of organic ions [21]. The SLC17 family contains nine genes that can be functionally divided into four groups (i) type I phosphate transporters, SLC17A1-4, (ii) vesicular excitatory amino acid transporter, SLC17A5 (previously known as sialin) (iii) vesicular glutamate
transporters (VGLUT), SLC17A6-17A8, and (iv) vesicular nucleotide transporter (VNUT), SLC17A9 [22].

The first group consists of 4 type I phosphate transporters which are known to co transport both sodium and phosphate [23] with the capacity to transport organic anions [24]. The ionic coupling properties and endogenous substrates for these transporters have not been identified. The second group includes only SLC17A5, which has now been identified as vesicular excitatory amino acid transporter (VEAT) [25]. This is one of the extensively studied member of this family, mainly because SLC17A5 has been implicated in Salla disease and infantile sialic acid storage disorder in humans [26]. Recently it has gained more importance as it is a vesicular transporter and might play a major role in neuronal signaling. SLC17A5 has a ubiquitous expression [26]; in the brain it is expressed mainly in the cerebral cortex, striatum and hippocampus [27, 28].

The next group contains 3 vesicular glutamate transporters, which are involved in loading glutamate into synaptic vesicles in glutamatergic cells. SLC17A6 (VGLUT2) and SLC17A7 (VGLUT1) has complementary expression pattern in the CNS with limited overlaps [29, 30]. SLC17A8 (VGLUT3) is relatively less abundant in the CNS and are found to co-localize in non-glutamatergic cells [31, 32]. SLC17A7 and 17A6 has peripheral expression limited to the pancreatic islets whereas SLC17A8 is also expressed in the kidney and liver [31].

The last group includes a lone member SLC17A9, which has now been identified as a vesicular nucleotide transporter [22]. This revelation will provide further insights into purinergic neurotransmission. Northern blot analysis suggested its expression in various tissues with high expression in the brain, adrenals and the thyroid gland. A detailed study of the expression of this gene in different regions of the brain is lacking which might provide an insight about their involvement in different signaling pathway. In paper II we do an overall comparison of the tissue expression of all the genes in the SLC17 family and focus on the SLC17A9 expression in the mouse brain.

SLC38 family
The SLC38 family includes 11 amino acid transporters which contains 5 recently identified orphans SLC38A7-11 [33]. The known members of this family are SLC38A1-6 that has been functionally characterized as sodium-coupled neutral amino acid transporters, also known as SNATs. The known SNATs are further classified according to the N/A system of classification described in the following paragraph.
System A was named so because of its preference for alanine however they have an ability to transport a broad range of substrates [34]. These transporters are sodium dependent, low affinity, sensitive to lower pH, intolerant to substitution of Na+ by Li+ and exhibits unidirectional transport. The distinguishing feature of System A is its ability to transport N-methylated substrates such as the nonmetabolizable amino acid analogue α-(methylamino) isobutyric acid (MeAIB) [34-37]. System N also mediates a sodium dependent uptake of neutral amino acids with sensitivity at lower pH and exhibits tolerance to Li+. However they exhibit a bidirectional transport [38], limited substrate profile with preference to glutamine, asparagine and histidine, inability to transport MeAIB and predominant expression in liver and brain [39]. The SNATs that belong to system A are SNAT1[34] and SNAT2 [37] and system N includes SNAT3 [40] and SNAT5 [41]. The literature on SNAT4 (SLC38A4, ATA3, NAT3) is conflicting. Sugawara et.al explained that rat SLC38A4 is a system A transporter [42] whereas Gu et.al described human SLC38A4 to be system N transporter [43]. It’s unclear if the different classification is because of a species specific difference.

All the known SNATS with the exception of SNAT4 are predominantly expressed in the brain and have amino acid glutamine as a preferred substrate [35]. These features of the SNATS have prompted speculation that they are plausibly involved in the “glutamate/GABA-glutamine cycle” in the brain. The glutamate/GABA-glutamine cycle can be described as a metabolite shuttle that involves the release of neurotransmitters glutamate/GABA from the neurons; subsequent uptake into the astrocytes and in the astrocytes glutamate/GABA is converted into glutamine, which is transported back into the neuron as a neurotransmitter precursor. The existence of such a cycle is due to the inability of the neurons to perform de novo synthesis of glutamate/GABA [44]. The suggestion is that the system A transporters SNAT1 and SNAT2 that are predominantly expressed in the neurons, are involved in the uptake of glutamine into the neurons and the system N transporters, SNAT3 and SNAT5 expressed in the astrocytes facilitate the efflux of glutamine from astrocytes [35, 44].

The other members of SLC38 family including SLC38A6 and the recently identified orphans SLC38A7-38A10 have not been assigned to the N/A system of classification and lack a detailed expression mapping in the brain. A detailed characterization of these members will further our understanding of their plausible role in the glutamate/GABA-glutamine cycle. In paper III, we have functionally characterized one of the previously orphans SLC38A7 transporters.
G protein-coupled receptors

The cell surface or membrane receptor includes three main classes of receptors namely G protein-coupled receptors (GPCRs), enzyme-linked receptors and ion channel linked receptors (Figure 4). GPCRs are the largest family of integral membrane protein in humans accounting for about 27% of the proteome [13]. These membrane receptors can bind and respond to a highly diverse array of ligands that vary not only in size but also in physical and chemical properties. This diversity of ligands stimulate the activation and regulation of various physiological functions which includes sensory functions carried out by olfactory GPCRs and functions like energy homeostasis, endocrine secretions and neuronal modulation controlled by the non olfactory GPCRs. Mutation and genetic variations such as single nucleotide polymorphism (SNP) in the GPCRs has been linked to a multitude of diseases and disorders [45-47] thereby making them important pharmacological targets. Although, approximately 50% of all modern drugs are directed towards the GPCRs; it still correspond to only a small fraction of the non olfactory GPCRs [48]. Huge efforts are currently being employed to further exploit the potential of the remaining characterized and orphan GPCRs in drug discovery and development.

The basic structural architecture of all GPCRs is similar with seven membrane spanning domain, an extracellular amino terminus and an intercellular carboxy terminus. Yet a striking feature is that there is no sequence homology between the GPCR families. The earliest structural view on GPCRs were based on weak analogy to the bacteriorhodopsin [49, 50] until the first crystal structure of bovine rhodopsin [51] was solved. This structure clearly resolved our views about the transmembrane domains, the intracellular and extracellular loops, and gave a detailed view of the ligand binding, providing the basis for further GPCR modeling. The structural knowledge was further strengthened recently by resolving the crystal structures of the β2-adrenergic, β1-adrenergic, and adenosine α2A receptor [52-54]. These structure details will help us in understanding of the underlying mechanisms in ligand binding, downstream signaling and functions of GPCRs.
Figure 4. Schematic representation of different cell surface (membrane) receptors which includes the three main classes: Enzyme-linked receptor, Ion channel linked receptor and G protein coupled-receptor.

The downstream signaling for a vast majority of GPCRs is transduced via coupling with the heterotrimeric G-proteins. The three subunits of the heterotrimeric protein are $\alpha$, $\beta$ and $\gamma$. The $\beta\gamma$-subunits are tightly linked and work as a single functional unit. The activation and inactivation of all G-proteins are carried out in the same fashion. The inactive heterotrimer of G-protein is formed by the association of GDP bound $\alpha$ subunit to the $\beta\gamma$-subunits. The binding of the agonists to the receptor changes its conformation and increases the binding of G-protein to its receptor. This in turn results in the replacement of GDP to GTP in the $\alpha$-subunit, reducing the affinity of the $\alpha$-subunit to the $\beta\gamma$-subunit leading to its functional dissociation [55]. This dissociated subunit can activate several downstream signaling cascades such as cAMP pathway or phosphotidylinositol pathway. However, it is important to note here that G-protein independent signaling via interaction of intercellular motifs present in the intercellular loops of GPCRs with the scaffolding protein also exists.

Functional studies on GPCRs

In the GPCR research, several approaches have been taken to assess its function. Initially studies were based on ligand binding studies, pharmacological activation and inactivation of receptors by agonists and antagonist. Phylogenetic analyses have been performed to provide us with insights not only about the origin of GPCRs but also on structure-function relations on ligand binding and signaling. Structural studies so far have provided insights into the molecular mechanisms involved in the regulation of GPCRs and assisted in the modeling of ligand binding pockets for orphan GPCRs. Studies on expression levels and distribution pattern of the receptors have provided
clues on the possible physiological roles. Another traditional approach to study the GPCR function in the CNS is by blocking the receptor function using antisense oligodeoxynucleotides. Advances in gene targeting techniques have lead to development of transgenic mice lacking one or more receptor protein and study of its effect. However in this thesis (paper IV and V), the approaches that we have taken are phylogenetic analyses, expression studies, inhibition of GPCR by antisense oligodeoxynucleotides followed by behavioral studies.

**GPCR evolution and classification**

GPCRs are an evolutionarily old and successful gene family which is reflected by its abundance in most eukaryotic species. Phylogenetic analyses revealed that the 7TM core is a highly conserved structural feature and is a hallmark for its identification in genomic sequences. Despite this strong evolutionary conservation, there is no overlap in their gene structure that allows for the identification of common ancestral 7TM domain in eukaryotic evolution. On the other hand, the extracellular and intracellular domains of the receptor vary greatly in length and sequence which facilitates the interaction with an array of extracellular ligands and intracellular signaling molecules. A closer look at the GPCR gene repertoires in various genomes revealed a strong correlation between the GPCR families and the complexity of the organism. The vertebrate genome shows a strong expansion in the number and diversity of the receptor compared to the invertebrate genome. The expansion of the genes is a result of various genetic mechanisms. The most prevalent mechanism in GPCR expansion is suggested to be gene duplication followed by an independent evolution of the gene copies.

The importance of the GPCRs is further exemplified by the numerous classification schemes proposed. These classification schemes were based on the structural and physiological properties of GPCRs. The first attempt to classify GPCRs was based on primary sequence similarity [56]. The other attempts to classify GPCRs was based on structural and ligand binding properties [57-59]. The complete sequencing of the human genome [60] provided a comprehensive view of the human GPCR repertoire which led to a more detailed phylogenetic classification called GRAFS system [61]. Based on this classification the human GPCRs can be divided into five families namely Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled/Taste2 (F), and Secretin (S). The GRAFS system of classification has taken advantage of the relatively conserved 7TM regions and additionally relied on the well defined functional domains present in the N-and C- termini. These five structurally and phylogenetically distinct families have evolved since early metazoan evolution. The papers included in this thesis describes two orphan Rhodopsin
GPCRs, hence a detailed description of the *Rhodopsin* GPCR is provided while only a brief overview of the other GPCR families is presented.

The *Glutamate* family is a phylogenetically old GPCR family which predates metazoan and fungal evolution [62, 63]. However, glutamate activation of these receptors may have arisen only in early metazoans [64]. The human genome includes 22 members of this family and they exhibit relatively specific ligand binding. Majority of receptor are specific to glutamate, while the other receptors bind to GABA, amino acids, cations, small organic molecules or carbohydrates. Distinctive feature of this group is the presence of ligand binding domain in the N-terminal called Venus fly trap module (VFTM) and a cysteine rich domain also in the N-terminus.

The *Adhesion* family is the second largest GPCR family in humans with 33 members [61]. They are characterized by the presence of long and diverse N-termini [65] which are highly glycosylated and has functional domain which have adhesive properties [66]. Another striking feature is the presence of a GPCR proteolytic cleavage site (GPS) in the extracellular region of the receptor [67, 68]. This *Adhesion* GPCRs are present in metazoans and sequence distantly related to them are found in plants and fungal genome, hence this family has evolved before the metazoan evolution [69]. The ligands for this family of receptors are extracellular matrix molecules. This is the only family of GPCRs that shows resemblance to other membrane proteins such as tyrosine kinase receptors, cadherins or integrins.

The *Secretin* family is a small group of receptor with 15 human members [61]. This family was earlier grouped with *Adhesion* and *Methuselah* family to form a larger group of class B receptors. *Secretin* family is suggested to descend from the *Adhesion* family prior to the split of protostome-deuterostome. The members of this family have an extracellular hormone binding domain (HBD) which confirms its specificity to peptide hormones.

The *Frizzled/Taste2* family includes two classes of receptor namely frizzled and taste2 receptors. The first frizzled receptor was identified in drosophila [70] and till date ten human homologues have been identified. The frizzled receptors (FZDs) bind to a family of Wnt glycoprotein through the wnt binding domain. SMO (smoothened receptor) also belongs to this family as they are structurally similar to the FZDs [71]. The human genome includes 25 functional taste 2 receptor (T2R) genes. The members of this family have only been identified in the vertebrate lineage [72]. T2R, a 7TM membrane protein was discovered to induce G-protein mediated signaling in 2000 [73] and hence classified among the GPCRs. T2R has no known functional binding domain in the N-terminus. The ligands for the T2Rs are small organic compounds.
**Rhodopsin family**

The *Rhodopsins* are the largest class of GPCRs with 672 human receptor proteins and 388 of these receptors are olfactory receptors [74]. *Rhodopsins* have been the most thriving family in evolutionary terms which is substantiated by the abundance of these proteins in bilateral species [72, 74, 75]. These receptors are not only present in all bilateral species, but also in cnidarians implying its evolution before the protostome-dueterostome split [69]. Rhodopsins were further phylogenetically divided into four main groups (α, β, γ and δ) with 13 sub-branches [61].

These receptors are highly variable in their primary structure and ligand specificity. Unlike other GPCR families the ligand specificity is not restricted to the N-terminus having short stretch of amino acids but occurs in the extracellular portion of the TM domain. The TM domain however features conserved signature motifs such as the E/DRY in between the TM3 and ICL2 (intercellular loop 2) and N/DP(X)n Y on TM7, which is present in almost all members of this family [51]. The diverse set of ligands modulating *Rhodopsins* are peptides, lipids, ions, neurotransmitters, nucleotides, pheromones, tastes and odors. Surprisingly, there is no complete correlation between the phylogenetic group the receptor belongs and their endogenous ligands. However some phylogenetic clusters in the sub-branches bind similar type of ligands [61].

The α-group is the largest among the *Rhodopsin* family including 101 human receptors. This group includes five main clusters: 1) prostaglandin receptors, 2) amine receptors, 3) opsin receptors, 4) melatonin receptors and 5) MECA receptors (includes melanocortin, endothelial differentiation GPCRs, cannabinoid and adenosine binding receptor) [61]. The two GPCRs Gpr162 and Gpr153 studied in this thesis belong to the amine receptor cluster of the α-group of *Rhodopsins*. The α-group is the only group among the *Rhodopsins* that has specificity to biogenic amines conferring this group to be major drug targets [76].

**Functions of Rhodopsins in the CNS**

Many of the *Rhodopsins* are highly expressed in the CNS and several studies have linked these receptors in regulation of behaviors ranging from food intake to motor control, learning, memory and reward pathways. Most of the functional data obtained are from animal knockout studies. The amine receptor cluster mainly includes serotonin receptors, dopamine receptors, muscarinic receptors and histamine receptors [61]. The members of all the four receptor family mentioned above have been implicated in basic physiological processes such as food intake and energy homeostasis [77]. Another be-
behavior that stood out was differences in locomotary behavior and anxiety levels. Hence, we decided to study the effect of Gpr153 and Gpr162 anti-sense knockdown rats on food intake and other basic behavioral test.
Aim

The overall aim of the thesis was to provide a basic understanding of some of the novel transmembrane proteins (SLCs and GPCRs) with respect to its phylogeny, expression profile, transport properties and functional and behavioral studies in animal models.

Paper I

- Classification of novel atypical SLCs based Pfam classification system
- Phylogenetic analysis of the SLCs to determine evolutionary conservation
- Basic overall expression profiling of all the 14 atypical SLCs

Paper II

- Phylogenetic analysis of the entire SLC17 family
- Expression profiling of the entire family
- Detailed expression study of SLC17A9, a novel vesicular nucleotide transporter

Paper III

- Detailed expression analysis of SLC38A7 in the mouse brain
- Investigate the cell type localization of SLC38A7 in the CNS
- Examine the sub cellular localization of SLC38A7
- Characterization of the substrate profile and uptake properties of SLC38A7
Paper IV

- Detailed expression analysis of Gpr162 in the mouse brain
- Study of feeding behavior in antisense oligodeoxynucleotide knock-down rats
- Investigation of basic behavior pattern of Gpr162 knockdown rats in open field tests and plus maze
- Determination of possible links to energy homeostasis in human genetic variants of Gpr162

Paper V

- Phylogenetic characterization of the Gpr153 receptor
- Detailed expression analysis of Gpr153 in the mouse brain
- Study of feeding behavior in antisense oligodeoxynucleotides knockdown rats
- Investigation of basic behavior pattern of Gpr153 knockdown rats in open field tests and plus maze
Methodological review

Phylogenetic analysis

By definition, phylogenetic analysis refers to the study of inherited molecular difference, analyzed by the comparison of either DNA or protein sequences that provide information on an organism’s evolutionary relationship that cannot be inferred by morphology. Furthermore phylogeny also involves the evolutionary relationship between genes and gene families both within an organism (orthologous) or between multiple organisms’ (paralogous). This is crucial for early prediction of the putative role of a newly discovered gene. In this thesis we have attempted to establish possible phylogenetic relation between novel genes and in some cases the entire gene family. The fundamental requirement for such an analysis is availability of reliable genomic sequence data from different organisms. Today, this is not a problem as a number of whole genome sequences from various species are publically available in databases. We have mined our sequences mainly from two widely popular and accepted databases: NCBI (http://www.ncbi.nlm.nih.gov) and Ensembl (http://www.ensembl.org/index.html)

Phylogenetic analysis can be performed either by comparing the degree of similarity between the sequences (conservation) or by studying their differences (changes or mutations in the sequences introduced by deletion, insertion or rearrangements). In our studies we have focused on the degree of conservation between sequences as the key and a good multiple alignment of the sequences is a critical step in such phylogenetic analysis. In our studies we have used two different multiple alignment software packages called T-coffee [78] in paper I and II and MAFFT [79] in paper V. The alignments were manually examined and incomplete sequences were removed. Phylogenetic trees in Paper I and II were constructed using maximum parsimony method using the Phylip 3.6 package and plotted using Tree view. The bootstrap consensus tree assessing the robustness of each node is made with 1000 replicates. In paper V the phylogenetic tree was constructed using MrBayes 3.1.2 and the tree was drawn in Figtree 1.3.1
Gene expression studies

Quantitative real time PCR (qPCR)

qPCR is the most powerful tool for quantitative analysis of nucleic acids with its simultaneous amplification and detection. More precisely, it’s a kinetic approach in which the PCR reaction is monitored in the early stages when it’s still linear. This technique is a modification of the original PCR developed by Kary Mullis in the early 1980s and he was honored with the Nobel Prize in chemistry (1993) for this achievement. One of the major limitations of PCR was the inability to quantify the initial amount of DNA template present. This limitation was overcome in 1992, by the development of qPCR by Higuchi [80, 81]. In real time PCR the amount of the product formed is monitored constantly during the course of the reaction by monitoring the fluorescence of the dyes or probes (we use SYBR green) used in the reaction that is proportional to the amount of product formed. Together with the number of amplification cycles to obtain detectable amount of product (corresponds to the threshold fluorescence level-Ct value) and assumption of certain amplification efficiency will aid to calculate the number of template DNA molecule that was initially present in the sample.

In this work qPCR was used in mRNA quantification of our gene of interest in various tissues isolated from mouse and rat. cDNA (complimentary DNA) is synthesized from the mRNA extracted from the isolated tissues. Random hexameric primers are used during cDNA synthesis to ensure amplification of all the RNA in the sample. This cDNA is then used as template along with gene specific primers for qPCR reaction. An important point here is to compensate for variation in the total cDNA in different samples due to discrepancies during isolation, sample handling, RNA extraction, and cDNA synthesis (efficiency of reverse transcriptase); by using an internal standard often called reference gene. An ideal reference gene in a given tissue should have the same copy number in all cells (preferably not too high or too low), be expressed in all cells and should not undergo significant changes due to experimental variables. The most commonly used reference genes are housekeeping genes (HKG), which codes for proteins required for the basic maintenance of all kind of cells. However, extensive studies in this field have confirmed that there is no universal reference gene with a constant expression in all tissues. Hence in our studies we use more than two HKG to determine the normalization factor, which is calculated by software called GeNorm [82].

This method has been used in papers I, II, IV and V. A detailed explanation of the reference genes used, primer sequences, qPCR reaction condition and data analysis is mentioned in the respective papers.
In situ hybridization (ISH)

The word *in situ* is derived from the Latin meaning ‘in position’ and hybridization comes also from the Latin word *hybrida* meaning ‘offspring’s of parents from unlike races or culture’. This technique was first described simultaneously in 1969 by John et al and Gall & Pardue. The technique of *in situ* hybridization allows the detection of specific nucleic acid sequences (genes/mRNA) in their own cellular environment. The principle behind this technique is the specific binding of labeled nucleic acid probe to its complimentary sequence in fixed tissues followed by the visualization of the position of the hybridized labeled probe [83, 84]. Initially, probes were radioactively labeled whereas now researchers around the world prefer to use non-radioactively labeled probes [85]. In our studies we have only used non-radioactively labeled probes such as digoxigenin (non-fluorescent) and FITC-fluorescein isothiocyanate (fluorescent). The labeled probe is then recognized by an antibody coupled to an enzyme and the addition of a substrate for this enzyme helps in the visualization of the entire complex (mRNA-probe-antibody-enzyme complex).

The success of ISH depends on optimal fixation of the cell/tissue for retention of target nucleotide sequences. The fixative that we have used in all our studies is buffered 4% paraformaldehyde which meets the three basic requirements, namely; preserves the integrity of the tissue, good retention of mRNA within the tissue and provides efficient access of the probe to the RNA [85]. The embedding of the tissue depends on the thickness of the section required for the study. We use agarose embedded tissue for thicker sections (60-70µm) whereas paraffin sections for thinner sections (7-8 µm). In our studies we have mainly used brain tissues from perfused mice to investigate the specific localization of our genes of interest in various regions of the brain.

Immunohistochemistry

Immunohistochemistry by definition is a ‘technique for identifying cellular and tissue constituents (antigens) by means of antigen-antibody interaction, this site of antibody binding is then visualized by either direct or indirect (secondary) labeling of the antibody’. In 1941, Coons et al were the first to develop a fluorescent labeled antibody technique to identify pneumococcus [86]. Since then there has been a significant advancements in this technique, enzyme labels have been introduced such as peroxidase [87] and alkaline phosphatase [88, 89] which are routinely used in our *in situ* hybridization studies. An immense progress in microscopy has reinforced the use of fluorescent labeled antibodies which we use in all our studies. In this thesis, we have used this technique to determine the localization and co-localization of
our proteins. In paper III, we have performed double IHC, with custom made polyclonal antibody for SLC38A7 and used cell type specific markers and synaptic markers to investigate the cell type specificity and co localization in the synaptic terminal respectively.

Functional Studies

Transport assay in *Xenopus leavis* oocytes

*Xenopus leavis* oocytes have been used for several years as a versatile system for expression of heterologous mRNA *in vivo*. The unique feature of the oocytes is its synthetically active yet resilient physiology. The large size of the oocytes aids in handling, maintenance and performing experimental procedures. Another important advantage of using the oocytes for studying transport proteins is emphasized by its ability to express the foreign transport protein in the oocytes plasma membrane. Oocytes have been used to functionally characterize mammalian transporters by uptake studies using radiolabelled substrate or electrophysiological recordings of substrate evoked current. In our studies, we have done uptake assay using radiolabelled substrates. The details of the assay are mentioned in paper III.

Antisense oligodeoxynucleotide technology

Antisense oligodeoxynucleotides as the name indicates are complimentary (antisense) sequences to the target (sense) mRNA sequence. Within the cells, these antisense oligos bind to the target mRNA strand and prevent their translation thereby resulting in the reduced expression of the corresponding protein. This inhibition was first observed in 1978 in Rous sarcoma viral RNA which was inhibited by a tridecamer oligodeoxynucleotide [90]. The principle behind the prevention of translation is that the binding of the antisense oligo to the target mRNA sequence form a RNA/DNA hybrid. This hybrid is then recognized by and endonucleases, RNase H and results in the cleavage of the mRNA sequence of the complex. Several modifications have been introduced in the 3’ and 5’ end of the antisense oligos to protect them from degradation by exonucleases and also increase the efficiency of cleavage by endonucleases. The antisense oligodeoxynucleotide analogs that have been used are 2’-deoxyoligonucleotide methyl phosphonate [91], 2’ deoxyoligonucleotide phosphorothioate , 2’-Deoxy- and ribo oligodeoxynucleotide analogs with either phosphate modifications or nonphosphate linkages, 2’-0-alkyl analogs, α-anomers, and many more. The most widely used antisense oligo analog is 2’ deoxyoligonucleotide phosphorothioate [92] that was used in our studies.
This technology was initially used in cell culture were the antisense oligos were microinjected, transfected or electroporated into the cells. However, various studies have suggested that antisense oligos can be directly administered in vivo in animal models resulting in control of gene expression. Several animal models have been successfully studied including rats, pigs, mice, rabbit and chick embryo [92]. The different modes of administration are intraluminal delivery by infusion /catheter, infusion by subcutaneously implanted osmotic pumps, intravenous injections etc [92]. In paper IV and V, the antisense oligodeoxynucotide is modified by phosphorothioate, the animal model used is rat and the mode of administration is via infusion by subcutaneously implanted osmotic pump. Further details are explained in the papers.

Feeding study
In Paper IV and V, we have performed feeding studies on knockdown rat models. The main idea of the feeding study is to monitor the total food intake and body weight of all the rats used in the experiment. In this thesis we measure the food intake of all rats used in the study before the surgery to establish a baseline. After surgery and infusion of the antisense oligos in the rats, the feeding study is continued for 7 days to see if the antisense oligodeoxynucleotide inhibition has any effect on food intake or body weight.

Behavioral studies
**Open field (OF)**
OF as first introduced in 1934 by Calvin Hall, who used defecation as an index of timidity/emotionality in rats. Till today, OF is one of the most commonly used behavior tool to measure anxiety related behavior in rodents. The popularity of this test is due to the simplicity of the apparatus, straightforward measurement of well defined behavior and a wide acceptance of the interpretation of the measured behavior. The size and shape of the apparatus are varied, from a circular to a square or rectangular shaped area. This test is normally conducted in well illuminated place and the naive animal is placed in centre of the field. Rodents tend to avoid the central unprotected area and move around near the walls. Measurement of central exploration is regarded as an index of anxiety/risk associated behavior [93-95]. A description of our experiment is provided in paper IV and V.

**Elevated plus maze (EPM)**
EPM is another widely used validated behavioral assay to test for anxiety. EPM consists of a plus shaped maze, elevated from the floor with two open arms and two enclosed arms opposite each other interconnected by a central
platform. The open arm activity that corresponds to the time spent in the unprotected area and percentage of entries in the open arm in relation to the total number of arm entries reflects the measure of anxiety. General locomotion is measured by the number of entries into the closed arm [94-98]. Another study suggested that the time spent in the centre could be a measure of decision making [99]. A description of both our behavioral studies is provided in paper IV and V.
Results and Discussion

Paper I

The main idea of this paper was to address the basic features of the 14 atypical SLCs that we identified in our previous study [18]. Firstly, we looked at a broader classification for these atypical SLCs by using the Pfam classification system. Based on the HMM alignment of the sequences to the Pfam database, all of these SLCs could be assigned into specific Pfam clans. Among the 14 atypical SLCs, 9 of them were distant members to the Major Facilitator Superfamily (MFS) clan. The remaining 5 transporters were classified into the APC clan (2 sequences), the DMT clan (1 sequence), the CPA_AT clan (1 sequence) and IT clan (1 sequence).

Secondly, the focus was on the phylogenetic analysis of these genes to see if they were evolutionarily conserved. These genes were found to be highly evolutionarily conserved and likely to be present in most bilateral species, except for SLC21A21 that was found only in mammals. However, it is important to note here that few of these genes lack orthologs in some of the species that was investigated. We speculate that the reason for the lack of orthologs is because it is either not essential for animal function and that their functions can be taken over by other proteins, most likely other solute carriers which in many cases are promiscuous towards their substrates and many substrates have multiple transporters.

Finally, we investigated the general expression profile of these genes. All these genes displayed a differential expression pattern in the CNS except for SLC15A5 and SLC21A21. It was notable that many of the MFS transporters such as MFSD1, MFSD9 and MFSD10 displayed relatively low expression in all the tissues tested suggesting that these transporters are either highly expressed in tissue that were not included in the panel or that they are expressed during early developmental stages. In conclusion this paper provides fundamental information such as classification, evolution and expression profile of the newly identified atypical SLCs, which might provide lead information on the functionality of these genes. However, further studies in detail about each of these genes are mandatory to reveal their functions.
**Paper II**

The objective of this paper was a global phylogenetic analysis and expression profiling of the entire SLC17 family. The additional focus of the paper was to study the expression pattern of SLC17A9 gene, a recently identified vesicular nucleotide transporter, in the mouse brain.

The sequences of the entire SLC17 family was thoroughly mined from eight different species and used for the phylogenetic analysis. The analysis revealed that the SLC17 family forms four main clades. We noticed that one of these is found only in *C. elegans* and suggest that this family be called SLC17A10 family (clade II). It was important to note that SLC17A9, known as a vesicular nucleotide transporter [8], forms a separate clade (clade I) and places most closely to the SLC17A10 clade. Clade III contains the human SLC17A6, A7 and A8, the vesicular glutamate transporters. The phylogenetic analysis shows that the three vesicular glutamate transporters found in humans originate from a common ancestor before the split of gnathostomes. The largest clade contains five human SLC17 sequences (clade IV), SLC17A1-5, where SLC17A5 is clearly the most basal member.

The quantitative PCR analysis on all the nine members of the SLC17 family on the rat tissues panel confirmed that expression of Slc17a1-4 only in the peripheral tissues but not in the CNS, while Slc17a5-9 is highly expressed in both the CNS and periphery. The detailed study of Slc17a9 in the mouse brain by *in situ* hybridization detected a wide spread expression of this gene in the brain. High expression was observed throughout the cerebral cortex, certain areas in the hippocampus and in specific nuclei of the hypothalamus and thalamus. Some of the regions with high expression, such as the medial habenula and the dentate gyrus of the hippocampus, are important sites for purinergic neurotransmission. However, other areas relying on purine mediated signaling, such as the molecular layer of the dentate gyrus and the periaqueductal gray, lack or have a very low expression of Slc17a9, suggesting that there could be another nucleotide transporter in these regions.

**Paper III**

The focus of this study was to functionally characterize the SLC38A7 with regards to its expression, localization and substrate profile. A detailed *in situ* hybridization on the mouse brain detected abundantly high expression of Slc38a7 mRNA in the brain. A strong expression of Slc38a7 was detected in ventromedial hypothalamus (VMH), basolateral amygdala (BLA), ventral tegmental area (VTA) and locus coeruleus (LC), areas known to be involved in many different physiological functions, including feeding [100, 101]. In
the cortex, expression of Slc38a7 appears in layers, and in the hippocampus. The gene was expressed in granular layer of dentate gyrus cells (GrDG) and pyramidal cell layer of hippocampus (Py); areas that predominantly contain neurons. Slc38a7 is also expressed in the layer of Purkinje cells of cerebellum, known to be chiefly GABAergic [102].

The next step was to investigate the localization of Slc38a7 to a particular cell type in the brain. Double immunohischemistry and combined in situ /immunohistochemistry was performed with either the SLC38A7 antibody or the Slc38a7 probe with the neuronal marker NeuN or glial marker GFAP. The results from this experiments confirmed that SLC38A7 is localized in the neuron and are absent in the astrocytes. Further, it was confirmed that SLC38A7 is present in both glutamatergic and GABAergic neurons.

Finally, SLC38A7 protein was over expressed in Xenopus laevis oocytes, to characterize the substrate profile and the substrate uptake properties. An initial screen with [3H]-labeled L-aminoacids revealed that uptake for SLC38A7 were specific for L-glutamine, L-arginine and L-alanine. L-glutamine was used as the substrate to study the time course and sodium dependency. We found that L-glutamine uptake by SLC38A7 increases with time. In presence of sodium the uptake of L-glutamine was significantly higher than that of control oocytes. However, replacement of sodium with lithium completely ceased the L-glutamine uptake whereas choline was able, to a lower extent (23% of sodium driven transport), drive transport of L-glutamine. Inhibition studies suggested that the substrate profile of SLC38A7 obtained by inhibiting the [3H]-L-glutamine uptake was ranked: L-glutamine > L-histidine > L-serine > L-alanine > L-aspargine > L-aspartic acid > L-glutamic acid > L-methionine > L-leucine > L-glycine. We can conclude that SLC38A7 transports polar amino acids and alanine. Based on our findings, we speculate that the SLC38A7 transporter plays a role in the glutamine-glutamate cycle. Further, the characterization of novel amino acid transporters, such as SLC38A7, may provide insight in key events in neurobiology, including neuronal metabolism and neurotransmitter cycling.

Paper IV

In this paper various approaches are taken to decipher the function of the orphan Gpr162 that was mined in one of our previous studies [103]. Initially we observed the overall expression profile of Gpr162 and found that this gene was predominantly expressed in the CNS and almost negligible in the peripheral tissue tested. A careful examination of the mouse brain by in situ hybridization confirmed dense expression in several brain regions involved in feeding and stress circuitry such as hypothalamus, amygdala, substantia
nigra and ventral tegmental area (VTA), among other regions. It was interesting to observe that Gpr162 was expressed exclusively in the gray matter and a complete lack of expression in the white matter suggesting the absence of this receptor in the oligodendrocytes.

The feeding behavior was further analyzed in antisense oligodeoxynucleotide induced knockdown rats which showed a significant reduction in the food intake when compared with the control group. The effect towards reduction in bodyweight was not as pronounced, however there was a strong trend towards reduction. Probably, a longer term down regulation of the Gpr162 receptor would have positively affected the reduction in body weight. Our basic behavioral data suggested that the Gpr162 knockdown rats were more active and demonstrated increased rearing, which is a measure of exploratory behavior as compared to the control. We have also done human genetic studies which imply that genetic variants of the Gpr162 have an effect on glucose homeostasis. In summary, we have found that the orphan Gpr162 is linked to the regulation of food intake related behavior.

**Paper V**

We used a similar approach as in paper IV to study the other orphan Rhodopsin Gpr153 that we also mined in the same study as mentioned earlier [103]. In addition, we also performed a phylogenetic analysis of the two closely related genes Gpr162 and Gpr153 and in the process identified an ortholog for Gpr153 in the elephant shark genome. The evolutionary study indicated that both Gpr153 and Gpr162 originated from a common ancestor before the split of the amphibians, somewhere between the teleost and amphibians. These results were complimented with the synteny analysis which confirmed our previous result. Our results also suggest that Gpr162 evolved much more rapidly than the mammalian Gpr153 which is indicated by their longer branch length and also by accumulation of higher number of residue substitutions. Consequently, mammalian Gpr153 is more probable to have retained the original function whereas Gpr162 might have been subjected to neo or sub-fictionalization after the gene duplication event.

The overall expression profile was distinct from that of Gpr162; Gpr153 was ubiquitously expressed in all tissues tested with a slight predominance in the CNS. This predominance in CNS expression was further confirmed by performing *in situ* hybridization on the mouse brain sections. Here, we observed dense expression in the thalamus, striatum and cerebral cortex. In the hypothalamus the expression was restricted to the arcuate nucleus and the paraventricular hypothalamic nucleus. The similarity between Gpr153 and
Gpr162 was represented by their exclusive expression in the gray matter and complete absence in the white matter.

One of the reason feeding behavior was analyzed is the relatedness to the serotonergic and adrenergic receptors that are known to be involved in energy homeostasis. The feeding was analyzed in antisense oligodeoxynucleotide induced knockdown rats, which suggested that there was only a slight trend in reduction of food intake and no effect on body weight. Our behavioral studies on plus maze and open field did not specify anxiety like behavior. However, we observe a significant reduction in the percentage of time spent in the central square which is a probable measure of decision making. This observation is supported by our in situ hybridization data which shows high expression in regions related to decision making such as cingulated cortex, prefrontal cortex and striatum.
Work in progress

The purpose of this part is only to give perspective of other related work, outside of this thesis, that has been worked on simultaneously with the studies presented here. The details of the methods and specificity of the gene is therefore not specified.

Generation and functional analysis of a novel transporter SLCZ1 mutant mice

The present study involves the characterization of a novel transporter, preliminarily named SLCZ1. SLCZ1 is an evolutionary highly conserved transporter and has a single copy in the genomes from mouse, rat, chicken, zebrafish, roundworm and fruitfly. Our expression analysis reveals that SLCZ1 is highly expressed in the central nervous system, with predominance in the hypothalamus. However, they are also expressed in some of the peripheral tissues especially in the lungs. Based on these results, we hypothesize that SLCZ1 might be involved in fundamental physiological functions; however the predominant expression in the CNS may also suggest its plausible involvement in neuronal circuitry. To investigate these hypotheses, we have generated SLCZ1 conditional mutant mice.

Generation of SLCZ1 mutant mice

We generated the Slcz1 deficient allele by replacing part of the Slcz1 gene with a targeting construct by homologous recombination in ES cells (Figure 5). Successfully targeted ES cells produced a recombinant allele with two lox p sites floxing exon 3, 4 and 5 on either side followed by neomycin cassette enclosed within the frt sites which were screened using southern and PCR strategy. Two positive clones of ES cells were selected for injection into the blastocyst and further transferred into foster mother to generate chimeric mice. These were bred with C57BL/6 mice to generate heterozygous mice carrying one floxed allele (Slcz1f/+), which were then intercrossed to produce homozygous mice (Slcz1f/f). The neo cassette was removed by crossing Slcz1f/+ mice to Deleter-FlpE mice [104] and the flipped Slcz1f/f were viable and fertile. These flipped mice were crossed to PGK-Cre mice [105]
to delete the targeted region and generate null mutant mice (Slcz1fc/fc). We performed western blot on homogenate from brain tissue from both control (wild type) and knockout (Slcz1fc/fc) to detect SLCZ1 protein. We could detect the SLCZ1 protein in the wild type homogenate but the band was completely absent in the knockout homogenate (Figure 5). This suggests that deletion of the targeted region results in the complete absence of SLCZ1 protein product in null mutant mice.

Figure 5. Gene targeting of the SLCZ1 locus results in specific loss of SLCZ1 protein expression. A) The gene targeting strategy shows exons 3, 4 and 5 in the SLCZ1 locus flanked by 5’ and 3’ loxP site followed by neo cassette flanked by frt sites resulting in recombinant allele (f). The location of the PCR primers used in the screening is labeled as P1, P2 and P3. The flipped allele is produced by crossing the heterozygous floxed mice with the deleter; Del-FlpE mice. The flipped mice are further crossed with PGK-cre mice to generate SLCZ1 null mutant mice. B) The PCR screen of the flipped and the null mutant mice with wild type mice and heterozygous mice using the primers illustrated in A. C) Western blot to detect the SLCZ1 protein in wild type and knock brain homogenate. The 48KD band in the wild type corresponds to the SLCZ1 protein and β-actin was used as loading control. D) A schematic view of the SLCZ1 transporter with 12 transmembrane domains. The targeted region corresponds to the transmembrane domain 2, 3 and 4, and loops 2, 3 and 4.
Analysis of SLCZ1 null mutant mice

The SLCZ1 null mutant mice were viable, fertile and showed no obvious developmental, behavioral or morphological abnormalities. We have performed preliminary in house behavioral test such as plus maze, rotorod and activity test. The plus maze test did not show any difference in the anxiety like behavior in the null mutant mice. The motor co-ordination was also normal based on the rotorod test. From the body weight measurement studies, we could monitor that the SLCZ1 null mutant mice were different in weight compared with their wild type control. The activity of the null mutant mice in the home cage was monitored with the activity measurement boxes (locoboxes) and there was no significant effect on cumulative total activity in the SLCZ1 null mutant. However, we observed that the total activity of the null mutant mice decreased rapidly when compared to the wild type control mice. We also treated the null mutant animals with psycho stimulant drug-amphetamine, an anxiolytic drug- diazepam and saline for control and monitored their activity in the locoboxes. Here we observed that there was no effect between the null mutant and wild type between the saline and the amphetamine group. However there was a significant reduction in activity between the saline and diazepam group. We also observed that there was a significant difference between the wild type and the null mutant mice, where the reduction in activity was not as pronounced as the wild type.

We further used the operant chambers to observe consummatory behavior and also to perform motivation studies. The mutant mice did not show any change in consummatory behavior but differed in motivation studies. The null mutant mice demonstrated a strong trend towards an increased motivation level measured by the number of active nosepokes. This trend was confirmed when a reinstatement was done after a break of two days and here we observed a significant increase in motivation level of the null mutant mice in comparison with their wild type controls.

There are many studies in progress which would further strengthen our findings and would probably provide a better functional understanding of this novel transporter, SLCZ1.
Conclusions and perspectives

In this thesis, we have provided fundamental information for some of the novel SLCs and GPCRs which were previously unknown and for some of them we have attempted to characterize their functional properties. The main findings from this work include:

- Majority of atypical SLCs belonged to the MFS type transporters that were evolutionarily well conserved and exhibited a ubiquitous expression profile. The only exception being SLC21A21, a liver specific MFS that was found only in mouse and humans.

- SLC17 family which originated from the MFS type transporters consists of four independent evolutionary clades which also correspond with their currently known substrates. One of the clades includes the eight novel genes identified in *C.elegans* and named Slc17a10.

- The recently identified Slc17a9 (VNUT) exhibits significant expression in some of the regions associated with purinergic neurotransmission. Few other regions such as dentate gyrus and PAG also important regions for purinergic neurotransmission lacks Slc17a9 indicating the possibility of another nucleotide transporter in these regions.

- Novel transporter SLC38A7 which belongs to the amino acid transporter family SLC38 was functionally characterized. Slc38a7 found to be highly expressed in the mouse brain was present in all the neurons but absent in the astrocytes. Slc38a7 was also co localized with both GABAergic and glutamatergic neurons.

- SLC38A7 exhibited specificity to L-glutamine and other amino acids with polar side chains. Based on their specificity to L-glutamine and insensitivity to MeAIB, they can be grouped with the System N transporters.

- The orphan GPCR, GPR153 and GPR162 have been studied in detail. Both the receptor are predominantly expressed in the CNS with
Gpr153 being ubiquitous. Gpr162 seems to have originated from Gpr153 through a gene duplication event before the divergence of amphibians.

- Studies on rodent models and human genetics of Gpr162 revealed changes in food intake related behavior or energy metabolism whereas the effect of Gpr153 on food intake was not as pronounced in the rodent model for Gpr153. The behavioral studies on rodent model for both the genes Gpr162 and 153 did not exhibit anxiety like behavior in OF and EPM tests.

The findings from this work revealed the need to further study some of these genes using different approaches, thus providing further insights into the physiological roles of these proteins.

In paper I, we have shown that a majority of the atypical SLCs are present in several animal species and expressed ubiquitously hinting that these proteins may be involved in some basic physiological functions and there were some atypical SLCs that were tissues specific such as Slc21a21 (liver specific transporter). A detailed individual analysis of these proteins with respect to their substrate specificity and expression of these proteins in specific cell types in particular tissues will provide a better understanding of their specific physiological role.

Paper II confirmed the expression of Slc17a9 in certain regions in the mouse brain associated with purinergic neurotransmission which is in agreement with its substrate specificity to nucleotides [22]. Further studies on the specific neuronal type expressing Slc17a9 will reveal whether ATP is coreleased with other neurotransmitter or if they form a separate type of neurons specific only for purinergic neurotransmission. We also found a complete lack of expression in certain regions confirmed to be related to purinergic neurotransmission and hence we suggest that there could be another vesicular nucleotide transporter. The SLC17 family has still four transporters whose substrates are unidentified and hence a study of nucleotide transport in these proteins would be a good starting point to look for possible vesicular nucleotide transporter.

SLC38A7, a novel member of the Slc38 family of amino acid transporters has been characterized in paper III. In this paper we have successfully used Xenopus laevis oocytes to study the uptake properties of SLC38A7. Hence, we will take advantage of the Xenopus laevis oocytes model system to characterize three, of the other novel member of this family, SLC38A8-10.
In paper IV and V, we have attempted to study the functional role of GPCR, Gpr162 and Gpr153 using antisense induced knockdown rat models. The main idea of knocking down this receptor in the CNS was to provide hints on possible effects on food intake and energy metabolism. In these knockdown models we were unable to establish the degree of silencing of this receptor (lack of antibody) and hence the results are based on the antisense induced change in functionality. These results can be further validated by studies on the cell types expressing these receptors and looking for interacting partners and downstream signaling of these receptors.

GPCRs and solute carriers are the two important classes of membrane proteins and both of them play a significant role in drug discovery and therapy. The solute carriers that transport xenobiotics among others are crucial for drug disposition and GPCRs are the most common drug targets. Hence the characterization of novel proteins in both these classes is important for the advancements in the field of medicine and pharmacology. A general perspective of deciphering the function of novel membrane proteins (GPCRs and SLCs) is to use several different approaches from phylogenetic analysis, expression profiling, and biochemical characterization to generation and analysis of knockout mouse models.
Acknowledgements

I hope I am able to pen down my feelings. This is the most important part of the thesis regarding some of the most amazing people who have stood by me through this entire process. I would like to say a word of ‘thank you’ and acknowledge them all.

My supervisor, Helgi Schiöth for believing in me and giving me challenging projects. For pushing me to write papers, teaching and developing my writing skills. For healthy criticisms which has always forced me to do better. For being a kind person and helping me adjust to the life in Sweden, even providing me an opportunity to learn Swedish (which I did not utilize). Thank you!!!

Robert Fredriksson, my co-supervisor without whom this work would have been quiet impossible. You have not just been a supervisor but also have worked in the lab and shared the excitements and disappointments. Thankyou for patiently listening to my never ending cribbing and questions. I wish someday I will become at least half as calm and patient as you are.

Klas Kullander, for introducing me to the research in Uppsala. He has guided and mentored me through the initial months, patiently familiarizing me to mice work and provided expert advice in generation of knockout mice. Thankyou for always having time to say a warm hello and spreading your vibrance and enthusiasm. I cannot describe in words the respect towards you.

I would like to acknowledge all the co-authors of our paper for their contribution and help during the whole process and especially during writing of the manuscripts.

There have been many more people in my group who have significantly contributed towards the completion of this work. A special thank you to all my project students, Ali (for all the help, putting up with me and spreading fun and laughter in the lab. You are a wonderful person inside out be the same), Anica (now a fellow PhD student, for all the help in the animal house, never ending tagging, tailing and genotyping. Thankyou for all the non scientific talks and baby talks), Atul (for assisting me with the mice and helping out in building knockout constructs and being a calm and organized student), Edwin (for being extremely independent and taking over my re-
sponsibilities during my maternity leave; wish I could have worked more
with you), Anirudhha (another extremely independent person, thank you for
helping me out with carrying on the behavioral studies and for some nice
conversations and discussions) and Sofie (thank you for finally getting the
western to work for our knockout mice and all the hard work).

Josefine, thank you is too small a word for you; a very lovable office mate
/friend, who has always been there and had the time to help me with every-
thing possible from recharging my mobile to completing the formalities of
this thesis. Maria, thank you for sharing the frustrations and excitements of
working with the frog eggs, interesting scientific discussions and proof read-
ing my thesis. I admire and envy your organized way of working, hope I
will learn from you some day. Markus, thank you for helping me fix my
laptop several times and helping me with all the bioinformatics analysis and
always having time to answer my questions. Johan, you are an amazing
person and a nice company in the lab; thank you for introducing me to your
favorite operant chambers and collaborating to do motivation studies. Åke,
my present officemate, thank you for sharing the excitement of your work
with me. Luca, my other officemate for a few months, for spreading the
smile and warmth in the lab. All my other fellow PhD students and seniors;
Mathias, Sahar, Anica, Kalle, Tatjana, Tobias, Samantha, Christian,
Madeliene thank you for creating a nice and cordial work environment. Ol-
ga, our RT PCR expert, thank you for all the work you have done in our
papers. Pawel, thank you for all the help with writing the manuscripts, edit-
ing pictures in photoshop, advice on all the scientific and professional mat-
ters and most of all for the long discussions. Rahul, thank you for company
in wee hours during thesis time. All the present and past members of the lab,
for creating a wonderful work atmosphere.

Nadine, my first friend here in Uppsala, a straight forward and honest per-
son. I have always admired how you balance work and home and excel in
both. Thankyou for introducing me to the world of baking and accepting me
as I am. You will always remain close to my heart. Fatima, a beautiful per-
son inside out, thank you for being there and listening to me. Kasia, the most
determined girl that I have met, thank you for all the small talks we have
had. All the three of you for the wonderful time we had during the girls’
night out.

There have been so many other people in Klas lab and the other groups in
the department, who have made my stay here in the department a memorable
one. Anders, my first tutor here at the department thank you for all the help.
I admire your knowledge about almost everything. Ingrid and Bo for nice
company in the radioactive lab. Thankyou, Karin, Lina, Christiane, He-
nrik G, Henrik B, Malin, Lucas, Soejong, Shahrzad, Daniel, Helena,
Gorel, Nicole, Greta, Aparna and Chetan for all the help and enjoyable talks.

Ulla, Emma, Brigitta, Maria, Marita and Lena for all the administrative help and a warm smile whenever we meet. Sussanne, Jenny, Sussie and Eva for taking care of the mice. Maud and Qun, for providing excellent transgenic facility and a friendly working relation. All the faculty members and the head of the department for providing a great research atmosphere.

A few friends in BMC outside the department, (Nikhil, Mumtaz and Junaid) for all the wonderful discussion, chat and lunch. My big gang of friends in uppsala outside the lab, who have made my stay here in Upp Sala an amazing experience; thankyou all for all the support, help, wonderful lunch and dinners, get together, X-mas and New year celebrations and more importantly the warmth and affection. Pratima, Pandeyji and Prashanth (Thanyou for sharing the most wonderful moment of your life with me), Puneet (for being a warm and caring person), Jitu mama (for teaching all of us to take life easy), Faizaan, Fatima & Zidu (for all the food and amazing food), Sonchita, Bhupi & Adi (for all the good times, celebrations and help), Sonchita’s mom and dad (for taking care of me during my early days of motherhood), Varghese, Julie and Evilin (for all the support and celebrations), Shalini, Siju and Amma (for spreading the smile and laughter), Meena, Chandra, Neha and Sanju (for great get togethers, homely and friendly occasions), Vijay and Neha (thanyou for the warm welcome to Sweden and nice company), Rajeev sir and Ranju chechi (for all the support and help). Chandu, Geetha & Bhaskar, Tanmoy & Sanhita, Noopur, Uma Shankar & Daksh, Gaurav, Prasoon, PK Singh Uncle, Nelson, Brinda & Surajit, Narenbhai, Remya, Venu sir and a lot of other friends (whose name I might have missed).

I have an extended family here in Uppsala, who make sure that I dont miss home. Stephy, Prakash, Mathu and Greta (we have managed to stay without u for a few months now but there is a vacuum that cannot be filled, miss u all), Rajesh and Nisha (For becoming a part of our family and all the love and support), Kali and Sulena (for everything that you guys do, for the unconditional love that u give Adi and all the help and support), Rashmi, Sarosh & Maanu (I have no words to say how much u all are a part of our life. Rashu, after meeting u, I no more complain that I dont have a sister to share all my feelings), Rashmi’s mom (for her love and affection), Navya and Sandeep (what can I say… for being there always, taking care and loving adi soo much, Navya, I think thank you will be a plain word for you, u have become one my closest friend for life).

Back home in India, there have been a lot of people who have guided me and inspired me to do Science. All my teachers from college and university
(NHM sir, VRD sir, CSK sir & KRS sir) who have developed my interests. **Jaffar** sir, my first research guide, for believing that a pure biology student can do biophysics. I still love to talk about Optical tweezers. I have always admired the love and care you show towards us students. Ramanan Sir, a person I have always looked upto, a simple man with a great mind. A whole bunch of amazing people at AU-KBC, who thought me dreams have no limit: **Sabna** (Ummmmmmmmma, the most lovable person I have met), **Madhavan, Tamil, Rajesh, Masood, Satish, Vipin, Anu and Viji. Kannan**, my friend from college because of whom I studied Biochemistry (thankyou for all those wonderful time in college). **Rakesh**, my little bro for all the love and affection.Few of my very close friends who believed in me more than I do myself: **Suchi, Ashu, Neethu, Ashwin and Reshmi. Siju** (a great friend, an amazing human being – its been a pleasure to know you and spend so many vacations together). I always feel blessed to have soo many loving and caring people around me all the time.

**Mummy & Pappa**, thankyou for everything, leaving our home in India for more than a year and staying here with us, in the most terrible winter times without complaining, for taking care of me as your daughter and encouraging me to study without worrying about household work. Without both of you, I do not know if we could have brought up Adi so well. **Unniaettan, Sinichechi and Shradha molu** for all the love and affection.

**My grandmothers** for their unconditional love. **Unnimama and Vijichechi** for encouraging me to pursue my dreams. **Padma Chechi** for taking care of me and making my life as comfortable as possible.

**Sree** (for considering me more as ur friend) and **Aryankuttan** (a wonderful inclusion into our family). **Sunilaetta**, (Thankyou for believing in all my decisions and supporting me as a friend, as a brother and for a wonderful childhood)

**Amma & Achan**, without you this wouldn't have been possible. For all the happiness, pleasure, and joy that you have given me through the years without expecting anything in return. For being the most kind, helpful and wonderful human beings. For teaching me that life is all about loving others and finding happiness in others joy. **Amma- UR the best. Achan- My role model (love u both)**

**Adikutta**, my little one- Ummmmmmmmmmm, Sorry! for not giving you enough time. **Nimmutta – truly my better half.** What else can I say.... My best friend, my love, my strength and pride....... My vocabulary falls short of words to describe my feelings towards you. Ur the best part of my life, my joy and happiness; we (Adikuttan and myself) are lucky and blessed to have you in our lives.
References


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 689

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine.

Distribution: publications.uu.se
urn:nbn:se:uu:diva-156832