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Functional and Biochemical Consequences of Disease Variants in Neurotransmitter Transporters: A Special Emphasis on Folding and Trafficking Deficits

Shreyas Bhat, **Ali El-Kasaby**, **Michael Freissmuth**, **Sonja Sucic***
Institute of Pharmacology and the Gaston H. Glock Research Laboratories for Exploratory Drug Development, Center of Physiology and Pharmacology, Medical University of Vienna, A-1090 Vienna, Austria

Abstract

Neurotransmitters, such as γ-aminobutyric acid, glutamate, acetyl choline, glycine and the monoamines, facilitate the crosstalk within the central nervous system. The designated neurotransmitter transporters (NTTs) both release and take up neurotransmitters to and from the synaptic cleft. NTT dysfunction can lead to severe pathophysiological consequences, e.g. epilepsy, intellectual disability, or Parkinson's disease. Genetic point mutations in NTTs have recently been associated with the onset of various neurological disorders. Some of these mutations trigger folding defects in the NTT proteins. Correct folding is a prerequisite for the export of NTTs from the endoplasmic reticulum (ER) and the subsequent trafficking to their pertinent site of action, typically at the plasma membrane. Recent studies have uncovered some of the key features in the molecular machinery responsible for transporter protein folding, e.g., the role of heat shock proteins in fine-tuning the ER quality control mechanisms in cells. The therapeutic significance of understanding these events is apparent from the rising number of reports, which directly link different pathological conditions to NTT misfolding. For instance, folding-deficient variants of the human transporters for dopamine or GABA lead to infantile parkinsonism/dystonia and epilepsy, respectively. From a therapeutic point of view, some folding-deficient NTTs are amenable to functional rescue by small molecules, known as chemical and pharmacological chaperones.

Keywords

Neurotransmitter; Transporter; *SLC6*; Disease variants; Folding; Pharmacochaperoning

1 Introduction

Neurotransmitter transporters (NTTs) belong to the solute carrier (SLC) superfamily of membrane transporters, which is comprised of 65 families (Hu, Tao, Cao, & Chen,

*Corresponding author at: Institute of Pharmacology, Medical University of Vienna, Währingerstrasse 13A, A-1090 Vienna, Austria. sonja.sucic@meduniwien.ac.at (S. Sucic).

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2020). NTTs are responsible for terminating the action of neurotransmitters via rapid reuptake of neurotransmitter molecules from the synaptic cleft, and their subsequent reloading into synaptic vesicles (Nelson, 1998). According to their pertinent sites of action, NTTs can be classified into two major superfamilies (Masson et al., 1999). (i) Plasma membrane transporters, which are further subdivided into the $Na^+/K^+/H^+$-dependent glutamate transporters (SLC1 gene family), choline-Na^+ symporter (*SLC5A7* gene), Na^+/Cl^--dependent transporters for dopamine, γ-aminobutyric acid (GABA), glycine, norepinephrine, serotonin (*SLC6* gene family), Na^+-independent organic cation transporters (*SLC22* gene family), equilibrative nucleoside transporters (*SLC29* gene family) and the choline transporter-like proteins (*SLC44* gene family). (ii) The second group encompasses the intracellular H^+-dependent vesicular transporters, which are divided into three subclasses: vesicular glutamate transporters (*SLC17* gene family), vesicular amine transporters (*SLC18* gene family), and the vesicular inhibitory amino acid transporter (*SLC32*). A phylogenetic tree based on amino acid sequence homology of the protein families belonging to the NTT superfamily is shown in Fig. 1.

By definition, NTTs have a ligand-binding pocket, which affords specificity, selectivity and flexibility to accommodate the cognate substrates and co-substrates and to allow for the conformational transition associated with substrate translocation. Hence, they are posited to be druggable. In fact, several NTTs are prominent pharmacological drug targets. The transporters of the *SLC6* family, for instance, are the sites of action of numerous exogenous compounds: e.g. the antiepileptic drug tiagabine blocks the GABA transporter 1 (GAT1, *SLC6A1*); antide-pressants (tricyclic compounds like imipramine, desipramine and amitryptyline; SSRIs/selective serotonin reuptake inhibitors, SNRIs/serotonin and noreprinephrine reuptake inhibitors), psychostimulants (e.g. cocaine and amphetamines) and anti-addictive drugs (e.g. ibogaine) act on the monoamine transporters for norepinephrine (NET, *SLC6A2*), dopamine (DAT, *SLC6A3*), and serotonin (SERT, *SLC6A4*) (Iversen, 2000; Rask-Andersen, Masuram, Fredriksson, & Schiöth, 2013). The indol alkaloid reserpine and tetrabenazine act on the vesicular monoamine transporters VMATs (e.g. *SLC18A2*); reserpine was introduced in the treatment of arterial hypertension in the 1950s. It has been abandoned in most countries, because the advent of effective inhibitors of angiotensin II formation and of angiotensin II-receptor anatagonists made reserpine dispensable: reserpine causes severe side effects including depression (due to depletion of serotonin and norepinephrine) and Parkinsonism (due to depletion of dopamine). Tetrabenazine, which is selective for VMAT2/*SLC18A2*, is still the treatment of choice for chorea/Huntington's disease (Rask-Andersen et al., 2013).

Mutations in NTTs have been linked to a broad range of pathologic conditions in people over the past two decades (Fig. 2). Recent research from our group revealed that point mutations in *SLC6* transporters can trigger aberrant protein folding, causing their retention in the endoplasmic reticulum (ER) compartment and hence precluding the delivery of variant transporters to their site(s) of action (e.g. in case of the *SLC6* family, at the cell surface). As a consequence, protein misfolding gives rise to severe clinical phenotypes, e.g. folding-deficient variants of the DAT trigger infantile/juvenile parkinsonism-dystonia, of the creatine transporter 1 (CRT1, *SLC6A8*) cause severe X-linked intellectual disability and of GAT1 cause myoclonic-atonic and other generalized epilepsies. Understanding the

molecular mechanisms that lead to protein misfolding and that underlie treatment avenues for folding deficits in NTT disease variants by pharmacological and chemical chaperones have potential for clinical impact.

2 *SLC1* family: EAAT and ASCT transporters

2.1 Glutamate transporters

Glutamate is the most abundant neurotransmitter in the mammalian central nervous system (CNS) (Meldrum, 2000). Glutamate clearance from the synaptic cleft is mediated by glutamate transporters, which are expressed both in neurons and astrocytes in the brain (Grewer, Gameiro, & Rauen, 2014). Astrocytes clear a large fraction of glutamate from the synapse; they convert glutamate to glutamine, which is returned into neurons by *SLC38* transporters; this glutamate-glutamine shuttle precludes extracellular glutamate mediated excito-toxicity (Bhutia & Ganapathy, 2016). In neurons, glutamine is converted to glutamate, which is refilled into synaptic vesicles. Glutamate transporters include 5 plasmalemmal excitatory amino acid transporters (EAAT1 (*SLC1A3*), EAAT2 (*SLC1A2*), EAAT3 (*SLC1A1*), EAAT4 (*SLC1A6*), EAAT5 (*SLC1A7*)) and 2 neutral amino acid transporters (ASCT1/*SLC1A4*) and (ASCT2/*SLC1A5*). Glutamate transport is primarily mediated by EAATs, while ASCTs accomplish transport at a low pH (Vadgama & Christensen, 1984). EAATs also have an inherent chloride conductance that may positively influence the concentrative power of these transporters (Vandenberg & Ryan, 2013). ASCT1 and ASCT2 mainly transport neutral amino acids such as alanine, serine, cysteine, and threonine in glial cells and neurons (Utsunomiya-Tate, Endou, & Kanai, 1996; Zerangue & Kavanaugh, 1996). ASCT1 is the major transporter that retrieves D-serine in neurons (Kaplan et al., 2018). ASCT2 has also been shown to retrieve glutamine and asparagine with high affinity, and is proposed to be an active participant in the glutamate-glutamine cycle between glial cells and neurons in the brain (Scalise, Pochini, Console, Losso, & Indiveri, 2018). Owing to the importance of glutamate transporters in glutamate homeostasis, it is plausible that their malfunction plays a role in many neurological diseases (reviewed in Vandenberg & Ryan, 2013; Kanai et al., 2013; O'Donovan et al., 2017). Table 1 lists the disorders arising from point mutations in plasmalemmal glutamate *SLC1* transporters, described in further detail below.

2.2 EAAT1 (GLAST; SLC1A3)

EAAT1 knockout mice (EAAT1$^{-/-}$) do not exhibit spontaneous epileptic seizures, but pentetrazol-induced seizures are enhanced both in their severity and in duration (Watanabe et al., 1999). They also show neurological symptoms commensurate with the prominent expression of EAAT1 in the cerebellum (i.e. reduced motor coordination), the inner ear (hearing loss) and retina (impaired vision). The mice display poor nesting behavior, abnormal sociability and anhedonia (Karlsson et al., 2009; Watase et al., 1998).

Point mutations in EAAT1 (*SLC1A3*) trigger primary episodic ataxia (EA) in patients harboring the mutations (Jen and Wan, 2018). Episodic ataxia is initiated by paroxysmal cerebellar dysfunction leading to a myriad of symptoms. Episodic ataxia is classified into 8 subtypes (EA1-8) based on the genetic loci affected. Common symptoms in these patients

include ataxia with dizziness, migraine, seizures, slurred speech, vertigo, hemiplegia and postural/gait imbalance set off by physical and emotional stress (Choi & Choi, 2016). EAAT1 mutations (Fig. 3a) are categorised as episodic ataxia subtype 6 (EA6). The first EAAT1 mutation associated with EA6, was a heterozygous de novo substitution of proline 290 by arginine (p.(P290R)) (Jen et al., 2005). Functional studies revealed a ~ 90% reduction in radiolabeled glutamate uptake in COS-7 cells expressing the p.(P290R) mutant alone. Co-transfecting the plasmids encoding wild type EAAT1 and p.(P290R) also caused reduced surface trafficking and uptake, indicative of a dominant-negative effect of the mutant on the wild type allele. Despite its low plasma membrane expression, surface-resident p.(P290R) had increased chloride conductance, due to a higher open probability of the chloride channel, contrasting the wild type transporter (Winter, Kovermann, & Fahlke, 2012). This gain-of-function in anionic conductance may explain the more severe symptoms triggered by this variant in people, compared to EAAT1$^{-/-}$ mice or flies (Parinejad et al., 2016). Another ataxia-associated mutation, the p.(C186S) heterozygous variant (De Vries et al., 2009), shows only 18% reduction in glutamate uptake compared to wild type EAAT1, when expressed in COS-7 cells. The mutation appears to disrupt trafficking, rather than the uptake activity of EAAT1 (Hayashi & Yasui, 2015). A patient harboring another EAAT1 variant, p.(T387P), suffered from relatively mild hemiplegic migraine (Kovermann et al., 2017). Biochemical analysis of the mutant revealed reduced trafficking to the plasma membrane. Moreover, an abrogated glutamate transport by p.(T387P), inferred from electrophysiological measurements, impinged on impaired intracellular K^+ binding.

Additional episodic ataxia variants in EAAT1 (Table 1 and Fig. 3a) are yet to be subjected to biochemical and functional characterization (Choi et al., 2017; Choi et al., 2017; D'Adamo et al., 2015; Iwama et al., 2018; Pyle et al., 2015). Most of these variants have been classified as pathogenic, based on the segregation among the affected families and on computational tools. In some cases, EAAT1 mutations are co-inherited with mutations in other genes implicated in the onset of episodic ataxia (Choi, Kim, et al., 2017; D'Adamo et al., 2015). The uncharacterized A329T mutation gives rise to episodic ataxia (Choi, Kim, et al., 2017) and to benign essential blepharospasm (Dong et al., 2020). On the other end of the spectrum, the gain-of-function p.(E219D) EAAT1 variant occurs at higher frequencies in individuals with Tourette syndrome, relative to the general population (Adamczyk et al., 2011). Together, these observations denote that: (i) uncharacterized variants necessitate experimental investigation to unveil their impact on trafficking and the transport cycle, and (ii) in some instances, modifying genes might contribute to the variable penetrance in episodic ataxia.

2.3 EAAT2 (GLT-1; SLC1A2)

EAAT2 is the most abundant glutamate transporter expressed throughout the CNS, accounting for approximately 1% of all brain proteins and 90% of the total glutamate uptake (O'Donovan et al., 2017). EAAT2 knockout mice (EAAT2$^{-/-}$) are phenotypically normal at birth. The symptoms of hyperactivity and epileptic seizures appear around 3 weeks of age, accompanied by stunted growth relative to wild type littermates. Half of all EAAT2$^{-/-}$ mice die within 4 weeks. These mice exhibit a 95% reduction in glutamate clearance from the synapse, leading to higher extracellular glutamate levels (Tanaka et

al., 1997). Heterozygous (EAAT2$^{+/-}$) mice show milder neurological symptoms, but they have an increased susceptibility to neuronal loss upon traumatic spinal cord injury (Kiryk et al., 2008). Random mutagenesis screening in zebrafish led to the discovery of techno trousers (tnt) locomotor mutants (Granato et al., 1996): the mutant fish larvae show an abnormal escape response with exaggerated body bends in response to touch 2 days after after fertilization. At 4 days post fertilization, they are paralyzed (McKeown et al., 2012). The phenotype is accounted for by a conservative point mutation (A393V) located in TM7b of EAAT2 and can be phenocopied by an EAAT2 inhibitor (McKeown et al., 2012). Collectively, these findings highlight the role of glial EAAT2 in controling neuronal excitability and preventing excitotoxicity by excess synaptic glutamate.

The very first point mutation in human EAAT2 (*SLC1A2*) was identified in a patient afflicted with sporadic amyotrophic lateral sclerosis (ALS), i.e. the p.(N206S) variant (Aoki et al., 1998) illustrated in Fig. 3b. ALS is a progressive neurodegenerative disease, leading to the loss of voluntary muscle control due to the death of motor neurons in the brain and spinal cord. N206 is highly conserved among EAATs and is a putative site for N-glycosylation. Its substitution by serine impedes glycosylation, trafficking from the ER and Golgi to the plasma membrane, resulting in a loss-of-function phenotype (Trotti et al., 2001).

In addition, EAAT2 point mutations have been identified in several cases of epileptic encephalopathy (EE). Almost all of these mutations (Table 1, Fig. 3b) are inherited in an autosomal dominant fashion. Early onset EEs are epilepsies with heterogeneous genetic causes characterized by several treatment-resistant seizure types, frequent epileptiform activity in the electroenchepalogram (EEG) and global/neuronal developmental delay (McTague et al., 2016). The p.(L85P) variant is recurrent in patients suffering from EE (Epi4K Consortium, 2016; Stergachis et al., 2019). Biochemical analysis revealed that EAAT2-L85P mediates <10% uptake and imposes a dominant negative effect on the wild type transporter. L85P appears to impair either transporter folding, trafficking or assembly of the mixed trimeric transporter complexes as a functional unit (Stergachis et al., 2019). While the EE-associated p.(P289R) variant is yet to be characterized, it is equivalent to and expected to mimic the properties of the p.(P290R) EAAT1 variant, discovered in a patient with episodic ataxia (described above).

Apart from EE, dysregulation of EAAT2 has also been implicated in the development of other neurological and neuropsychiatric disorders, such as hereditary spastic paraplegia, Alzheimer's disease, depression, autism, schizophrenia and bipolar depression. EAAT2 variants that have not been functionally or biochemically examined are compiled in Table 1 and Fig. 3b (Epi4K Consortium, 2013; Epi4K Consortium, 2016; Fiorentino, Sharp, & McQuillin, 2015; Guella et al., 2017; Meyer et al., 1998; Stergachis et al., 2019; Wagner et al., 2017). In the absence of functional data, their phenotypic repercussions can currently not be surmised.

2.4 EAAT3 (EAAC1, SLC1A1)

EAAT3 is expressed predominantly in neurons of the neocortex, hippocampus and some midbrain regions (Vandenberg & Ryan, 2013). EAAT3 knockout mice (EAAT3$^{-/-}$) do not

exhibit any spontaneous epileptic seizures or neurodegeneration, but they show reduced spontaneous locomotion in an open field test, and are subject to premature ageing. The mice present dicarboxylic aminoaciduria (DA) with increased urinary excretion of aspartate and glutamate (Peghini et al., 1997).

Point mutations in human EAAT3 also lead to DA, an autosomal recessive disorder, manifested by an abnormal excretion of urinary glutamate and aspartate due to the lack of reabsorption of anionic amino acids in the kidney. The first EAAT3 DA mutation, p.(R445W), was identified in an adult patient who, along with his brother, bore kidney stones in early adulthood and had abnormally high urine levels of glutamate and aspartate (Bailey et al., 2011). The second mutation was found in a child harboring a 3-base pair (TCA) deletion at positions c.1184– 1186, leading to the deletion of isoleucine 395 (p.(I395del)). Functional characterization revealed both variants to be trafficking-deficient: the lack of plasma membrane expression was associated with an almost complete abrogation of substrate transport and of transport-associated currents. The patient harboring the p.(R445W) mutation (Fig. 3c) also exhibited an undiagnosed obsessive-compulsive disorder (OCD)-like trait, suggestive of EAAT3 mutations playing a role in the onset of neurological disorders. This is further endorsed by the discovery of uncharacterized EAAT3 variants in patients suffering from severe OCD and bipolar depression (p.(T164A), Wang et al., 2010), schizophrenia/schizotypal personality disorder (p.(R280C), Demily et al., 2018) and autism (p.(E48*), Doan et al., 2019). In fact, EAAT3 was proposed as a novel susceptibility locus in the pathogenesis of schizophrenia and bipolar disorders (Afshari et al., 2015; Myles-Worsley et al., 2013; Rees et al., 2014). Thus, the existing evidence links EAAT3 mutations to a broad spectrum of mental disorders. The actual impact of the uncharacterized EAAT3 mutations on its trafficking and transport cycle ought to be assessed for explaining the phenotypic variation, which may also be influenced by other genes.

2.5 EAAT4 (SLC1A6) and EAAT5 (SLC1A7)

EAAT4 is expressed primarily in Purkinje cells of the cerebellar cortex. It is also sporadically present in the cerebral cortex, vestibular hair cells and calyx endings. EAAT5 is exclusively localized to the retina, with presynaptic expression at the synaptic terminals of cone, rod and bipolar rod cells (Vandenberg & Ryan, 2013). Notably, compared to other transporters, EAAT4 and EAAT5 have a reduced rate of glutamate turnover and a high chloride conductance. Thus, it has been argued that they serve as (inhibitory) glutamate-receptors, rather than as glutamate transporters (Dehnes et al., 1998; Veruki, Mørkve, & Hartveit, 2006). Knocking out EAAT4 in mice ($EAAT4^{-/-}$) triggers age-dependent progressive motor deficits and ataxia due to Purkinje cell death in the cerebellum (Perkins et al., 2018). Currently, there are no reports on EAAT5 knockout mice or on human disease mutations in either EAAT4 or EAAT5.

2.6 ASCT1 (SLC1A4) and ASCT2 (SLC1A5)

The distribution of ASCT1 and ASCT2 in neurons and glial cells has been well described (Gliddon, Shao, LeMaistre, & Anderson, 2009; Weiss, Derazi, Kilberg, & Anderson, 2001). ASCT1 knockout ($ASCT1^{-/-}$) or ASCT2 knockout ($ASCT2^{-/-}$) mice are viable and fertile (Kaplan et al., 2018). $ASCT2^{-/-}$ mice show no behavioral changes. Conversely,

ASCT1$^{-/-}$ mice display sensorimotor, behavioral and learning deficits. These phenotypic differences in ASCT1$^{-/-}$ and ASCT2$^{-/-}$ mice also hold true in the corresponding human ASCTs. Point mutations in ASCT1 cause early onset spastic tetraplegia, thin corpus callosum, and progressive microcephaly (SPATCCM), inherited in an autosomal recessive manner (OMIM# 616657). Of the ASCT1 variants (listed in Table 1 and Fig. 3d), Y191* (Abdelrahman, Al-Shamsi, John, Ali, & Al-Gazali, 2019), L315Hfs*42 (Damseh et al., 2015) and W453* (Conroy et al., 2016) code for truncated transporters. While no biochemical characterization data is available, the variants are predicted to have folding and/ or trafficking deficits. The phenotype of other mutants, e.g. the recurrent p.(E256K) (Damseh et al., 2015; Heimer et al., 2015; Srour et al., 2015) and p.(R457W) variants, can be accounted for by their impaired transport cycles. Immunoblots of the surface-labelled protein fractions suggested normal delivery of these mutants to the plasma membrane (Damseh et al., 2015). Trafficking of the p.(R457W) ASCT1 variant to the cell surface is peculiar, since the equivalent mutation in EAAT3 (p. (R445W) Bailey et al., 2011), is in fact ER-retained. The p.(E256K) variant showed markedly reduced levels of uptake of tritiated serine and alanine. Substrate uptake by p.(R457W) was abolished, which rationalizes the more severe clinical symptoms in the patient harboring the latter mutation. Another ASCT1 variant p.(G381R) was predicted to be damaging by in silico modeling, but without further experimental validation (Pironti et al., 2018).

3 *SLC5* and *SLC44* families: choline transporters

3.1 Choline transporters

Acetylcholine (ACh) exerts its neurotransmitter action in the central and peripheral nervous systems via the cognate nicotinic and muscarinic acetylcholine receptors (Brown, 2019). Upon synaptic release, ACh is degraded to choline and acetate by acetylcholinesterases residing in post-synaptic membranes. While acetate diffuses into the surrounding extracellular space, choline is transported into presynaptic neurons by the designated choline transporters. Choline transporters encompass the high-affinity choline transporter, with a K_M of ~2 μM (CHT1, *SLC5A7*) (Sarter & Parikh, 2005) and the Na^+-independent intermediate affinity *SLC44* (choline transporter-like proteins, CTLs) transporters (Traiffort, O'Regan, & Ruat, 2013). The latter group is comprised of 5 members (*SLC44A1–5*), of which CTL3 (*SLC44A3*) and CTL5 (*SLC44A5*) are only poorly characterized (Traiffort et al., 2013).

3.2 CHT1 (SLC5A7)

CHT1 is a Na^+/Cl^--dependent secondary active transporter (Apparsundaram, Ferguson, George Jr, & Blakely, 2000), with highly regulated assembly and trafficking to the cell membrane (for details see: Ribeiro et al., 2005; Matsuo et al., 2011; Cuddy et al., 2012; Okuda et al., 2012). Choline uptake by CHT1 is crucial: 1) Neurons cannot synthesize choline de novo; i.e., uptake by CHT1 is the main source of choline. 2) Choline provided by CHT1 is converted to ACh by choline acetyltransferase, with CHT1-mediated choline uptake being the ratelimiting step in ACh synthesis (Ferguson & Blakely, 2004; Okuda and Haga, 2003; Ribeiro et al., 2006). 3) CHT1 knockout (CHT1$^{-/-}$) mice die within 1 h of birth as a result of impaired breathing (Ferguson et al., 2004), 4) Point mutations in CHT1 lead

to inheritable neuromuscular junction disorders, transmitted in both, autosomal recessive and dominant manner (Wortmann & Mayr, 2019).

Loss-of-function point mutations in CHT1, inherited in an autosomal dominant manner, lead to the onset of one of the many types of distal hereditary motor neuronopathy (dHMN, for details see Irobi et al., 2006). CHT1 mutations (Table 2 and Fig. 3e) are grouped as type VIIa dHMNs (OMIM#158580), characterized by distal muscular atrophy and unilateral or bilateral vocal cord paralysis (McEntagart et al., 2001). Multiple members of 2 unrelated Welsh kindred, suffering from type VIIa dHMNs, were found to harbor a heterozygous frame shift mutation, leading to the p.(K499Nfs*13) variant (Barwick et al., 2012; Ingram et al., 2016). This truncated variant lacks the cytoplasmic C-terminal domain and the concomitant motifs required for CHT1 trafficking from the ER to the membrane. Unsurprisingly, studies of this mutant in heterologous systems revealed reduced protein abundance/translation and impaired surface trafficking, which led to diminished choline uptake (to ~30% of wild type) upon co-transfection with the wild type CHT1 plasmid. Interestingly, mice with a monoallelic knockout of CHT1 ($CHT1^{+/-}$) are viable and fertile, with no discernible differences to wild type mice in standard behavioral tests (Bazalakova et al., 2007; Ferguson et al., 2004). These findings are indicative of a dominant negative effect of p.(K499Nfs*13) on the wild type CHT1. Subsequently, 3 additional frameshift variants (p.(P509Lfs*3), p.(H521Qfs*2) and p. (K510Nfs*2)) were identified in patients afflicted with type VIIa dHMNs (Hamanaka et al., 2018; Salter et al., 2018). These variants are expected to be trafficking-deficient due to their truncated C-termini.

Patients with homozygous or compound heterozygous mutations in the CHT1 gene suffer from a distinct type of congenital myasthenic syndrome (CMS20, OMIM#617143; Rodríguez Cruz, Palace, & Beeson, 2018). Manifested as susceptibility to fatigue and muscle weakness, these mutations lead to a phenotypic spectrum in patients; while some exhibit classical neonatal onset of CMS accompanied by treatable episodic apneas and a good prognosis, others develop brain atrophy, intellectual disability and developmental delay. Some mutations are lethal and lead to antenatal or infantile lethal arthrogryposis (congenital joint contracture), severe hypotonia and/or akinesia (Banerjee et al., 2018; Bauché et al., 2016; Kashevarova et al., 2014; McMacken et al., 2018; Pardal-Fernández et al., 2018; Wang et al., 2017). Of the 17 variants reported to date (Table 2 and Fig. 3e), the p.(S94R), p.(V112E) and p. (P210L) variants have defective trafficking and accumulate in intracellular compartments (for details, see Section 9). This leads to dramatic reductions in choline uptake (Wang et al., 2017). The remaining characterized mutants (Banerjee et al., 2018; Bauché et al., 2016), all preserved membrane trafficking and had protein levels and stability comparable to the wild type transporter (shown by immunoblots), but had severely reduced choline uptake. This is indicative of intrinsic deficits introduced by mutations in conformational transitions required for substrate translocation. Other, as yet uncharacterized CHT1 mutants (Bauché et al., 2016; McMacken et al., 2018; Pardal-Fernández et al., 2018) are listed in Table 2. Of note, a non-synonymous CHT1 polymorphism coding for a p.(I89V) variant leads to a 40 –50% reduction in maximal choline uptake compared to the wild type CHT1 (Okuda et al., 2002). This particular polymorphism has been implicated in distraction vulnerability (Berry et al., 2014), ADHD (English et al., 2009) and major depressive disorders (Hahn et al., 2008).

3.3 Choline transporter-like proteins (CTL 1–5; *SLC44* A1-5)

The cellular distribution of CTL1 (*SLC44A1*), has been studied in detail (Michel and Bakovic, 2012): there is a plasma membrane fraction, which plays an important role in providing choline for phospholipid synthesis at the cell surface, and a mitochondrial fraction, which provides choline for betaine production in the mitochondria. CTL1 is expressed in neurons as well as in myelinating and mature oligodendrocytes, but it does not overlap with markers of cholinergic neurotransmission (Traiffort, Ruat, O'Regan, & Meunier, 2005). Point mutations in CTL1 lead to an autosomal recessive early onset neurodegeneration with ataxia, tremor, optic atrophy and cognitive decline or CONATOC (OMIM# 618868). Other symptoms of CONATOC include macrocephaly, swallowing difficulties, truncal muscle weakness and hypotonia. So far, 3 CTL1 variants (Table 2 and Fig. 3f) have been identi fied in affected individuals from 3 unrelated consanguineous families (Fagerberg et al., 2020; Reuter et al., 2017). The p.(D517Mfs*19) variant is a truncated protein lacking 2 transmembrane domains and the C-terminus. Despite the truncation, it is trafficked to the cell surface at levels comparable to wild type CTL1, but its choline uptake ability is reduced by 70–80% (Fagerberg et al., 2020). This is indicative of deficits in the catalytic transport activity triggered by the mutation. In contrast, the p.(K90Mfs*18) and p.(S126Mfs*8) variants both resulted in too short a protein fragment to possess any functional capacity. In addition, patient fibroblasts expressing the p.(D517Mfs*19) mutant had altered homeostasis of phosphatidylethanolamine, phosphatidylserine, iron and mitochondrial fatty acids at the cost incurred for maintaining normal levels of phosphatidylcholine. These defects were accompanied by ultrastructural abnormalities, as shown by electron microscopy: an elongated ER, increased mitochondria and small vesicles, but a reduced number of free ribosomes. Interestingly, the pleotropic consequences were reversed by supplementation of choline at high concentrations (Fagerberg et al., 2020), presumably because organic cation transporters, with their low affinity for choline, supported adequate levels of influx.

The cognate substrate of CTL2 (*SLC44*A2) is not known: circumstantial evidence suggests that CTL2 does transport choline, albeit to a lesser extent than CTL1 (Kommareddi et al., 2010; Nakamura et al., 2010). CTL2 was first identified as an inner ear antigen, targeted by autoantibodies implicated in autoimmune hearing loss (Nair et al., 2004). The expression of CTL2 in glial and sensory cells (=hair cells) of the inner ear is consistent with the hypothesis that CTL2 is required for hair cell survival (Beyer et al., 2011). This conjecture was verified in CTL2 knockout ($CTL2^{-/-}$) mice (Kommareddi et al., 2015): the mice show agedependent loss of hearing; initially, their perception of high frequencies is impaired, but as they age, the loss of hair cells and neurons in the spiral ganglion progresses, resulting in reduced auditory perception across the entire spectrum of frequencies. Since CTL2 is required for the integrity of hair cells, it is a candidate gene in the screening for Menière's disease (OMIM# 156000), a disorder associated with episodic (rotational) vertigo, impaired hearing and tinnitus (Nair et al., 2016).

CTL4 (*SLC44A4*) transports choline and thiamine across plasma membranes (Nabokina et al., 2014; Traiffort et al., 2013). Transcripts are barely detected in the brain, but show pronounced expression in the inner ear, intestine, stomach and kidney (Ma et al., 2017;

Traiffort et al., 2013). CTL4, like CTL2, has also been implicated in sensorineural hearing loss. A loss-of-function point mutation p.(M156V) (Fig. 3f and Table 2), inherited in an autosomal dominant manner, was found in multiple members of a Chinese family, who suffered from sensorineural hearing loss, accentuated in the middle frequencies (OMIM# 617606, Ma et al., 2017). Functional characterization of the p.(M156V) variant in heterologous cells revealed significant reduction in choline uptake and subsequent acetyl choline synthesis and secretion. However, cellular trafficking consequences of this mutation were not examined. In the same study, the disruption of CTL4 activity in a zebrafish model led to a significant reduction of hair cells and neuromasts, impairing balance and hearing responses. This is indicative of a vital role for CTL4-mediated choline uptake in the physiology of hair cells in the inner ear. A CTL4 variant p.(D47V) was also screened with other causative genes in patients suffering from age-related macular degeneration and blindness (Cheng et al., 2015). This mutation has not been functionally characterized.

4 *SLC6* family: monogenic diseases associated with transporters for γ-aminobutyric acid (GABA), glycine and monoamines

4.1 GABA transporters (GAT1-3, BGT1; SLC6A1,11-13)

GABA is the key inhibitory neurotransmitter in the brain. Similar to glutamate, low levels of extracellular GABA must be maintained to afford a large dynamic range. This is accomplished by plasmalemmal GABA transporters (GATs). The GABA transporter family is comprised of 4 high affinity Na^+- and Cl^--coupled symporters: GAT1 (*SLC6A1*), GAT2 (*SLC6A13*), GAT3 (*SLC6A11*) and BGT1 (Betaine transporter, *SLC6A12*). GAT1 and GAT3 are the most predominant isoforms in the brain; high intensity staining for both isoforms can be seen in the cerebellum (molecular layer), basal ganglia (ventral pallidum, globus pallidus), olfactory bulb (glomerular layer) and the retina (for references see Zhou & Danbolt, 2013; Scimemi, 2014). GAT2 and BGT1, on the other hand, are expressed primarily in the liver and the kidney. GAT1 resides primarily in the axon terminals of GABAergic neurons. GAT3 is predominantly expressed in glial cells.

Phenotypic consequences of non-functional GAT1 were first postulated in patients with a heterozygous microdeletion at 3p25.3 spanning *SLC6A1* and *SLC6A11* (the genes encoding GAT1 and GAT3, respectively). These patients suffer from developmental delay, intellectually disabilities (ID), ataxia and stereotypic hand movements (Carvill et al., 2015; Dikow et al., 2014; Rauch et al., 2012). In addition, duplication in this locus is implicated in the onset of the Prader-Willi syndrome (Bittel, Kibiryeva, Dasouki, Knoll, & Butler, 2006). Of the >100 GAT1 variants deposited in the ENSEMBL database, 45 point mutations (including 12 frameshift/truncating and 28 missense) are displayed in Fig. 3g and Table 3 (Cai et al., 2019; Carvill et al., 2015; Halvorsen et al., 2015; Islam et al., 2018; Johannesen et al., 2018; Mattison et al., 2018; Palmer et al., 2016; Posar & Visconti, 2019; Rauch et al., 2012; Wang et al., 2020; Zech et al., 2017). The mutations are linked to impaired cognitive development, several epileptic seizure types and mild to moderate ID (mostly language impairment) in the afflicted individuals. Most patients exhibit absence seizures, with the characteristic spike-wave discharges in the electroencephalogram, and behavioral disorders. These symptoms are reminiscent of the phenotypes observed in *SLC6A1* $^{-/-}$ mice

(Chen et al., 2015; Cope et al., 2009). Myoclonic atonic/astatic epilepsy (Doose Syndrome or MAE, OMIM#:616421) was seen in roughly half the patients, while the others either exhibited different types of generalized or focal epilepsies. Behavioral consequences include aggressiveness, irritability, hyperactivity, ASD and attention deficits (Johannesen et al., 2018). Several de novo missense variants associated with GAT1 have also been implicated in increased susceptibility to autism and schizophrenia (Rees et al., 2020; Satterstrom et al., 2018). Most of the mutations associated with epileptic seizures are de novo, but some are transmitted in an autosomal dominant manner (Carvill et al., 2015; Halvorsen et al., 2015; Johannesen et al., 2018). The dominant negative effect can be rationalized by GAT1 forming constitutive oligomers (see Section 9 for further details, Schmid et al., 2001).

Only a fraction of all GAT1 mutations, reported to date, has been characterized. For instance, the p.(G94E), p.(W235R), p.(F270S), p. (I272del), p.(Y445C), p.(W496*) and p.(G550R) variants showed uptake levels ranging from 0 to 27% of wild type activity (Mattison et al., 2018). Their trafficking is yet to be assessed, i.e. the loss-of-function phenotypes may be due to ER-retention or inactivity at the plasma membrane. Complete functional and trafficking data are available for the p.(G234S) variant identified in a patient with the Lennox-Gastaut syndrome (Cai et al., 2019). Its cell surface expression and GABA uptake were reduced by 30% and 70%, respectively, indicative reduced protein stability, surface trafficking and catalytic transport cycle. The p. (P361T) variant, reported in a patient afflicted with absence and atonic seizures and ASD, is folding-deficient (Wang et al., 2020), it has decreased protein expression, increased ER-retention (determined by immunoblots and confocal microscopy) and > 70% reduction in GABA uptake. Variants such as p.(G362R) (Halvorsen et al., 2015), p.(R44Q) and p.(A288V)(Carvill et al., 2015), are predicted to mimic the synthetic loss-of-function mutations at equivalent residues (Ben-Yona & Kanner, 2013; Rosenberg & Kanner, 2008; Zomot & Kanner, 2003).

The remainder of the known GAT1 variants (Fig. 3g and Table 3) compel functional and biochemical analyses. Mutations that abolish GAT1 activity can compromise synaptic inhibition, as vesicle refilling is contingent on synaptic GABA-uptake. Thus, the depletion of vesicular GABA-stores and the subsequent loss of inhibitory input becomes a possible long-term consequence of GAT1 dysfunction. This prompts one to ruminate over the diversity in phenotypic consequences among the GAT1 variants. It is conceivable that mutations induce variable effects on substrate translocation rates, transport-associated currents, GAT1 delivery to the presynaptic specialization, its internalization from the cell surface and possibly affected interactions with regulatory proteins. This calls for a detailed characterization of pathogenic GAT1 mutants. At this point, no disease relevant mutations have been identified in GAT2, GAT3 and BGT1.

4.2 Norepinephrine transporter (NET; *SLC6A2*)

The human NET was the first *SLC6* transporter, in which a point mutation was reported to trigger protein misfolding with accompanying functional defects (Shannon et al., 2000). The patient (and her identical twin) harbored a loss-of-function p.(A457P) variant, associated with chronic orthostatic intolerance (OMIM# 604715). This condition is manifested by excessive sympathetic activation on physiological cues, inducing the following symptoms:

abnormally high plasma norepinephrine concentrations (particularly when standing upright) leading to postural tachycardia, syncope, light-headedness and altered mental activity. The patient was heterozygous for the mutation, indicative of an autosomal dominant mode of inheritance. This was also confirmed biochemically; the p.(A457P) (Table 3) mutant exhibited a severely reduced norepinephrine uptake (<2% of wild type NET) and exerted a dominant-negative effect on wild type NET uptake activity (Hahn, Robertson, & Blakely, 2003). Knock-in mice harboring one allele of p. (A457P) recapitulate the tachycardia (postural hypotension is difficult to reproduce in a 4-legged animal); in addition, they are more anxious, when tested in typical paradigms (elevated plus maze, open field test), but show less signs of behavioral despair in the tail suspension test (Shirey-Rice et al., 2013). Interestingly, in $NET^{+/-}$ mice, substrate uptake is essentially comparable to that seen in wild type animals, despite NET protein levels being reduced by 50%. This alludes to compensatory activation of the transporter by an unknown mechanism. Despite the (near) normal transport activity, $NET^{+/-}$ mice are more anxious, when challenged in the open-field and elevated plus maze tests (Fentress et al., 2013). Similar to p.(A457P) knock-in mice, homozygous $NET^{-/-}$ mice showed less behavioral despair in the forced swim and tail-suspension tests (Xu et al., 2000). In rodents, this response can be elicited by acute administration of antidepressants, which block NET and/or SERT. In fact, this antidepressant-like behavior of $NET^{-/-}$ mice is not further enhanced upon administration of NET-selective antipressants or by an SSRI (selective serotonin-reuptake inhibitor) (Xu et al., 2000). Taken together, the data highlight the complex liaison between transporter activity, maintenance of vesicular stores and synaptic transmission. In NET deficiency, vesicular stores appear to be adequately sustained; the phenotypic consequences are hence more likely to arise from altered rates of norepinehrine clearance from the synaptic cleft.

Apart from p.(A457P) variant, there are several single nucleotide polymorphisms (SNPs) in NET, with altered functional activity and clinical relevance to cardiovascular disorders and the long-QT syndrome (reviewed by Hahn & Blakely, 2002, 2007). One of the SNPs p.(A369P), present at a minor allele frequency of 5%, leads to abolished surface NET expression and concomitant NE clearance presumably due to misfolding (Hahn, Mazei-Robison, & Blakely, 2005). An ADHD-associated variant p.(T283R) (present at a frequency of 0.42%) shows a 30–50% reduction in substrate transport (Hahn, Steele, Couch, Stein, & Krueger, 2009). The trafficking of this variant is yet to be examined.

4.3 Dopamine transporter (DAT; *SLC6A3*)

DAT knockout ($DAT^{-/-}$) mice are prone to early death (Giros, Jaber, Jones, Wightman, & Caron, 1996) and require a high-calory diet to reach adulthood. They maintain vesicular dopamine stores by upregulating dopamine synthesis (Efimova, Gainetdinov, Budygin, & Sotnikova, 2016; Gainetdinov, 2008). Their hyperactivity can be accounted for by increased dopamine levels in the synaptic cleft. Approximately a third of all $DAT^{-/-}$ mice sporadically develop a progressive locomotor disorder, characterized by a loss of striatal GABAergic medium-sized spiny neurons. The previously described hyperactive animals developed atactic gait abnormalities, which progress to tremor, weight loss and subsequent death (Cyr et al., 2003). *Drosophila melanogaster* harboring disrupted dDATs are termed fumin

(Japanese for sleepless): they are hyperactive and have a greatly reduced rest or “sleep” time (Kume et al., 2005).

Loss-of-function point mutations in human DAT trigger the syndrome of Parkinsonism and dystonia, with either infantile, juvenile or adult disease onset (Kurian et al., 2009). The infantile and juvenile forms are collectively known as the dopamine transporter deficiency syndrome (DTDS, OMIM#613135). DTDS was first discovered in infants, who manifested abnormal motor development (resting tremor, muscle rigidity, slow dystonic limb movements, abnormal muscle contractions, difficulties in feeding and global developmental delay). So far, at least 14 non-synonymous exonic variants, 3 truncated exonic variants, 7 intronic (including splice site) variants and 1 in frame variant have been identified among DTDS patients (Baga et al., 2020; Heidari, Razmara, Hosseinpour, Tavasoli, & Garshasbi, 2020; Kurian et al., 2009; Kurian et al., 2011; Kuster et al., 2018; Ng et al., 2014; Puffenberger et al., 2012; Yildiz, Pektas, Tokatli, & Haliloglu, 2017). All DTDS mutations (Fig. 3h and Table 3) are inherited in an autosomal recessive manner; patients being either homozygotes or compound heterozygotes. With the exception of p.(R219G/S) and p.(G380_K384delinsE), all exonic mutations have been fully characterized (Asjad et al., 2017; Beerepoot, Lam, & Salahpour, 2016). These variants are folding-deficient, evident from increased complex formation of variants DATs with the cytosolic chaperone HSP70-1A (for details, see Section 9).

The age of onset and the phenotypic spectrum of DTDS appear to be governed by the basal uptake levels of the mutants: e.g., patients harboring the A314V mutation presented DTDS-associated symptoms in adolescence (11 years). This is attributable to A314V having the highest uptake capacity among the known DTDS variants (i.e. 8% of wild type levels) (Ng et al., 2014). This conjecture is further supported by the discovery of a DAT variant identified in a patient with adult onset of Parkinsonism and ADHD (Hansen et al., 2014). The compound heterozygote patient, expressed p.(I312F) and p.(D421N) mutations (Fig. 3h). Individually, p.(I312F) shows ~50%, while p.(D421N) shows only <10% of wild type DAT uptake levels. If both mutants are co-expressed, uptake is reduced to ~30%. The mutants traffic normally to the cell surface and the lack of catalytic activity is caused by perturbed binding of the ligand and Na^+ ions to p.(D421N). Both mutants additionally exhibit gain-of-function electrophysiological properties: p.(I312F) shows a large anion conductance and p.(D421N) a large constitutive leak current (Herborg, Andreassen, Berlin, Loland, & Gether, 2018) that may exacerbate reduced DAT function. In addition, p.(D421N) supports anomalous dopamine efflux. Another variant with preserved trafficking, but functional deficits is p.(ΔN336), an in-frame deletion of N336 recently identified in a heterozygous patient suffering from ASD (Campbell et al., 2019). This variant is thought to assume a rate limiting “half-open and inward facing state”, that leads to abrogated dopamine uptake.

A knock-in model in *Drosophila melanogaster* displayed hyperactivity, impaired social interactions and other behavioral equivalents of ASD. The ADHD-associated SNP, encoding the p.(V382A) variant shows significantly reduced uptake and ligand binding compared to the wild type DAT (Lin and Uhl, 2003). This variant is likely misfolded, trafficking-deficient

and impedes wild type DAT delivery to the cell surface, suggestive of a dominant variant effect.

In addition to the loss-of-function mutations, other DAT variants showing defects unrelated to trafficking and catalytic transport have been reported in a clinical setting (Fig. 3h and Table 3). For instance, the p.(A559V) and p.(T356M) variants were screened in patients with bipolar disorder, ASD or ADHD (Bowton et al., 2014; Grünhage et al., 2000; Hamilton et al., 2013; Mazei-Robison, Couch, et al., 2005). These variants exhibit spontaneous anomalous dopamine efflux (Hamilton et al., 2015; Herborg et al., 2018; Horschitz, Hummerich, Lau, Rietschel, & Schloss, 2005; Mazei-Robison, Bowton, et al., 2005). This functional feature was validated in behavioral phenotypes of knock-in animal models (Davis et al., 2018; DiCarlo et al., 2019; Mergy et al., 2014). The functional phenotype of the p.(E602G) variant, identified in a patient with bipolar disorder (Grünhage et al., 2000), is still unknown. There is conflicting evidence in the literature regarding the functional consequences of this variant (Herborg et al., 2018; Horschitz et al., 2005). The variant p.(R615C) (Fig. 3h) was reported in a heterozygous ADHD patient. It supports dopamine uptake to levels comparable to wild type DAT, but underogoes more rapid constitutive and amphetamine-insensitive endocytosis and cell surface recycling. Interestingly, the variant localizes in membrane microdomains different from those inhabited by the wild type DAT (Herborg et al., 2018; Kovtun et al., 2015; Sakrikar et al., 2012). These alterations lead to a gain-of-function effect, reversible by an endocytic brake through cdc42-activated non-receptor tyrosine kinase Ack1 (Wu, Bellve, Fogarty, & Melikian, 2015).

4.4 Serotonin transporter (SERT; *SLC6A4*)

SERT knockout ($SERT^{-/-}$) mice are viable and do not have any gross developmental abnormalites (Bengel et al., 1998). They show virtually no serotonin uptake and 60–80% reduction in serotonin levels in various brain regions. These mice are insensitive to the psychostimulant action of 3,4-methylenedioxymethamphetamine (MDMA). $SERT^{-/-}$ mice exhibit anxiety-related behavior, hypolocomotion, predisposition to stress and show less aggression and social interaction (Holmes, Murphy, & Crawley, 2003; Kalueff et al., 2007). The behavioral changes in these mice provide insights into the role of SERT in the progression of affective disorders and other neuropsychiatric diseases in people (Holmes et al., 2003; Kalueff et al., 2007). The only human SERT variant that leads to loss of transport activity is the rare p.(P339L) variant (Glatt et al., 2001), which has dramatically reduced surface expression and serotonin uptake due to misfolding and ER retention (Prasad et al., 2005). Yet, this variant has not been causally linked to any human disease. However, many novel molecular features involved in the folding and trafficking of SERT have been revealed from studies on synthetic misfolded SERT mutants (for details, see Section 9).

Disease relevant point mutations in human SERT are hypermorphic. The mutations were identified by screening patients afflicted with OCD, ASD, eating disorders and other neuropsychiatric disorders (Camarena, González, Hernández, & Caballero, 2012; Delorme et al., 2004; Hernández-Muñoz et al., 2020; Moya et al., 2013; Ozaki et al., 2003; Sutcliffe et al., 2005; Voyiaziakis et al., 2011; Wendland et al., 2008). They are displayed in Fig. 3i and Table 3, and most have been extensively characterized (Kilic et al., 2003; Prasad et al.,

2005; Prasad et al., 2009). They show increased rates of serotonin uptake by: 1) increased substrate affinity and/or 2) maximal uptake velocity and/or 3) altered patterns of surface regulation. For instance, the p.(I425V) variant increased the binding and catalytic activity of SERT (reduced K_M for 5-HT transport and higher Bmax of radioligand binding), but its trafficking profile remained unchanged relative to wild type (revealed by immunobloting). These properties render p.(I425V) constitutively active in a nitric oxide-independent manner (Kilic et al., 2003). The p.(G56A) variant exhibits elevated basal phosphorylation (Prasad et al., 2009) and assumes a high affinity conformation, which causes an insensitivity to regulation by phosphatases (Quinlan et al., 2019; Quinlan et al., 2020). Experiments on knock-in p.(G56A) mice indicated behavioral responses consistent with autistic symptoms in people, i.e. repetitive behavior, deficits in social interactions and in multisensory processing (Siemann et al., 2017; Veenstra-VanderWeele et al., 2012). Other SERT variants such as p.(F465L) and p.(L550V) preserve their sensitivity to phosphorylation similar to wild type SERT, but display increased trafficking to the cell surface, consequently augmenting catalytic serotonin uptake (Prasad et al., 2009). Uncharacterized SERT variants comprise p.(L90F), p.(N211S), p.(V274I) and p.(F474L).

4.5 Glycine transporter-1 (GlyT1; SLC6A9) and -2 (GlyT2; SLC6A5)

In the brain, GlyT1 is predominantly expressed in astrocytes. GlyT1 knockout mice ($GlyT1^{-/-}$) are born without any gross abnormalities. Most of these mice die some 6–14 h after birth, largely due to wasting and dehydration caused by their inability to suckle (Eulenburg, Retiounskaia, Papadopoulos, Gomeza, & Betz, 2010; Gomeza et al., 2003; Tsai et al., 2004). Glycine uptake in the frontal brain, brain stem and spinal cord is also greatly diminished in $GlyT1^{-/-}$ mice. In addition, they show severe deficits in motosensory functions, breathing and respiratory rhythmic activity at the pre-Bötzinger complex, arising due to excess glycine receptor activation in the respiratory neurons. Some of the conditional knockout mice survive the critical postnatal period and can have a normal life span. The shocked (sho) gene encodes a mutated GlyT1 gene (G81D in TM2) in zebrafish: the mutation abrogates glycine transport. Homozygous fish embryos do not swim and fail to mount a normal escape response to tactile stimuli (Cui et al., 2005; Mongeon et al., 2008).

Deficits in $GlyT1^{-/-}$ mice parallel the symptoms in people suffering from glycine encephalopathy or non-ketotic hyperglycinemia (NKH), an autosomal recessive disorder with a defective glycine cleavage enzyme complex (Applegarth & Toone, 2006). NKH patients, who were negative for mutations in genes involved in the glycine cleavage system, were subsequently screened for mutations in GlyT1 (Alfadhel et al., 2016; Kurolap et al., 2016): the GlyT1 variants, p.(K310Ffs*31), p. (Q573*) and p.(S407G) (Fig. 3j) were identified in homozygous individuals from 3 unrelated consanguineous families (Table 3). 3 of the 6 affected individuals died within 7 months of age (one death was pre-natal) due to respiratory failure. All patients manifest encephalopathy, shallow breathing, hyperekplexia (exaggerated startle reflex, see below), hypotonia, which paradoxically progresses to muscle hypertonicity resulting in arthrogryposis (joint contracture), and facial dysmorphism (Alfallaj & Alfadhel, 2019). The 2 surviving patients overcame respiratory failure, in a manner similar to some $GlyT1^{-/-}$ mice (see above). Though none of the mutants have been characterized, p. (K310Ffs*31) and p.(Q573*) are predicted to be non-functional

transporters, with the former truncated after the 4th transmembrane domain and the latter harboring a premature stop codon in the 11th transmembrane domain.

In contrast to GlyT1, GlyT2 is expressed exclusively in neurons. It is delivered to presynaptic glycinergic terminals, where it retrieves glycine from the synaptic space into the presynaptic neurons. GlyT2 knockout ($GlyT2^{-/-}$) mice exhibit complex neuromotor deficits that culminate in premature death, after the second post-natal week (Gomeza et al., 2003): on postantal day 10 (P10), $GlyT2^{-/-}$ mice display strong and spontaneous tremor and muscular rigidity. They fail to right themselves when turned on their back. When suspended from their tails, they respond with hind feet clasping. Death occurs due to troubled feeding, desiccation and continued convulsions. These deficits can rationalized by the reduction of glycinergic control in motoneurons.

The complex neuromotor phenotype in $GlyT2^{-/-}$ mice is reminiscent of hyperekplexia (startle disease) in people and other mammals harboring loss-of-function mutations in the key players of glycinergic neurotransmission (Davies et al., 2010; Gill et al., 2011, 2012; Harvey, Topf, Harvey, & Rees, 2008). Hereditary hyperekplexia (human startle disease) is an inherited and genetically heterogenous neurological disorder characterized by an exaggerated startle response to unexpected stimuli, general stiffness following startle events, impaired central pain modulation and other symptoms depending on the gene mutated (Thomas et al., 2013). Thus, startle disease patients were screened for exonic variants in the pre-synaptic neuronal GlyT2 (Carta et al., 2012; Dafsari et al., 2019; Eulenburg et al., 2006; Giménez et al., 2012; Kitzenmaier et al., 2019; Masri et al., 2017; Mineyko et al., 2011; Rees et al., 2006). These studies led to the identification of ~35 disease variants (Fig. 3j and Table 3). In addition to hyperekplexia, most patients with GlyT2 mutations also exhibit hypotonia, recurrent infantile apneas and delayed development (Thomas et al., 2013). The majority of these patients are either homozygotes or compound heterozygotes. Some patients inherit GlyT2 mutations in an autosomal dominant manner (López-Corcuera et al., 2019). The dominant negative effect of the p. (S512R) variants can be rationalized by GlyT2 forming constitutive oligomers (for further details see Section 9). The autosomal dominant p. (Y705C) variant, on the other hand, shows milder functional phenotypes (Giménez et al., 2012). Functional defects, in this variant, arise from the substituted cysteine forming aberrant disulfide bonds with other cysteines in the transporter (as confirmed by cysteine accessibility experiments). Further evidence in support of this proposed mechanism is the rescue of p.(Y705C) to wild type uptake levels by reducing agents (e.g. dithiothreitol) that break such erroneous disulfide bonds (Giménez et al., 2012).

A considerable fraction of GlyT2 disease variants arise from nonsense or frame shift mutations: these truncated transporters are ER-retained (shown by confocal miscroscopy and immunoblotting) and consequently show no glycine uptake (Carta et al., 2012; Eulenburg et al., 2006; Rees et al., 2006). Furthermore, many variants can readily traffic to the plasma membrane, but impair the catalytic function of GlyT2. For instance, the defects in p.(W482R), p.(N509S) and p. (A275T) GlyT2 variants were characterized electrophysiologically. The p.(W482R) variant, showed Na^+-dependent transient currents, but no glycine induced currents (Rees et al., 2006). This is indicative of normal surface expression, but perturbed glycine binding in this variant. The p. (N509S) and p.(A275T)

variants, on the other hand, showed reduced and voltage-dependent potencies in mediating glycine-induced currents, relative to wild type GlyT2 (Carta et al., 2012; Rees et al., 2006). These variants evidently affect Na^+ binding and glycine-Na^+ coupling, which are essential for the GlyT2 transport cycle. Molecular modeling studies determined the effects of mutations on the GlyT2 catalytic cycle, revealing that the amino acid transition in the p.(S513I) variant precludes Cl^- binding (Carta et al., 2012). Other variants are yet to be characterized.

4.6 Pathological variants in other SLC6 transporters

SLC6A6 encodes TauT, a plasmalemmal transporter which primarily mediates uptake of taurine and, to a lesser extent, GABA (Ramamoorthy et al., 1994; Tomi, Tajima, Tachikawa, & Hosoya, 2008; Uchida et al., 1992). Taurine is a sulfur containing amino acid that is mainly supplied by dietary intake and its entry into various tissues is achieved by TauT (Schuller-Levis & Park, 2003). Taurine is abundantly present in many organs including the brain and retina. It acts as an osmolyte and anti-oxidant in cells; underlying its pleiotropic action in the immune system and neurons. $TauT^{-/-}$mice exhibit negligible levels of taurine uptake in all tissues, resulting in multisystem failure, lower body mass, impaired fertility, cardiomyopathy, progressive and severe retinal degeneration, liver fibrosis, age-dependent hearing loss and reduced exercise capacity by skeletal muscles (Heller-Stilb et al., 2002; Ito et al., 2008; Warskulat et al., 2007). While the role of TauT deletion in the advent of the 3p-syndrome (OMIM#613792, Patel et al., 1995; Han, Budreau, Chesney, & Sturman, 2000) is unclear, disease relevant loss-of-function mutations in TauT mimic the phenotypic features of $TauT^{-/-}$mice. Homozygous deletion of the splice site between exon 8 and 9 of TauT was associated with dilated cardiomyopathy in one patient (Shakeel, Irfan, & Khan, 2018). Biallelic mutations in TauT that encode the p.(A78E) and p.(G399V) variants (Table 4) were identified in siblings from 2 unrelated families suffering from rapidly progressive childhood retinal degeneration (Ansar et al., 2020; Preising et al., 2019), accompanied by cardiomyopathy (Ansar et al., 2020). TauT-A78E and G399V were correctly delivered to the cell surface, but their transport activity was impaired (5–15% uptake of wild type TauT). Molecular modeling predicts that glutamate E78 substitution forms an anomalous salt bridge with arginine R284. This salt bridge may stabilize transporter conformations that do not affect folding and trafficking but may perturb taurine and co-substrate binding (Preising et al., 2019). Molecular modeling of the p.(G399V) variant predicted that the valine substitution indirectly slows down conformational transitions needed for the progression along the Taut transport cycle (Ansar et al., 2020). Patients harboring this variant were orally administered high taurine doses, which improved visual performance and remedied the cardiomyopathy symptoms due to taurine supplementation by an unknown transporter.

SLC6A7 encodes the transporter for L-proline (ProT) in the human brain (Shafqat et al., 1995). Proline is a non-essential amino acid, playing a crucial role in several metabolic pathways (e.g. the synthesis of arginine and glutamate in mitochondria). Many human inherited disorders associated with dysfunction in proline metabolism have been reported, some with neurological consequences (Wyse & Netto, 2011). There are no known disease-associated variants in ProT to date, but a large deletion of the 5q32 region,

encompassing the *SLC6A7* gene, has been identified in patients with intellectual disabilities and mandibulofacial dysostosis (Vincent et al., 2014).

SLC6A8 encodes the creatine transporter-1 (CRT1, Nash et al., 1994; Dai, Vinnakota, Qian, Kunze, & Sarkar, 1999). Creatine, a precursor of phosphocreatine, acts as an energy buffer to maintain cellular ATP levels (Rae & Bröer, 2015). Over 80 point mutations in the human CRT1 have been identified (Table 4), a large fraction of which code for missense variants (Farr, El-Kasaby, Freissmuth, & Sucic, 2020). Loss-of-function mutations in CRT1 lead to an X-linked creatine transporter deficiency (CTD), clinically manifested as mild to severe intellectual disability, developmental delay, epilepsy and ASD (OMIM#300352, Salomons et al., 2001; Van De Kamp, Mancini, & Salomons, 2014). To date, only a few studies have assessed the molecular features of CTD, by examining their folding and trafficking profiles (Betsalel et al., 2012; Salazar et al., 2020; Uemura et al., 2017; Valayannopoulos et al., 2013). We recently classified 16 loss-of-function missense CRT1 variants as misfolded (see Section 9, El-Kasaby et al., 2019). CRT1 knockout ($CrT^{-/y}$)micehaveim-paired learning and memory acquisition reflecting the global intellectual deficits in CTD patients (Skelton et al., 2011). *SLC6A10* or CRT2, located on chromosome 16, is a pseudogene paralogous to CRT1. *SLC6A14* encodes a transporter for neutral, dipolar and basic amino acids (ATB^{0+}), primarily expressed in peripheral tissues (Sloan & Mager, 1999). No disease mutations have been identified in ATB^{0+} thus far.

The remaining 6 *SLC6* transporters (*SLC6A15–20*) predominantly transport neutral amino acids (Bröer, 2009). Interestingly, of these neutral amino acid transporters, *SLC6A17* codes for the vesicular transporter NTT4, which is also abundantly expressed in both dendritic and post- synaptic sites of predominantly glutamatergic and some GABAergic synapses (Hägglund et al., 2013; Iqbal et al., 2015). NTT4 mediates Na^+-coupled uptake of alanine, glycine, leucine, proline and glutamate (Parra et al., 2008; Zaia & Reimer, 2009). Disease relevant mutations in NTT4 include 2 missense variants; p.(G162R) and p.(P633R) (Table 4), discovered in unrelated families with an autosomal recessive form of intellectual disability accompanied by progressive tremors, speech impairment and abnormal behavior (OMIM#616269) (Iqbal et al., 2015). In transfected hippocampal neurons, these variants show different phenotypic profiles, evident from confocal microscopy images: 1) the p.(G162R) variant does not exhibit any trafficking deficits, but molecular modeling predicts structural perturbations, which likely impair its transport activity. Through unknown mechanisms, p.(G162R) also appears to suppress dendritic spine formation, alter dendritic morphology and induce global morphological changes in transfected neurons. 2) On the other hand, the trafficking of p.(P633R) to the dendrites is compromised. According to molecular modeling, the substitution of hydrophobic proline, which contacts the membrane lipids, by charged arginine induces anisotropic changes in protein folding. *SLC6A15* encodes the sodium-coupled branched-chain amino acid transporter (SBAT1), a protein abundantly expressed in the brain (Bröer et al., 2006; Takanaga, Mackenzie, Peng, & Hediger, 2005). No point mutations in *SLC6A15* have been reported, but polymorphisms, which reduce the expression of SBAT1 transcripts, have been linked to an enhanced risk for major depression and stress susceptibility (Kohli et al., 2011). *SLC6A16* encodes the poorly characterized NTT5, which is expressed in the brain, among many other tissues (Farmer et al., 2000); no known variants thereof are known as yet.

SLC6A18 (B^0AT3), *SLC6A19* (B^0AT1) and *SLC6A20* (XTRP3) encode amino acid transporters, which are expressed in the kidney and intestine, where they facilitate the absorption of glycine, proline, and neutral amino acids, respectively (Bröer, 2006Mutations in these genes, upon co-inheritance with *SLC36*A2 (proton-coupled amino acid transporter-2, PAT2) mutations, act as modifiers to the complex pathogenesis of digenic iminoglycinuria (OMIM#242600) or hyperglycinuria (OMIM#138500), which results in renal wasting of proline and glycine (Bröer et al., 2008). This study describes 4 B^0AT3 point mutations, including p.(G79S), p.(Y319X), p.(L478P) and p.(G496R), a B^0AT1 splice site variant and a XTRP3 missense variant p.(T199M). The expression of these variants was abstruse in heterologous systems for functional assays, but cRNA injections in oocytes made it possible to examine their trafficking. The p(L478P) variant was similar to wild type B^0AT3, while p.(G79S), p.(Y319X), and p.(G496R) were all trafficking-deficient. It is still unclear whether the missense mutations trigger protein misfolding, or eliminate interactions with collectrin or angiotensin-converting enzyme 2 (ACE2), essential for B^0AT3 trafficking to the plasma membrane (Singer et al., 2009). The p.(T199M) XTRP3 variant had an uncharacterized catalytic deficiency in terms of proline uptake with cellular expression resembling the wild type transporter. Besides, >20 B^0AT1 mutations (Table 4) inherited in an autosomal recessive manner have been identified in patients suffering from the Hartnup disease (OMIM#234500), a syndrome of renal aminoaciduria, variable manifestations of dermatitis, ataxia and seizures (Bröer, 2009; Kleta et al., 2004; Seow et al., 2004). Like B^0AT3, B^0AT1 also requires collectrin and ACE2 for proper surface expression. Only a few Hartnup disease-mutations characterized in functional or biochemical assays show ER-retention (due to misfolding) or trafficking deficits (due to altered interactions with collectrin and/or ACE-2) (Camargo et al., 2009).

5 *SLC17* family: vesicular transporters GLUTs, sialin and VNUT

The *SLC17* family includes 9 structurally related, but functionally divergent proteins. Members *SLC17A1,* 3 and 4, were originally classified as type 1 phosphate transporters; in fact, they transport organic anions including exogenous (e.g. non-steroidal anti-inflammatory drugs/ NSAIDs) and endogenous substrates (urate) (relevant references in Reimer, 2013; Togawa, Miyaji, Izawa, Omote, & Moriyama, 2012; Togawa et al., 2015). SCL17A2 is structurally different and has since been reclassified as a type 2 phosphate transporter (*SLC34A1*). *SLC17A1–4* transporters are expressed in non-neuronal cells of the kidney, liver and the digestive system. No neurological disorders have been linked to their dysfunction yet, whereas mutations in *SLC17A3* lead to hyperuricemia and gout susceptibility in people (OMIM#612671, Jutabha et al., 2010).

5.1 Sialin (AST, ISSD, NSD, SD, SIASD, SLD; SLC17A5)

The ubiquitous and lysosomal membrane-residing sialin (*SLC17A5*) was classically thought to be responsible for the cytosolic efflux of sialic acid from lysosomes (Reimer, 2013) to support recycling of sialylated proteins and lipids (Schauer, 2008). Of recently, sialin has been highlighted for its under-appreciated role in supporting plasmalemmal uptake of nitrate (Qin et al., 2012) and vesicular uptake of glutamate, aspartate and N-acetylaspartylglutamate (Miyaji, et al., 2008; Mochel et al., 2010: Lodder-Gadaczek et al., 2013). It is therefore

not suprising that sialin dysfunction has severe neurological consequences. Patients who recessively inherit point mutations in sialin suffer either from the less severe Salla disease (OMIM# 604369), the more acute intermediate severe Salla disease or the often-fatal infantile sialic acid storage disorder (ISSD, OMIM# 269920) (Aula et al., 2000; Verheijen et al., 1999). These patients commonly present cerebellar atrophy, hypotonia and intellectual disabilities; the symptoms are markedly more severe in ISSD patients that include motor retardation, coarse facial features, hepatospleno- and cardiomegaly, failure to thrive, gross developmental delay and death at age < 5 years (Aula et al., 2000; Varho et al., 2002).

Barring a few exceptions, the basal function of the mutants seems to determine the severity of disease symptoms (Aula, Jalanko, Aula, & Peltonen, 2002; Miyaji et al., 2008; Miyaji et al., 2011; Morin et al., 2004; Wreden, Wlizla, & Reimer, 2005). For instance, ISSD associated sialin variants such as p.(Δ268–272) and p.(H183R) show 1) appropriate targeting to lysosomal and (presumably) vesicular membranes, 2) indistinguishable glutamate and aspartate uptake features relative to wild type sialin and 3) complete loss of sialic uptake capacity. Hence, it is conceivable that the onset of severe phenotypes in ISSD may arise from a type of lysosomal storage disease (Sagné & Gasnier, 2008), wherein the faulty accumulation of sialic acid in lysosomes predominantly leads to fatal neuronal cell death. The Salla disease associated p.(R39C) variant, on the other hand, posesses 1) 10–50% of wild type sialin uptake activity, 2) a slow, yet appreciable trafficking, compared to wild type, to the lysosome at steady state and 3) little to no glutamate and aspartate uptake capacity. It is therefore plausible that neurological symptoms of Salla disease can be explained by lack of vesicular refilling of glutamate and aspartate by this variant. This may, in turn, thwart cognate neuronal signaling. Sialin is hence more than a mere lysosomal transporter; as it may play a key role in neurological function and disorders (Robak et al., 2017) and in phenotypic variability, i.e. from ISSD to Salla disease. These possibilities ought to be explored in future knock-in animal models of sialin variants. The sialin knockout (*SLC17A5*$^{-/-}$) mice display symptoms reminiscent of ISSD in people: gait abnormality, motor retardation, seizures, hypomyelination, leukoencephalopathy and premature death during the third postnatal week (Prolo et al., 2009; Stroobants et al., 2017).

5.2 VGLUT1-3 (1, 2: BNPI, DNPI; 3: DFNA25; *SLC17*A6-8)

Glutamate is packaged into synaptic vesicles by vesicular glutamate transporters (VGLUT 1–3). VGLUTs transport glutamate (L-enantiomer over D-), but not aspartate or glutamine. The K_M of VGLUTs for glutamate is ~2 mM, commensurate with its cytosolic levels in neurons. VGLUT1 (*SLC17A7*) was originally reported as a brain-specific Na^+-dependent inorganic phosphate transporter (BNPI, Ni et al., 1994). It was subsequently shown to be localized in pre-synaptic terminals of neurons, and to mediate vesicular glutamate uptake (Bellocchio, Reimer, Fremeau Jr, & Edwards, 2000; Takamori, Rhee, Rosenmund, & Jahn, 2000). VGLUT2 (*SLC17A6*) was first referred to as the differentiation-associated Na^+-dependent inorganic phosphate transporter (DNPI), because its expression levels were upregulated upon differentiating a rat pancreatic tumour cell to neuronal cells; Aihara et al., 20005). VGLUT2 is found primarily at vesicular membranes of gluta-matergic synapses, but its distribution in the brain is distinct from VGLUT1; in fact, their expression is, for most parts, mutually exclusive (El Mestikawy, Wallén-Mackenzie, Fortin, Descarries, & Trudeau,

2011; Fremeau Jr., Voglmaier, Seal, & Edwards, 2004). The presence of glutamate release in non-glutamatergic neurons led to the identification of VGLUT3 (*SLC17A8*) (Gras et al., 2002; Schäfer, Varoqui, Defamie, Weihe, & Erickson, 2002).

VGLUT1 knockout (VGLUT1$^{-/-}$) mice suckle and move normally in their first 2 postnatal weeks. However, after the third week, they fail tothrive(Fremeau Jr. et al., 2004; Wojcik et al., 2004): their body weight is lower than that of wild type and VGLUT1$^{+/-}$ heterozygous littermates. VGLUT1$^{-/-}$ mice die of progressive emaciation between 18 and 21 days post birth if not separated from their littermates (Fremeau Jr., Kam, et al., 2004). This coincides with the time period, where expression of VGLUT2 and of VGLUT1 is down- and upregulated, respectively (FremeauJr.,Kam,etal.,200 ; Wojcik et al., 2004). The surviving VGLUT1$^{-/-}$ mice progressively lose their motor coordination (climbing fibers in the cerelebellum are rich in VGLUT1), they turn blind and have a heightened startle response (Fremeau Jr., Kam, et al., 2004).

VGLUT2 knockout mice (VGLUT2$^{-/-}$) die at birth (Moechars et al., 2006; Wallén-Mackenzie et al., 2006), which is consistent with the high expression of VGLUT2 in early infancy (see above). Death is due to respiratory arrest: the mice fail to generate a central respiratory rhythm due to the lack of activity in neural circuits at the pre- Bötzinger (PBC) area (Moechars et al., 2006; Wallén-Mackenzie et al., 2006).

The VGLUT3 knockout (VGLUT3$^{-/-}$) phenotype was originally described in zebrafish (Obholzer et al., 2008): As in zebrafish models, VGLUT3$^{-/-}$ mice are deaf; their auditory nerves are not responsive to acoustic stimuli (Ruel et al., 2008; Seal et al., 2008). VGLUT3 is expressed in hair cells of the cochlea; in the absence of VGLUT3, the terminals of auditory nerve fail to be excited. Interestingly, contrary to asteroid zebrafish larvae, VGLUT3$^{-/-}$ mice have a normal vestibular response (Seal et al., 2008), but they develop epileptic (generalized, synchronous) discharges in the electroencephalogram. These seizure equivalents are, however, not accompanied by motor symptoms, or by behavioral changes (Seal et al., 2008).

Only one study reported human variants of VGLUT1 and their association with mental disorders: In a Taiwanese case-control study, a cohort of 376 schizophrenic patients was matched with 368 control subjects (Shen et al., 2009). 2 missense variants L516M and P551S (Fig. 3k), discovered in the coding region of VGLUT1, both located in exon 12 (Table 5), were detected explicitly in the patient cohort. The functional consequences of these mutations have not yet been investigated. The screen for VGLUT2 variants among the same cohort of patients did not disclose any coding variants in the *SLC17*A6 gene (Shen et al., 2010).

Point mutations in VGLUT3 were reported in patients suffering from autosomal dominant non-syndromic sensorineural deafness (DFNA, loci DFNA25, OMIM#605583), closely resembling presbycusis. The disorder is characterized by a late-onset hearing loss of predominantly high frequency sounds, which progressively intensifies with age (Greene et al., 2001). The VGLUT3 mutation p.(A211V) (Fig. 3k) was identified in several members of 2 supposedly unrelated American families, who probably shared a common ancestor

(Ruel et al., 2008). This variant caused a ~ 65% reduction in VGLUT3 protein levels in transfected cells in vitro and in vivo in neurons of the CNS, although its functional impact was modest (Rametetal.,201). The mutation appears to additionally alter the uniform distribution of VGLUT3 across the vesicular pools, explaining the reduced frequencies of mini-excitatory postsynaptic currents in transfected hippocampal autaptic neurons, relative to wild type VGLUT3 controls. While mechanistic insights from VGLUT3$^{-/-}$ mice and zebrafish (see above) can account for the hearing loss in patients afflicted with DFNA25, they do not readily rationalize the delayed onset, the slow progression and the preferential loss of function in hair cells at the base of the cochlea. Knock-in mice harboring 2 alleles for the homologous p.(A224V) recapitulate the slow onset of hearing loss and the predilection for the frequency range of 5–20 kHz; interestingly in these mice the perception of the ultrasonic range is not affected (Joshi et al., 2020). In contrast to the reduced expression in neurons (Rametetal.,201), the levels of p.(A224V) and its distribution in inner hair cells did not differ from the wild type protein. However, the stereocilia bundles were fused in those hair cells required to sense the 5–20 kHz range. This indicates that the primary defect is impaired mechanosensation rather than reduced glutametergic neurotransmission. The candidate link between the mutation and the abnormal assembly of sterocilia is one or several proteins, which – hypothetically – interact with the second intracellular loop (that harbors p.(A211/A224)) of VGLUT3 and is/are required for the assembly of the stereocilia. In 2016, screening for VGLUT3 variants in a South Korean cohort of 87 unrelated patients suffering from DFNA revealed a frameshift mutation (M206Nfs*4, Fig. 3k) in one patient (Table 5). The variant led to a truncated 209 amino acid residue VGLUT3 (Ryu et al., 2016). 2 additional non-synonymous variants were identified, I78V and A374S (Fig. 3k), albeit with minor allele frequencies of 0.1% and 0.2% (from the 1000 Genomes Project Database). The same group discovered a novel splicing variant in a Korean family with 3 generations of DFNA (Ryu et al., 2017). None of the variants have been functionally characterized. It is worth noting that VGLUT3 deficiency in mice results in many behavioral alterations, e.g. increased anxiety (Amilhon et al., 2010), reduced pain threshold (Seal et al., 2009) and increased propensity for cocaine abuse (Sakae et al., 2015). The equivalent changes have not been reported in individuals harboring the mutations.

5.3 VNUT (C20orf59, POROK8; SLC17A9)

SLC17A9 is a synaptic vesicular and lysosomal nucleotide transporter (VNUT), which is expressed in both neuronal and glial cells of the hippo-campus,midbrainandthecerebralcortex(Larssonetal.,2012).VNUTis also expressed in purinergic cells of endocrine glands, such as pancreas, adrenal and thyroid glands, liver and T-cells (Cao et al., 2014; Geisler et al., 2013; Sathe et al., 2011; Sawada et al., 2008; Tokunaga, Tsukimoto, Harada, Moriyama, & Kojima, 2010). VNUT accomplishes the refilling and maintainance of nucleotide (primarily ATP) concentrations at 0.1–1 mM in synaptic vesicles. ATP and other nucleotides are coreleased with neurotransmitters during exocytosis and mediate crucial downstream purinergic responses via purinoreceptors (Miras-Portugal et al., 2019). VNUT knockout mice (VNUT$^{-/-}$) do not display any gross neurological abnormalities or behavioral alterations (Sakamoto et al., 2014). ATP release is abolished and glutamate release reduced, when hippocampal neurons prepared from these animals are depolarized. The absence of ATP-storage and release in spinal dorsal horn

neurons protects against neuropathic pain resulting form peripheral nerve injury (Masuda et al., 2016).

Point mutations of human VNUT were identified in patients suffering from disseminated superficial actinic porokeratosis (DSAP, OMIM#616063; Cui et al., 2014). DSAP is a pre-cancerous skin condition characterized by red brown scaly spots (generally on the limbs) with coronoid lamellae (fine, thready rim on the edge of the spots). 2 VNUT variants, p.(R9C) and p.(R311N), were identified in two unrelated families, and transmissed in an autosomal dominant manner (Table 5). The mutants have not yet been functionally characterized.

6 *SLC18* family: vesicular monoamine, acetylcholine and polyamine transporters

The *SLC18* family of neurotransmitter transporters is comprised of 4 members: *SLC18A1* encodes the vesicular monoamine transporter-1 (VMAT1), *SLC18A2* encodes VMAT2, *SLC18A3* encodes the vesicular acetylcholine transporter (VAChT) and *SLC18B1* encodes the vesicular polyamine transporter (VPAT) (Lawal and Krantz, 2013). *SLC18A1/2* and *SLC18A3* operate in a relay with the plasmalemmal transporters for monoamines (*SLC6A2*, *SLC6A3*, *SLC6A4*) and choline (*SLC5A7*), respectively, to recycle neurotransmitters (norepinephrine, dopamine, serotonin) and the neurotransmitter precursor choline to refill synaptic vesicular stores. These transporters thus afford a constant vesicular pool for synaptic transmission events.

6.1 VMAT1 (*SLC18A1*) and VMAT2 (*SLC18A2*)

SLC18A1/VMAT1 is the predominant transporter in the peripheral nervous system (sympathetic ganglia including the adrenal medulla and some enteric neurons (Erickson, Schafer, Bonner, Eiden, & Weihe, 1996; Lawal and Krantz, 2013). VMAT2, on the other hand, is expressed exclusively in the CNS, particularly in the monoaminegic neurons of the brain stem. Outside the CNS, VMAT2 is also found in histaminergic and monoaminergic cells of the sympathtic ganglia, adrenall medulla, myenteric and submucosal plexus (Erickson et al., 1996; Lawal and Krantz, 2013). With a few notable exceptions (e.g. fenfluramine) VMAT2 has a higher affinity for serotonin, dopamine, norepinephrine, epinephrine, histamine and for exogenous ligands, such as, reserprine, tetrabenazine, amphetamines and the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) as compared to VMAT1; the difference in affinity varies from modest 2–3 fold (for serotonin, dopamine, (nor)epinephrine, MDMA and reserpine) to pronounced (20–30 fold for histamine and amphetamine), while tetrabenazine does not have any appreciable affinity for VMAT1 (Erickson et al., 1996).

VMAT2 knockout ($VMAT2^{-/-}$) mice die within days of birth (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997). Prior to their death, the mice feed poorly, display hypoactivity and prone to hypothermia. Their brains contain very low levels of monoamines (1–6% of wild type levels), but normal levels of monoamine metabolites indicating an exceedingly high turnover. Mice with one VMAT allele knocked out ($VMAT2^{+/-}$) or with hypomorphic

alleles are phenotypically comparable to wild type mice and survive into adulthood. However, they show 30–50% reduction in monoamine levels in the brain. In addition, their locomotor response to transport inhibition by cocaine (Wang et al., 1997) and to amphetamine-induced reverse transport are augmented (Mooslehner et al., 2001; Takahashi et al., 1997; Wang et al., 1997). This is justified by supersensitivity of (postsynaptic) dopamine receptors (Mooslehner et al., 2001; Wang et al., 1997). VMAT1 knockout ($VMAT1^{-/-}$) mice display neurocognitive deficits (Multani et al., 2013), suggesting that VMAT1 has physiological roles in the CNS, along with the previously established functions in peripheral neuroendocrine cells.

Although disease-relevant mutations have not been discovered in VMAT1 to date, polymorphisms and rare variants have been implicated in an increased risk for neurological disorders (Bly, 2005; Lohoff et al., 2006; Lohoff et al., 2014). For instance, the p.(T136I) polymorphism is present at higher frequencies in bipolar depression patients, relative to controls (Lohoff et al., 2006). The p.(S98T) variant, on the other hand, was identified in 2 schizophrenic individuals (Lohoff et al., 2014). Both variants significantly increase monoamine uptake in intracellular vesicular compartments of heterologous cells (Lohoff et al., 2014). The molecular causes of this increase in catalytic activity of these as well as other VMAT1 variants are still unknown. Point mutations in VMAT2 (Fig. 3k, Table 5), on the other hand, are known to have neurological consequences. These mutations are inherited in an autosomal recessive manner and lead to a form of infantile Parkinsonism and dystonia, referred to as brain monoamine vesicular transport disease (OMIM#618049). The first identified VMAT2 mutation encodes the p.(P387L) variant found in several members of an extended consanguineous family (Rilstone, Alkhater, & Minassian, 2013). The patients manifested a myriad of symptoms indicative of monoaminergic deficiencies, including dystonia and parkinsonism (reflecting a lack of dopamine), sleep and mood disorders (reflecting a lack of serotonin) and postural hypotension (reflecting a lack of norepinephrine). Immunoblotting did not reveal any differences in the levels and trafficking profiles between the wild type and p.(P387L) proteins. However, serotonin uptake by the mutant was remarkably reduced, alluding to a loss-of-function phenotype. Treatment with pramipexole (D2-receptor agonist) remedied the condition; it corrected the movement deficits and allowed for motor development. Similar responses were seen in additional studies (Jacobsen et al., 2016; Padmakumar et al., 2019; Rath et al., 2017), which identified p.(P237H) and p.(P316A) variants in children from unrelated families exhibiting the symptoms attributable to monoamine deficiency as mentioned above. The variants p.(P237H) and p.(316A), were not functionally characterized. The p.(P387L) variant was also identified in a patient afflicted with severe microphthalmia (an eye developmental disorder with abnormally small eyes), microcephaly, epilepsy, failure to thrive and global developmental delay (Patel et al., 2018). This observation suggests that the development of the human eye involves VMAT2. Upon knock-in of p.(P237H) or p. (P387L) in Caenorhabditis elegans transporter homologue (cat1), the feeding of transgenic worms was impaired (Young et al., 2018): they had reduced grazing and pharyngeal pumping responses, which are regulated by serotonin and dopamine. This points to these 2 VMAT2 variants being loss-of-function mutants.

6.2 VAChT (SLC18A3)

The vesicular acetylcholine transporter VAChT/*SLC18A3* is endoded by a single exon, embedded in intron 1 of the choline acetyltransferase (CHAT) gene (Erickson et al., 1994). Accordingly, their expression is linked and both are markers of cholinergic neurons. VAChT is expressed in the striatal magnocellular interneurons, in neurons of the basal forebrain and brain stem, preganglionic sympathetic neurons, pre- and postganglionic parasympathetic neurons and in the motorneurons of the ventral spinal cord (Erickson et al., 1994; Lawal and Krantz, 2013).

VAChT knockout mice ($VAChT^{-/-}$) die within minutes of birth (De Castro et al., 2009). The newborn pups do not survive due to respiratory failure, arising from the lack of vesicular acetylcholine storage and release at the neuromuscular junction. Targeting the 5′-untranslated region of the *SLC18A3* gene afforded the generation of mice with low expressing VAChT (Prado et al., 2006): in homozygous and heterozygous knockdown mutants ($VAChT^{KD}$), expression is reduced by 65 and 45%, respectively. Homozygous mice are myasthenic, i.e. their muscle strength is reduced and gets aggravated upon physical exertion. In heterozygous $VAChT^{KD}$, the level of VAChT suffices to maintain normal levels of quantal acetylcholine release at the neuromuscular junction (detected by recording miniature endplate potentials) and muscle strength. However, the reduction in VAChT/ synaptic refilling in some brain regions of these mice impairs cholinergic transmission to the extent visible in cognitive tasks, e.g. in paradigms of object recognition and social memory (Prado et al., 2006). This is consistent with cholinergic transmission in the brain being vulnerable and declined in early Alzheimer's disease (Grothe, Heinsen, & Teipel, 2012).

Deficiencies in VAChT function also have clinical repercussions in people. Patients with impaired VAChT function, arising from biallelic loss, suffer from congential myasthenic syndrome (CMS type 21, OMIM# 617239, Rodríguez Cruz et al., 2018). Earlier clues to this genotype-phenotype correlation were provided by screening of patients with deletions in the loci spanning 10q11.22 and 10q11.23, that encompass VAChT and CHAT (Stankiewicz et al., 2012). These patients present neurological symptoms such as intellectual disabilites, global developmental delay and CMS. Point mutations in VAChT (Fig. 3k and Table 5) linked to CMS were first identified in 2 patients, homozygous for either the p.(G186A) or for the p.(D398H) variant. In both cases, there were symptoms of impaired transmission at the motor endplate, i.e. ptosis, ophthalmoplegia, fatigable weakness and apneic crises (O'Grady et al., 2016). The loss-of-function phenotype of p.(G186A) and p.(D398H) was confirmed using an electrophysiological approach, wherein the patients exhibited a rapid decline in compound muscle action potential upon low-frequency repetitive stimulation. The homozygous p.(G360R) variant was identified in 2 brothers who had severe hypotonia and athrogryposis at birth. One died of respiratory failure 5 days after birth; the other (aged 4.5 years at the time of the study) relied on mechanical ventilation since birth (Aran et al., 2017). Heterologously expressed p.(G360R) was undetectable in immunoblots, although the transfected cells accumulated abundant mRNA levels (Aran et al., 2017). This is consistent with a protein folding defect. Comparably severe symptoms of respiratory distress were seen in a patient, who was hemizygous for the p.(V52F) variant and co-inherited a 10q11.2 deletion in the other allele (Schwartz et al., 2018). Mutations in VAChT can have detrimental

effects on fetal development, as they impair movement in utero. In 2 fetuses with akinesia deformation and lethal multiple pterygium syndrome (i.e. growth of skin webs over large joints), the defect was linked to a biallelic mutation which introduces a premature stop codon and hence truncates VAChT at C372 (Hakonen et al., 2019).

6.3 VPAT (SLC18B1)

The vesicular polyamine transporter (VPAT/*SLC18B1*) was initially annotated as C6ORF192 and subsequently assigned to the *SLC18* family, based on sequence homology (Jacobsson et al., 2010). When reconstituted in liposomes, VPAT takes up spermine and spermidine (and serotonin), driven by an antiport of H^+ (Hiasa et al., 2014). In the brain, VPUT expression is elevated in the hippocampus, cerebral cortex and the cerebellum. At the cellular level, it is predominantly localized to astrocytes, where it is found in vesicular structures spread throughout the cytoplasm (Hiasa et al., 2014). In mast cells, VPUT carries out the uptake of spermine and spermidine in secretory granules (Takeuchi et al., 2017). In the brain, polyamines are thought to regulate glutamatergic neurotransmission via NMDA-receptor modulation (Moriyama et al., 2020; Ogden and Traynelis, 2011). In VPUT knockout mice ($VPUT^{-/-}$), the levels of total polyamines in the brain are reduced (Fredriksson et al., 2019), translating to deficits in short- and long-term memories, possibly linked to reduced glutametergic and GABA-ergic signaling (Fredriksson et al., 2019). In addition, a link between SNPs in the human *SLC18*B1 gene and cognitive performance has been established (Fredriksson et al., 2019). However, no point mutations in VPAT have yet been identified in human disorders.

7 The ’uptake-2′ system: PMATs and OCTs

Extraneuronal biogenic amine uptake transporters, collectively known as the ‘uptake-2’ system, are hypothesized to play a role in the delayed onset of therapeutic action of antidepressant, which target the monoamine transporters (Schildkraut & Mooney, 2004). This system comprises 2 transporter families: organic cation transporters OCT1, 2 and 3 of the *SLC22* family (Koepsell et al., 2007)andthe plasma membrane monoamine transporter or PMAT of the *SLC29* family (Young, Yao, Baldwin, Cass, & Baldwin, 2013). The members of the uptake-2 system differ from the uptake-1 transporters (i.e. the plasmalemmal monoamine transporters SERT, DAT and NET) in 2 aspects. The fi rst is their extraneuronal distribution, compared to the distribution of the uptake-1 system, which is restricted to pre-synaptic neurons. Secondly, the uptake-2 system mediates Na^+-independent and low affinity substrate uptake, albeit with a substantially higher maximal velocity relative to uptake-1 transporters. In fact, it is this high capacity of uptake-2 transporters which is thought to counterpart the uptake efficiency of Na^+-dependent, high-affinity and low-capacity properties of uptake-1/monoamine transporters in rapidly terminating neurotransmission (Daws, 2009; Hensler et al., 2013).

OCT1 (*SLC22A1*),OCT2(*SLC22A2*) and OCT3 (*SLC22A3*) belong to the 13-member family of human *SLC22* transporters. These are putative 12 transmembrane polyspecific transporters that support uptake, elimination and distribution of a wide range of substrates encompassing cationic drugs, toxins and biogenic amines (Koepsell et al., 2007). The roles

of OCT2 and OCT3 in maintaining biogenic amine uptake have been well characterized in the brain (Koepsell, 2013; Yoshikawa & Yanai, 2016). Blocking OCT2 and 3 in mice, either by genetic inactivation or by high affinity antagonists, produces anti-depressant-like effects, which are additive when co-treated with selective serotonin or norepinephrine reuptake inhibitors (Hensler et al., 2013). This is suggestive of potential contribution of OCT2 and OCT3 in a delayed onset of antidepressant action (Schildkraut & Mooney, 2004). The role of OCT1 in the brain is restricted to allowing the passage of endogenous substrates and exogenous drugs across the blood-brain barrier (Koepsell, 2013).

PMAT (*SLC29A4*) belongs to the 4 member *SLC29* family of equilibrative nucleoside transporters (ENTs). ENTs 1–3 are primarily involved in the transport of purine and pyrimidine nucleosides (Young et al., 2013). In contrast, PMAT transports biogenic amines in the brain (Wang, 2016). The pharmacology of PMAT overlaps with that of OCTs indicating that PMAT may contribute to the uptake-2 system (Duan & Wang, 2010).

Murine knockouts of uptake-2 transporters are viable and fertile and show only subtle neurological deficits, indicative of redundancy and compensation, either by each another or by other transporters (Duan & Wang, 2013; Gilman et al., 2018; Jonker et al., 2001; Jonker et al., 2003; Zwart, Verhaagh, Buitelaar, Popp-Snijders, & Barlow, 2001). Most polymorphisms and functional consequences of variations in OCTs have been described in a context to inter-individual variations in response to non-CNS cationic drugs (such as anti-cancer drugs and the antidiabetic drug metformin) and non-CNS diseases (reviewed in Koepsell, 2020). Of note, mutations in OCT3 and PMAT (Table 5) have been identified in patients afflicted with OCD and autism, respectively. The p.(M370I) variant in OCT3 was identified in 3 members of a family suffering from OCD, inherited in an autosomal dominant manner (Lazar et al., 2008). When expressed alone, this variant was delivered to the cell surface at wild type levels, but its norepinephrine uptake was reducedby40%,presumablyduetodecreasedaffinityofthemutanttransporter for norepinephrine. In PMAT, the variants p.(D29G), p.(A138T) and p.(D326E) were identified in autistic patients (Adamsen et al., 2014). These mutants showed cell surface expression comparable to wild type PMAT, dermined by surface biotinylation and immunoblots. Uptake of serotonin, dopamine and MPP^+ was modestly reduced in heterologous cells expressing p.(A138T) and p.(D326E). For p.(D29G), the transport defect was restricted to MPP^+ (Adamsen et al., 2014). A lot of studies have emphasized the importance of OCT3 and PMAT in response to drugs targeting the monoaaminergic systems (Gasser, 2019; Haenisch & Bönisch, 2009; Hensler et al., 2013; Mayer et al., 2018). It is therefore feasible that genetic variations in uptake-2 play a role in many complex neuropsychiatric disorders and/or individual variability in response to therapeutic interventions.

8 *SLC32* family: vesicular GABA transporter (VGAT), vesicular inhibitory amino acid transporter (VIAAT)

The single member of the *SLC32* family, *SLC32A1* encodes the vesicular GABA transporter (VGAT)/vesicular inhibitory amino acid transporter (VIAAT). Its physiological role is to refill synaptic vesicles with glycine, GABA or β-alanine (Aubrey et al., 2007; Juge et al.,

2013; Wojcik et al., 2006). VIAAT exhibits low sequence similarity (of 10–20%) with *SLC17* and *SLC18* vesicular transporters. This transporter is primarily delivered to the synaptic vesicles of glycinergic and GABA-ergic neurons in the brain and spinal cord (Chaudhry et al., 1998; Gasnier, 2004; McIntire et al., 1997; Sagné et al., 1997). Knockout of VIAAT in mice leads to intrauterine death (Saito et al., 2010; Wojcik et al., 2006); similarly, VIAAT$^{-/-}$ *Drosophila melanogaster* fail to hatch (Fei et al., 2010). VIAAT$^{-/-}$ murine fetuses adopt a hunched posture due to muscular stiffness (Wojcik et al., 2006). When subjected to preterm delivery by cesarean section on E18.5, they fail to breath (Saito et al., 2010), an observation consistent with abnormal muscle control. Similar to glutamate decaboxylase 67-deficient mice, VIAAT$^{-/-}$ murine fetuses have a cleft palate, which argues for a role of tongue mobility in palate closure. The omphalocele (a prolaps into the umbilical cord of a peritoneal sac containing the gut), seen in VIAAT$^{-/-}$ murine fetuses, phenocopies defects seen in KCC2 (neuron-specific chloride potassium symporter-2, *SLC12*A5)-deficient mice (Hübner et al., 2001). This can be rationalized as follows: in the absence of KCC2, the chloride gradient in developing neurons is reversed; thus, stimulation of GABAA and glycine receptors is excitatory prior to KCC2 expression during brain development (Rivera et al., 1999; Stein, Hermans-Borgmeyer, Jentsch, & Hübner, 2004). Accordingly, the omphalocele reflects abnormal motor control, rather than structural defects. Point mutations in VIAAT have not yet been reported in human disorders.

9 NTT protein folding: lessons learnt from the *SLC6* transporters

Proteins have to adopt a precise 3-dimensional structure to acquire functional activity (Hartl, Bracher, & Hayer-Hartl, 2011). The “thermodynamic hypothesis” described by Christian Anfinsen, almost half a century ago, posits that the natively folded state is reached, when the protein has the lowest Gibbs free energy (Anfinsen, 1973). To attain this energetically favorable conformation, the protein must pass through a variety of intermediate folding states. The folding intermediates are assisted by proteinaceous chaperones, which shield the hydrophobic residues of the protein from the aqueous milieu and thus prevent aggregation with other proteins (Kim et al., 2013). The folding landscape resembles a funnel-shaped energy surface: The unfolded protein is routed along a downhill path towards its native state. However, the energy surface is often rugged, which results in transient accumulation of kinetically trapped conformations (Hartl et al., 2011). Proteinaceous chaperones smoothen the energy landscape, thereby promoting the progression towards the final, natively folded, state (Jahn and Radford, 2005).

The folding trajectory of *SLC* transporters can be envisioned as follows: like the majority of membrane proteins, *SLC* transporters co-translationally enter the ER membrane. The translocation process is initiated by the signal-recognition particle (SRP), which recognises the signal sequence of the nascent polypeptide chain as soon as it emerges from the ribosome. The ribosome-nascent-chain-SRP complex is next directed to the ER, where it binds to the SRP receptor. Subsequently, the polypeptide chain is inserted into the protein-conduction channel (the Sec61 translocon complex). Upon entering the channel, the hydrophobic segments are released individually or in pairs into the lipid phase via a lateral gate (Rapoport, 2007). On the lumenal side, the extracellular loop 2 of the nascent transporter is subject to N-linked core glycosylation (Freissmuth, Stockner, &

Sucic, 2018). After cleavage of the 2 terminal glucose moieties by α-glucosidase I and α-glucosidase II, the newly synthesized glycoprotein is recognized by the membrane-bound chaperone calnexin (Fig. 4). Calnexin acts as a folding sensor in the ER lumen and its release from the folding intermediate allows for removal of the third glucose moiety by α-glucosidase II (Hebert, Foellmer, & Helenius, 1995). Importantly, the release of calnexin also licenses the transporter to form oligomeric structures (Freissmuth et al., 2018), which is a prerequisite for ER export (Scholze, Freissmuth, & Sitte, 2002). However, if the transporter fails to adopt a proper folded state, it is re-glycosylated by the UDP-glucose: glycoprotein glucosyltransferase (UGT), which allows for a reassociation with calnexin and thus for an additional round of folding. The calnexin cycle continues until the protein either reaches a natively folded state or acquires a terminally misfolded architecture that is no longer recognized by UGT. In the latter case, the protein is eventually subject to proteasome-mediated degradation (Molinari et al., 2005). Concomitantly, the folding process is assisted from the cytosolic side by a relay of heat-shock proteins (HSPs). These molecular chaperones (i.e. an unidentified HSP40 isoform, HSP70-1A and HSP90ß) sequentially engage the C-terminus of the transporter and thus sterically cover the binding site for the coatomer complex II (COPII) machinery component Sec24 (Fig. 4). After reaching the natively folded state, HSP90ß is released, the binding site becomes available and subsequently the transporter is exported from the ER in a COPII-dependent manner (Freissmuth et al., 2018). However, if the transporter is not able to achieve a stable fold, it is ultimately destined for ER-associated degradation (Fig. 4, ERAD) by an unidentified E3-ligase (Chiba, Freissmuth, & Stockner, 2014). Importantly, COPII-mediated ER export is a prerequisite for correct axonal targeting: mutations that disrupt the binding site for Sec24, which acts as a cargo receptor, prevent enrichment of the transporter in the axonal compartment (Montgomery et al., 2014; Reiterer et al., 2008). There are 4 mammalian isoforms of this COPII machinery component, referred to as Sec24 A-D. In case of *SLC6* transporters, the relevant isoforms are Sec24D (GAT1, NET, DAT) and Sec24C (SERT, GAT3, GlyT2, BGT1, $ATB^{0,+}$) (Freissmuth et al., 2018; Kovalchuk et al., 2019; Sucic et al., 2011). Interestingly, the isoform preference between Sec24D and Sec24C, is determined by the residue located at the +2 position, downstream from the ER export motif. While hydrophobic residues (tyrosine or valine) at this position promote the recruitment of Sec24D, hydrophilic residues (lysine, asparagine or glutamine) support the engagement of Sec24 isoform C (Sucic et al., 2013).

Genetic mutations in *SLC* proteins can be transmitted either in a dominant or recessive manner. Dominant transmission can be rationalized by a model, where transporters form oligomeric structures prior to their exit from the ER. Accordingly, the mutated transporter oligomerizes with the product of the wild type allele and thus hinders its delivery to the target membrane. Circumstantial evidence in support of this model are autosomal dominant point mutations in glutamate transporters (*SLC1* family) and the high affinity choline transporter (CHT1, *SLC5A7*). CHT1 and most of the *SLC1* transporters form homooligomers (Okuda et al., 2012; Vandenberg & Ryan, 2013), which assemble in the ER prior to their export and trafficking to the cell surface as functional units. Several mutations, such as the ALS-linked p.(N206S) EAAT2 variant (Section 2.2), the episodic ataxia causing EAAT1 p. (P290R) variant (Section 2.1), and the dHMN p.(K499Nfs*13) CHT1 variant

(Section 3.2) display autosomal dominant features (Barwick et al., 2012; Jen et al., 2005; Trotti et al., 2001). Their co-transfection with wild type counterparts in heterologous cells, drastically reduces surface expression and function, compared to cells expressing wild type transporters alone. Among members of the *SLC6* family, the hallmark variant showing autosomal dominant transmission is p.(A457P) in human NET (Section 4.2) that exerts a dominant-negative effect on the wild type transporter (i.e. the product of the healthy allele) (Hahn et al., 2003). The same holds true for the dominant negative GlyT2 variants associated with hyperekplexia, p.(S512R-rodent/S510R-human) and p.(Y705C) (Section 4.5, Giménez et al., 2012; Arribas-González, de Juan-Sanz, Aragón, & López-Corcuera, 2015). These dominant negative effects could be overcome by exposure to the chemical chaperone 4-phenylbutyric acid (4-PBA) or by overexpressing the ER chaperone calnexin, for the p.(S512R) and p.(Y705C) variants, respectively. Such findings are consistent with the notion that *SLC* transporter oligomerization is a key requirement for their ER export (Anderluh et al., 2014; Farhan et al., 2004; Scholze et al., 2002).

Recessive transmission is readily rationalized by assuming that folding-deficient mutants are stalled in a complex with ER-associated chaperones, which preclude oligomer complex formation. *SLC* transporter folding is subject to a stringent quality control system, which relies on proteinaceous chaperones. From a teleological perspective, the goal of such a meticulous quality control is precluding the delivery of aggregation-prone proteins to the cell surface (model synapses are illustrated in Fig. 5). This conjecture has been widely explored for *SLC6* transporters. Our earlier efforts using synthetic GAT1, SERT and DAT mutants revealed that ER-retention is triggered by increased association of mutants with calnexin and HSPs (El-Kasaby et al., 2010; El-Kasaby, Koban, Sitte, Freissmuth, & Sucic, 2014; Kasture et al., 2016; Korkhov et al., 2008). These observations also apply to disease relevant varinants. For instance, point mutations in DAT lead to infantile/juvenile parkinsonism-dystonia (Kurian et al., 2009; Kurian et al., 2011; Ng et al., 2014). The vast majority of these cause folding defects in DAT, i.e. the mutated DATs accumulate as ER-resident core-glycosylated proteins, complexed with both calnexin and HSPs (Asjad, Kasture, et al., 2017). Missense variants in CRT1, linked to creatine transporter deficiency syndrome, are also known to be misfolded as demonstrated by increased transporter co-localization with calnexin in the ER (El-Kasaby et al., 2019). Consequently, this allows for the premise that alleviating the stringency of ER quality control mechanisms may promote the surface expression of folding-deficient variants. In fact, inhibition of individual components of the HSP relay by pharmacological means restores the cell surface expression of aberrantly folded variants of SERT (El-Kasaby et al., 2014), DAT (Asjad, Kasture, et al., 2017; Kasture et al., 2016) and CRT1 (El-Kasaby et al., 2019).

Some point mutations in NTTs lead to correctly folded proteins, which nonetheless fail to reach their target membranes due to trafficking defects. At present, only a few mutations appear to fit into this category: e.g. the p.(C186S) EAAT1 variant shows uptake levels comparable to wild type EAAT1 (De Vries et al., 2009), but its delivery to filopodia in transfected astrocytes is impaired (Hayashi & Yasui, 2015, Section 2.2). In addition, the loss-of-function p.(S94R), p. (V112E) and p.(P210L) variants in CHT1 fail to be sorted to nerve terminals, despite mutant and wild type protein levels being comparable in transfected cells (Wang et al., 2017). Similarly, despite being targeted to neuronal soma and dendrites,

the loss-of-function p.(P633R) NTT4 variant does not enter dendritic spines, as does the wild type NTT4 (Iqbal et al., 2015). Disrupted trafficking of transporter mutants can thus lead to either a loss-of-function phenotype or to alterations in their physiological roles (Kasture et al., 2019). Many recessive loss-of-function variants traffic to the cell surface in a manner indistinguishable from wild type proteins. In such instances, mutations most likely impair catalytic transport activity, e.g. altering the binding site/s of substrates and/or co-substrates ($Na^+/Cl^-/K^+/H^+$) or impeding the structural rearrangements necessary for completion of the physiological transport cycle. Elucidating the impaired step(s) is achievable by electrophysiological approaches, which allow for probing distinctive partial reactions of the transport cycle with unrivalled temporal resolution (Campbell et al., 2019; Carta et al., 2012; Herborg et al., 2018; Kovermann et al., 2017; Rees et al., 2006).

10 Pharmacological rescue of folding-deficient NTT variants

Folding-deficient mutants can be functionally rescued by chemical or pharmacological chaperones (Chaudhuri & Paul, 2006). The putative mechanisms of rescue are illustrated in Figs. 4 and 5. Chemical and pharmacological chaperones are small molecules that stabilize the misfolded protein, promote (re)folding and hence facilitate its delivery to the designated cellular locations (Loo and Clarke, 2007). The key difference between chemical and pharmacological chaperones is their substrate specificity: Chemical chaperones, such as glycerol, dimethyl sulfoxide (DMSO) and 4-PBA promote folding of a wide array of proteins (Perlmutter, 2002). In contrast, pharmacochaperones directly bind to and stabilize their cognate target proteins. Most of these small molecules are thought to facilitate the stabilization of the native state, thus enhancing protein expression (Marinko et al., 2019). Nonetheless, this mechanism does not account for the increased surface expression of monoamine transporters by ibogaine and its congeners (Bhat et al., 2020), which appears to occur via stabilization of a folding trajectory intermediate. In addition, the approved drug tafamidis is a pharmacochaperone, which restores the folded state of transthyretin (mutations in transthyretin cause a form of amyloidosis), by preventing protein aggregation and dissolution of aggregates (Coelho et al., 2016). Other prominent examples include migalastat and lumacaftor, which are clinically used in the treatment of Fabry disease (Germain, Hughes, Nicholls, Bichet, et al., 2016) and cystic fibrosis (Wainwright et al., 2015), respectively.

The action of pharmacological chaperones is restricted to specific target proteins (Ringe & Petsko, 2009). Ibogaine is the first pharmacochaperone found to be effective on SERT and several mutants thereof. Ibogaine binds to the inward-facing conformation of SERT and rescues a synthetic SERT-$RI^{607,608}$AA mutation, located at the ER export motif on the transporter's C terminus, i.e. the binding site for the COPII component cargo receptor SEC24C (El-Kasaby et al., 2010). The rescue effect was even more potent upon treatment with ibogaine's metabolite noribogaine (El-Kasaby et al., 2014). Other SERT ligands, including the inhibitor imipramine and substrates serotonin and amphetamine, which bind to the outward-facing and occluded transporter conformations, respectively, produced no rescue effects (El-Kasaby et al., 2010). Interestingly, second site suppressor mutations, which trap SERT in its inward-facing conformational state, can also promote the delivery of folding-deficient mutants to the plasma membrane (Koban et al., 2015). These observations

collectively indicate that the folding trajectory of SERT proceeds via the inward-facing conformation (Kasture et al., 2017). Notably, ibogaine, noribogaine and the atypical DAT inhibitor bupropion, can all correct misfolded DTDS variants in the closely related human DAT (Asjad, Kasture, et al., 2017; Beerepoot et al., 2016; Kasture et al., 2016). Ibogaine's hallucinogenic properties make it an inapt candidate for human therapeutics. However, a recent systematic investigation of partial substrates of monoamine transporters identified PAL1045 as another potent pharmacochaperone, acting on a synthetic folding-deficient SERT mutant $PG^{601,602}AA$ (Bhat et al., 2017). Since the complex ring system of ibogaine is amenable to structural variations and chemical modifications, a consequent fluorinated analog proved valuable in rescuing the equivalent $PG^{584,585}AA$ mutant in DAT, with a higher efficacy than the parent compound ibogaine. This analog also corrected folding defects in 6 DAT mutants associated with DTDS (Bhat et al., 2020).

The pharmacological repertoires of other NTT transporters, such as EAATs and CHT1, have been explored (Ennis & Blakely, 2016; Vandenberg & Ryan, 2013). It is pertinent to elucidate whether the folding trajectories of these transporters also ensue via the inwardfacing state. This conjecture ought to be probed because EAAT3 and CHT1 variants are of clinical interest (see Sections 2.3 and 3.2, respectively). Drugs that inhibit these transporters in a non-competitive manner and show preferential binding to the inward-facing transporter states (e.g., (+)-HIP-B for EAAT3 and ML352 for CHT1) may serve as lead compounds in the search for effective pharmacochaperones of misfolded disease variants in these transporter subfamilies (Callender, Gameiro, Pinto, De Micheli, & Grewer, 2012; Ennis et al., 2015).

Regretably, many *SLC* transporters do not have a rich pharmacology, confining the opportunities for initial pharmacochaperone screening. In such instances, chemical chaperones can be employed to assist protein folding, as shown for 4-PBA in rescuing misfolded variants of SERT (El-Kasaby et al., 2010), CRT1 (El-Kasaby et al., 2019), and GlyT2 (Arribas-González et al., 2015). Treatment of cells with a HSP70 inhibitor pifithrin-μ also rescued several folding-deficient variants of DAT (Asjad, Kasture, et al., 2017, Kasture et al., 2016). Moreover, both pifithrin-μ and a HSP90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) sensitized misfolded SERT mutants to the pharmacochaperoning action of noribogaine (El-Kasaby et al., 2014). HSP inhibitors have additionally been in the spotlight for the development of cytostatic agents (Neckers and Workman, 2012). Their action in alleviating folding deficits can be rationalized as follows: the progression of proteins along the folding trajectory is rigorously monitored by a HSP relay (Section 9). These proteinaceous chaperones must be released prior to the assembly of COPII vesicles and ER export of the protein cargo. HSP inhibition is thought to relax the stringent ER quality control, thus endorsing ER export (Asjad, Nasrollahi-Shirazi, Sucic, Freissmuth, & Nanoff, 2017; Freissmuth et al., 2018). The chemical chaperone 4-PBA prevents protein aggregation by interacting with hydrophobic domains of misfolded proteins. However, 4-PBA has other reputed modes of action, such as modulating HSC70, HSP70 and histone deacetylase levels, the latter leading to transcriptional regulation of genes in the unfolded protein response system (Cousens, Gallwitz, & Alberts, 1979; Rubenstein & Zeitlin, 2000).

Several allosteric modulators, which negatively or positively attune transporter activity, have recently caught attention in extending the already diverse expanse of NTT pharmacology (Hasenhuetl, Bhat, Freissmuth, & Sandtner, 2019; Niello et al., 2020). These drugs stabilize unique conformational states upon binding to their target transporters. As such, they hold significant therapeutic potential in the treatment of both misfolded and catalytically impaired transporter mutants. For instance, Zn^{2+} rescues the uptake of the ASD associated p.(T356M) DAT variant (see Section 4.3), while reducing spontaneous anomalous dopamine efflux leading to the partial correction of mutant DAT function (Hamilton et al., 2013; Hamilton et al., 2015). Ivacaftor, a renowned CFTR modulator, is in clinical use in the treatment of cystic fibrosis, administered in combination with the corrector molecule lumacaftor (Wainwright et al., 2015).

Pharmacochaperoning of misfolded *SLC* transporters is not limited to heterologous expression in cell lines. In 2016, Kasture et al. provided a proof-of-principle that folding-deficient mutants of DAT are amenable to rescue in vivo, i.e. utilizing *Drosophila melanogaster* as a resourceful animal model to demonstrate chemical- and pharmaco-chaperoning (Kasture et al., 2016). Living flies harboring the dDAT-G108Q mutation typically show a sleepless phenotype, reminiscent of a DAT knockout phenotype in flies (Wu, Koh, Yue, Joiner, & Sehgal, 2008). Upon treatment with noribogaine and/or HSP70 inhibitor pifithrin-μ, the mutant transporter reached the axonal compartments and normal sleep time was restored in these flies (Kasture et al., 2016). More importantly, this effect was replicated in flies expressing 2 human DAT variants (V158F and G327R), which give rise to infantile parkinsonism (Asjad, Kasture, et al., 2017) and in the synthetic folding variant DAT-$PG^{584,585}$AA (Bhat et al., 2020). To the best of our knowledge, pharmacochaperoning has not yet been tested in other animal models.

Pivotal insights into the molecular mechanisms underlying *SLC6* transporter folding were granted by in vitro experiments carried out on synthetic mutants, setting the stage for restoring the function of folding-deficient and loss-of-function disease variants in NTTs. Here, pharmacochaperoning may own major therapeutic significance in mending mutation-specific defects. In fact, even seemingly minor improvements in the basal activity of mutant transporters can lead to pronounced alleviation of disease phenotypes, substantiated by observations in DAT and sialin. That is, while only nominal rescue of DAT activity was measured in vitro, complete restoration of dopaminergic function was seen in knock-in flies harboring DTDS mutations (Asjad, Kasture, et al., 2017). Evidently, once the minimal threshold for functional rescue is achieved, increasing the efficacy of pharmacochaperoning in vitro by rational drug design has no further improvement on the behavioral output in vivo (Bhat et al., 2020).

In conclusion, recent findings from our group and others have shed new light onto the prospective uses of pharmacochaperoning in the treatment of many emerging folding diseases among NTT family members. The array of pathologic conditions triggered by anomalous folding in NTTs (Fig. 6) is ever ascending, necessitating the pursuit for novel chaperone compounds. The currently available state-of-the-art molecular dynamics simulations and electrophysiological approaches ought to provide valuable insights that bridge mutation-specific biophysical and structural effects to their biochemical and

pharmacological impacts. It is conceivable that such joined endeavours, in consortium with rational drug design, may lead to the development of effective treatment options for many folding diseases in NTTs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AChT	vesicular acetylcholine transporter
ASD	autism spectrum disorders
ASCT	alanine/serine/cysteine transporter
BGT	betaine/GABA transporter
CHT	high affinity choline transporter
CNX	calnexin
COPII	coatomer complex II
CRT	creatine transporter
C-terminus	carboxy terminus
CTL	choline transporter-like
d	*Drosophila melanogaster*
DAT	dopamine transporter
DMSO	dimethyl sulfoxide
EAAT	excitatory amino acid transporter
EEG	electroencephalography
ENT	equilibrative nucleoside transporter
ER	endoplasmic reticulum
ERAD	ER-associated degradation
GABA	γ-aminobutyric acid
GAT	GABA transporter

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GFP	green fluorescent protein
GlyT	glycine transporter
h	human
HEK	human embryonic kidney
HSP	heat shock protein
MAE	myoclonic-atonic epilepsy
MAT	monoamine transporter
NET	norepinephrine transporter
NTT	neurotransmitter transporter
N-terminus	amino terminus
SERT	serotonin transporter
SLC	solute carrier
SRP	signal-recognition particle
TM	transmembrane segment
UGT	UDP-glucose: glycoprotein glucosyltransferase
VGAT	vesicular GABA transporter
VIAAT	vesicular inhibitory amino acid transporter
VGLUT	vesicular glutamate transporter
VMAT	vesicular monoamine transporter
VNUT	vesicular nucleotide transporter
WT	wild type

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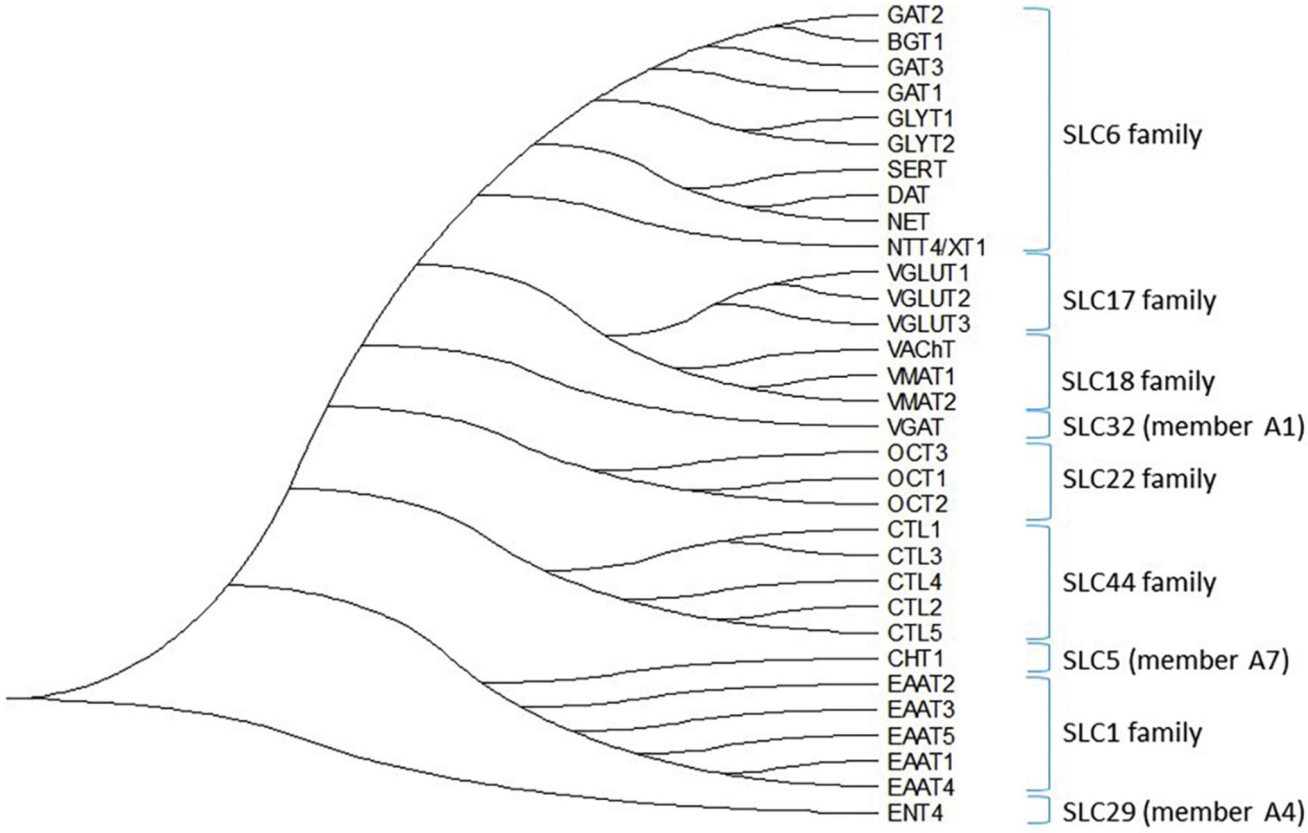

Fig. 1.

A phylogenetic tree of neurotransmitter transporter families. The evolutionary history was inferred using the maximum likelihood method and JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. This analysis involved 32 amino acid sequences. There were a total of 953 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

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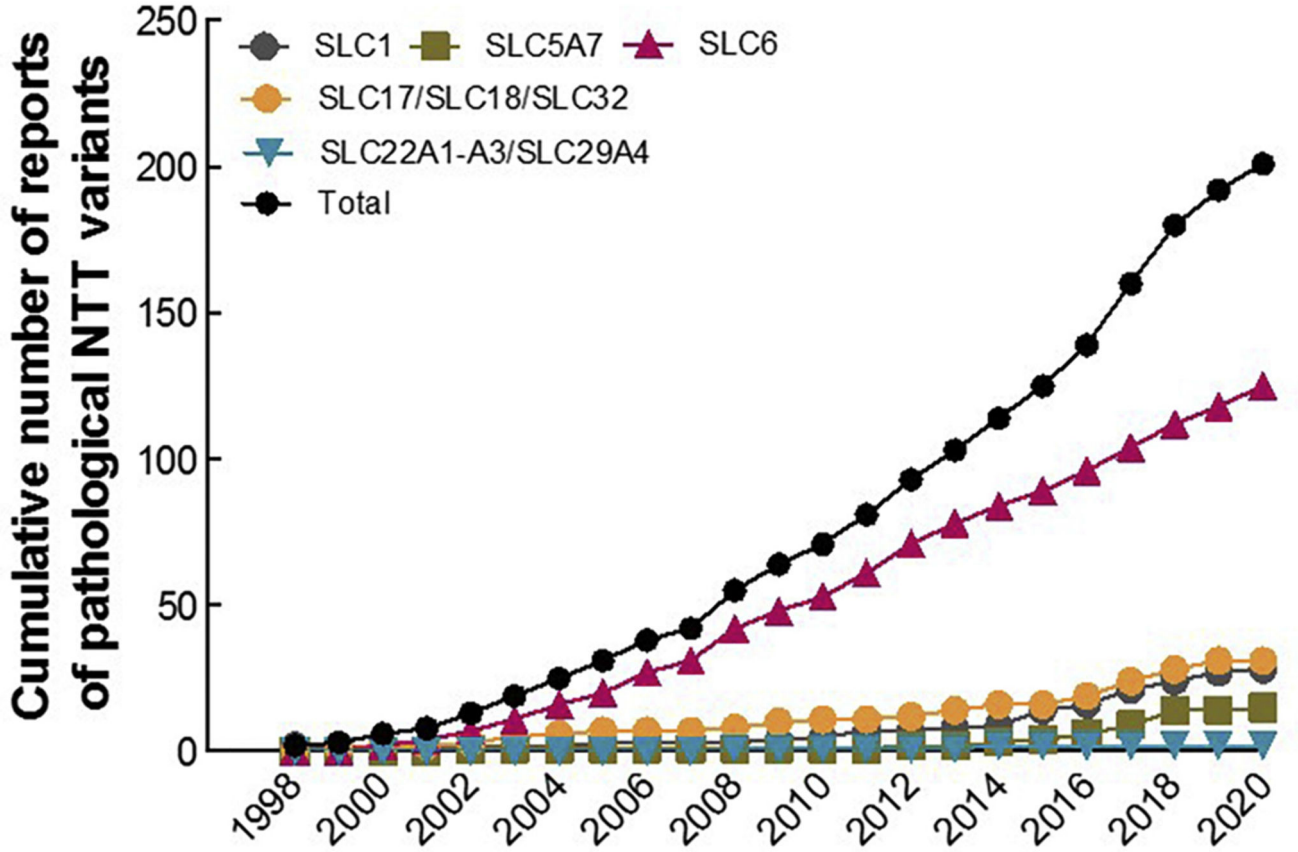

Fig. 2.

A cumulative number of misfolded disease variants reported among the NTT subfamilies. Numbers of reported pathological NTT variants, belonging to different subfamilies (color-coded), within the given time-span of two decades. The black line represents a total number of disease variants across the NTT protein family.

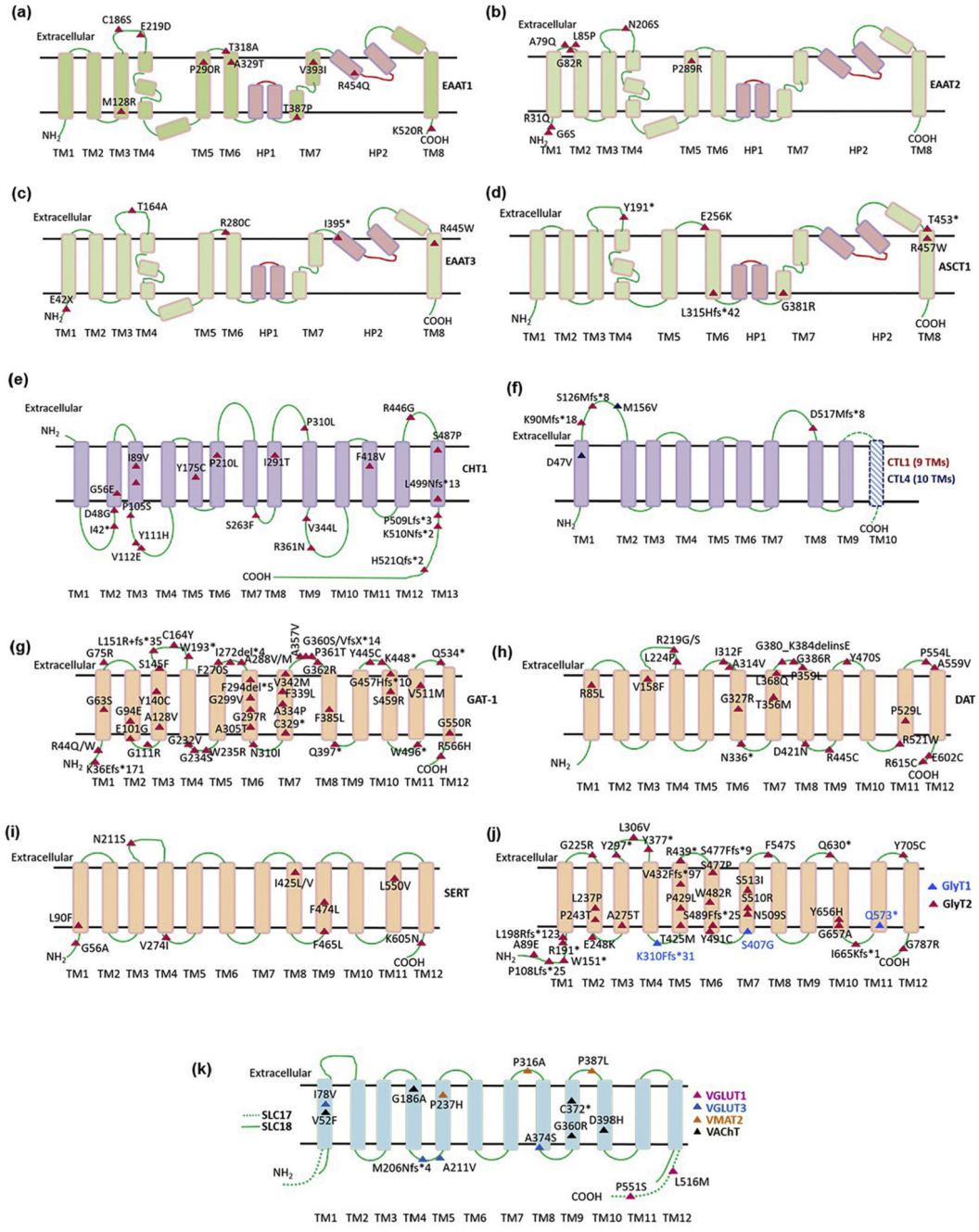

Fig. 3.

Disease-associated variants mapped onto topology diagrams of different NTT subfamilies. The location of point mutations, associated with human disease, are displayed for NTT family members: (a) EAAT1, (b) EAAT2, (c) EAAT3, (d) ASCT1, (e) CHT1, (f) CTL1 (in red) and CTL4 (in blue), (g) GAT1, (h) DAT, (i) SERT, (j) GlyT1 (in blue) andGlyT2(inblack)and (k) vesicular transporters; VGLUT1 (in magenta), VGLUT3 (in blue), VMAT (in orange) and VAChT (in black).

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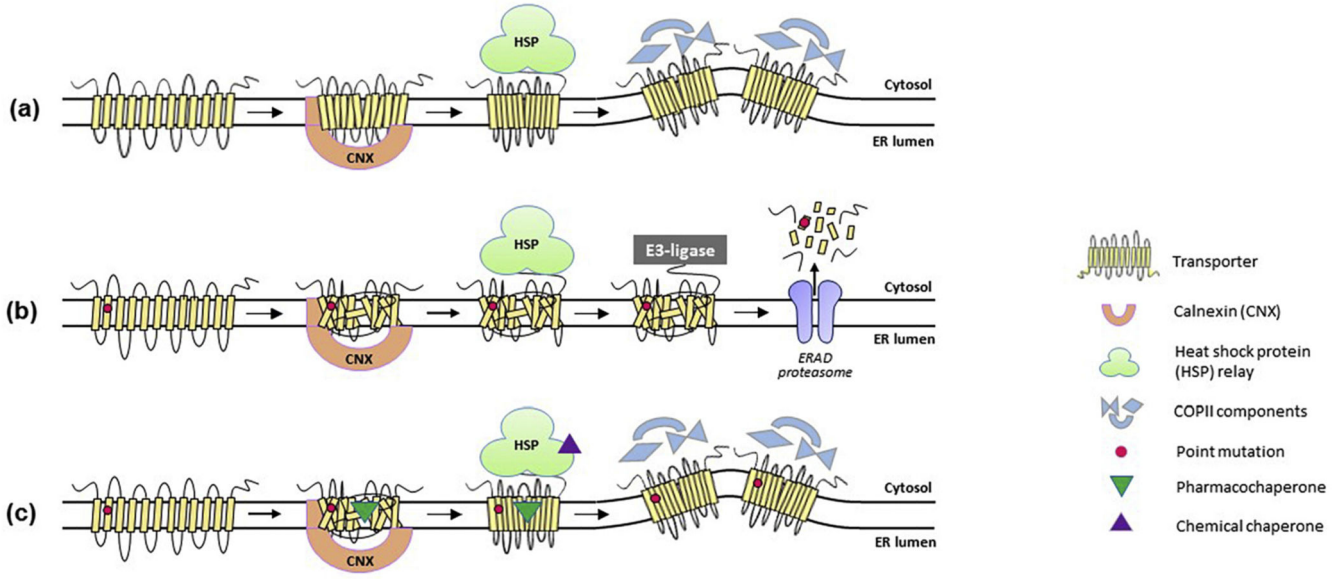

Fig. 4.

A magnifying glass view of the folding trajectory and molecular mechanisms involved in the rescue of misfolded NTT transporters. Upon insertion into the ER membrane, *SLC6* transporters are engaged by calnexin on the lumenal side and by a relay of HSPs on the cytosolic side. While release of calnexin (CNX) allows for transporter oligomerisation, dissociation of HSPs clears the C-terminal binding site for the COPII component Sec24 and thus allows for ER export via COPII-coated vesicles (a). However, point mutations can impair the folding process of the mutant proteins, causing retention in the ER. Folding-deficient transporters are then marked for ERAD by an unidentified E3-ligase (b). In some instances, such folding defects can be corrected by pharmacological means. Pharmacological chaperones selectively bind to the mutant transporter, which reduces the energy barrier along the folding trajectory and thus promotes native folding. In contrast, HSP inhibitors are thought to relax the stringent ER quality control, which results in improved ER exit (c).

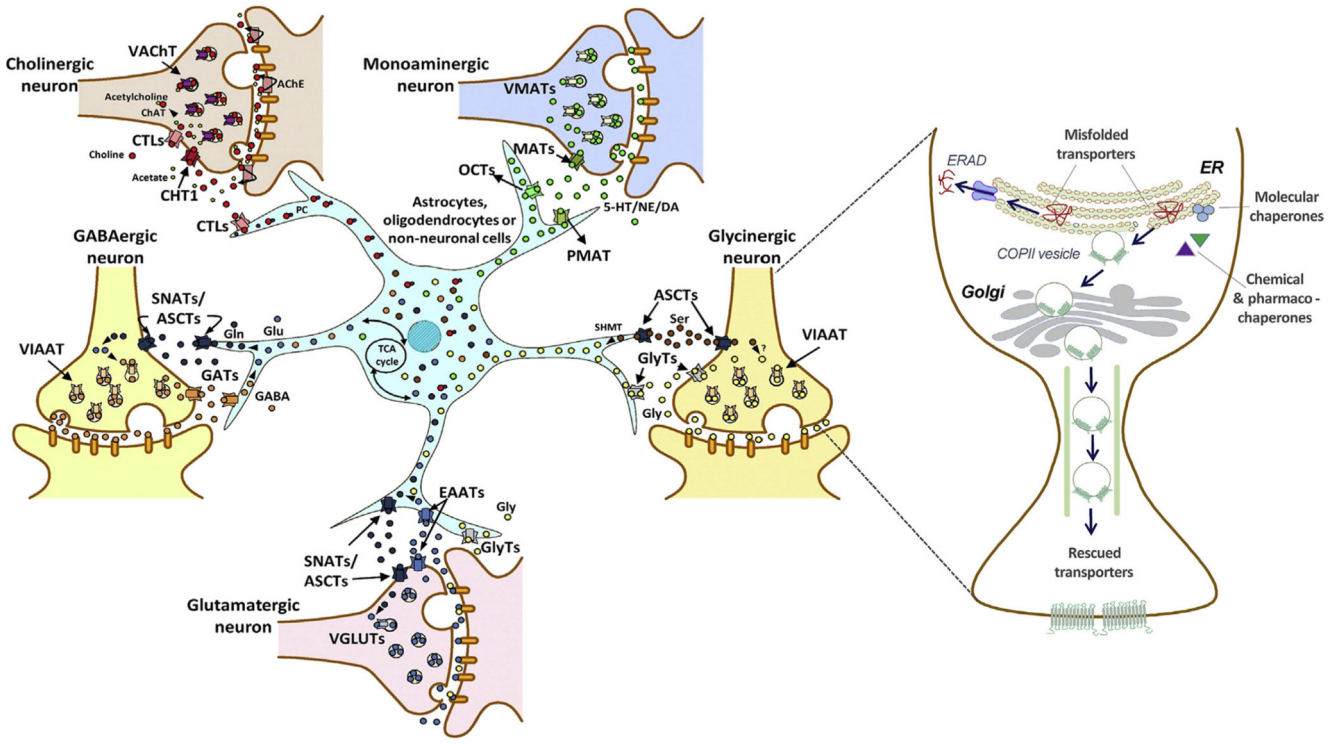

Fig. 5.

A basic synapse model of key NTT subfamilies and transporter rescue by pharmacochaperoning. A simplified representation of key synapse types, centered around a symbolic glial/ oligondedrocyte/non-neuronal cell: monoaminergic (in blue), glycinergic (in yellow), glutamatergic (in pink), GABAergic (in lime green) and cholinergic (in beige). Neurotransmitters are shown as circles, packaged into vesicles by the designated vesicular transporters, e.g. VMATs sequester biogenic amines dopamine, norepinephrine and serotonin to storage vesicles in monoaminergic neurons, setting them up for the next neurotransmitter release event. To the right, a magnifying glass view over pharmacochaperone action on misfolded NTTs: rescued transporters reach their eponymous site of action at the synapse, where they resume their physiological functions (left, model synapses). Abbreviations: 5-HT – 5-hydroxytryptamine, AChE-Acetylcholinesterase, ChAT-Choline acetyltransferase, DA-dopamine, Glu-glutamate, Gln-glutamine, Gly-glycine, Ser-serine, NE-norepinephrine, PC-phosphatidylcholine, SNATs-sodium-coupled neutral amino acid transporters/*SLC38*; SHMT-serine hydroxymethyltransferase.

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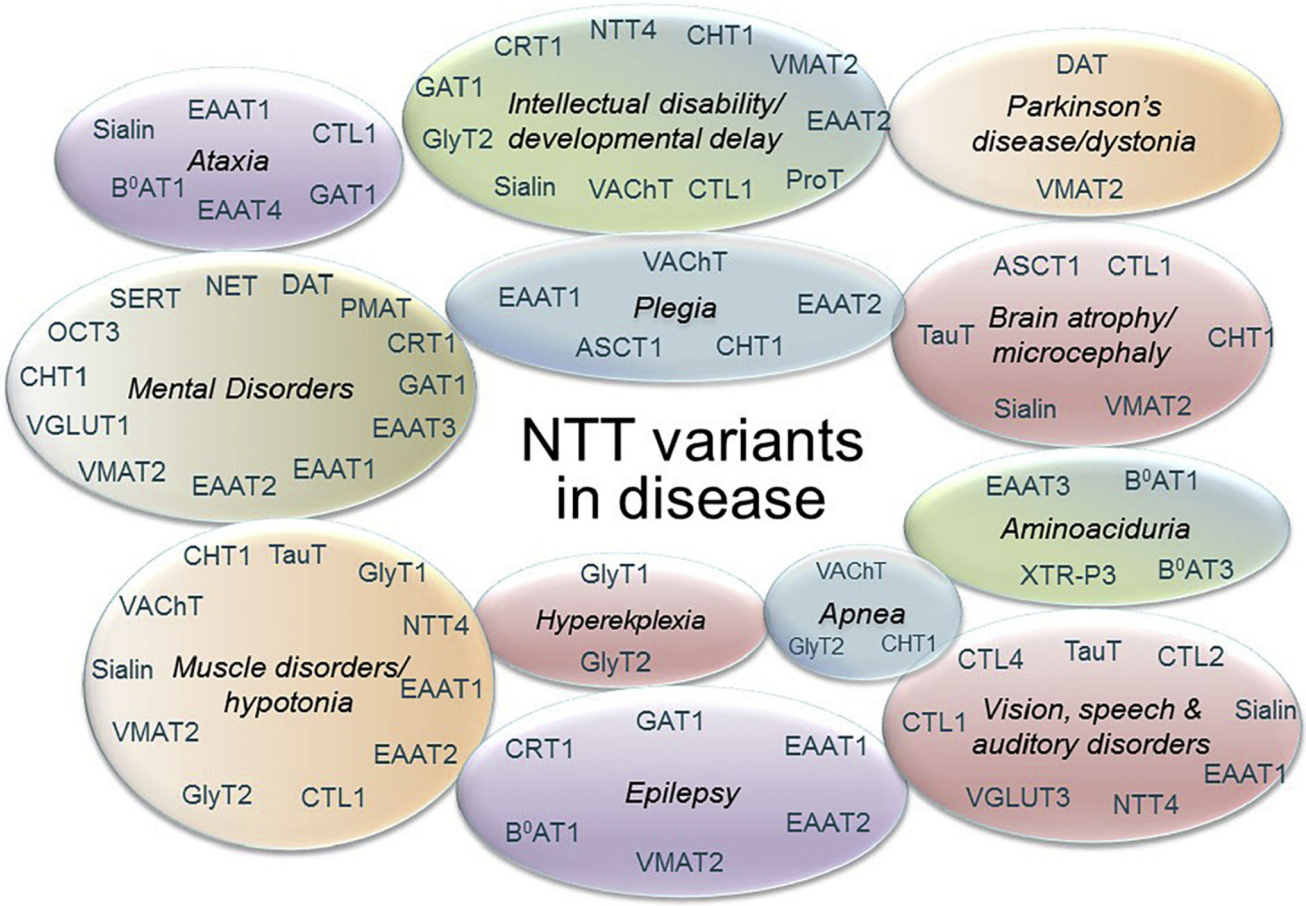

Fig. 6.

Folding diseases in NTT proteins. Folding diseases caused by mutations in NTT genes are displayed in the balloons, with the implicated transporters listed within the circular shapes representing different disorders. Apart from the conditions displayed in the figure, many other symptoms have been linked to specific transporters (e.g. CHT1: akinesia; CTL2: transfusion related acute lung injury; CTL4: sialidosis; EAAT1: vertigo, migraine, postural/ gait imbalance; GlyT1: encephalopathy; NET: postural hypotension and long QT syndrome; Sialin: cognitive dysfunction; VMAT2: postural hypotension; VNUT: porokeratosis).

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Table 1

Mutations in human plasmalemmal *SLC1* transporters.

Gene	Protein name	Missense coding variants	Transport phenotype	Traf ficking to target membrane	Disease phenotype	Mode of inheritance	OMIM#
SLC1A1	EAAT3	E48*, R445W, I395del	Loss-of-function	Reduced	Dicarboxylic aminoaciduria, autism	AR	222730
		T164A	?	?	Obsessive compulsive disorder (OCD) and bipolar disorder	AD	
		R280C	?	?	Schizotypal personality disorder	SM	615232
SLC1A2	EAAT2	N206S	Loss-of-function	Reduced	Sporadic amyotrophic lateral sclerosis	AD	
		L85P	Loss-of-function	Reduced	Epileptic encephalopathy	AD/CHet	617105
		G82R, P289R, L474*	?	?			
		A79G	?	?	Hereditary spastic paraplegia	AD	
		G6S, R31Q	?	?	Bipolar disorder and schizophrenia	AD	
SLC1A3	EAAT1	P290R	Loss-of-function	Reduced	Episodic ataxia	AD	612656
		C186S	Minimally reduced	Altered			
		M128R, T318A, A329T[1], V393I, R454Q, K520R	?	?			
		T387P	Loss-of-function	Reduced	Hemiplegia migraines with aura	AD	
		A329T[2]	?	?	Benign essential blepharospasm	AD	
		E219D	Gain-of-function	Increased	Tourette syndrome	AD	
SLC1A4	ASCT1	E256K, R457W	Loss-of-function	Unaltered	Spastic tetraplegia, thin corpus callosum, and progressive	AR	616657
		Y191*, L315Hfs*42, G381R,W453*	?	?	microcephaly; SPATCCM		

AR: Autosomal recessive; AD: Autosomal dominant; CHet: Compound heterozygotes; SM: somatic mosaicism.

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Table 2

Mutations in human plasmalemmal choline transporters.

Gene	Protein name	Missense coding variants	Transport phenotype	Trafficking to target membrane	Disease phenotype	Mode of inheritance	OMIM#
SLC5A7	CHT1	K499Nfs*13	Loss-of-function	Reduced	Distal hereditary motor neuronopathy	AD	158580
		P509Lfs*3, K510Nfs*2, H521Qfs*2	?	?			
		D48G, G65E, P105S, R361Q, R446G, S263F	Loss-of-function	No change	Congenital myasthenic syndrome	AR/CHet	617143
		S94R, V112E, P210L,	Loss-of-function	Altered			
		I42*, Y111H, Y175C, I291T, P310L, V344L, F418V, S487P	?	?			
*SLC44*A1	CTL1	D517Mfs*19 K90Mfs*18, S126Mfs*8	Loss-of-function	Unaltered Reduced	Childhood-onset neurodegeneration with ataxia, tremor, optic atrophy, and cognitive decline (CONATOC)	AR	618868
SLC44A4	CTL4	D47V	?	?	Age-related macular degeneration and blindness	?	
		M156V	Loss-of-function	?	Postlingual non-syndromic mid-frequency sensorineural hearing loss	AD	617606

AR: Autosomal recessive; AD: Autosomal dominant; CHet: Compound heterozygotes.

Table 3

Mutations in human plasmalemmal *SLC6* neurotransmitter transporters.

Gene	Protein name	Missense coding variants	Transport phenotype	Trafficking to target membrane	Disease phenotype	Mode of inheritance	OMIM#
SLC6A1	GAT1	9/45 variants characterized	Loss-of-function		Myoclonic-atonic epilepsy	AD	616421
		G94E, W235R, F270S, I272*, Y445C, W496*, G550R		?			
		G234S, P361T		Reduced			
SLC6A2	NET	A457P	Loss-of-function	Reduced	Chronic orthostatic intolerance	AD	604715
SLC6A3	DAT	A559V, E602G, R615C	Unaltered dopamine uptake, anomalous dopamine efflux	Unaltered	Attention deficit hyperactivity disorder (ADHD), bipolar disorder	AD	
		R85L, V158F, R219G, R219S, L224P, A314V, G327R, L368Q, G380_K384delinsE, G386R, P395L, R445C, Y470S, R521W, P529L, P554L	Loss-of-function	Reduced	Dopamine transporter defeciency syndrome (Infantile Parkinsonism/ dystonia)	AR	613135
		V382A			ADHD	AD	
		I312F, D421N		Unaltered	Adult Parkinsonism with ADHD	CHet	
		ΔN336, T356M		Unaltered	Autism spectrum disorder	AD	
SLC6A4	SERT	G56A, K605N	Gain-of-function	Unaltered	OCD, depression, Autism spectrum disorder, Asperger's syndrome, Tourette's syndrome	AD/AR	164230 (I425V)
		I425V, I425L, F465L, L550V		Increased			
		N211S, V274I, F474L	?	?			
		L90F	?	?	Anorexia nervosa-restrictive type	AD	
SLC6A5	GlyT2	G225R, Y297*, R439*, S477P, S477Ffs*9	?	?			
		P108Lfs*25, Y377*, V432Ifs*97, Q630*	Loss-of-function	Reduced			
		W151*, R191*, L198Rfs*123, L237P, P243T, E248K, S489Ffs*39, S513I, F547S, I655Kfs*1, Y656H, G657A		?	Hyperekplexia	AR	614618
		A89E, A275T, L306V, T425M, W482R, Y491C, N509S, G787R		Unaltered			
		P429L S510R, Y705C		Reduced		AD	
SLC6A9	GlyT1	K310Ffs*31, S407G, Q573*	?	?	Glycine encephalopathy	AR	617301

AR: Autosomal recessive; AD: Autosomal dominant; CHet: Compound heterozygotes.

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Table 4

Mutations in other human plasmalemmal *SLC6* transporters.

Gene	Protein name	Missense coding variants	Transport phenotype	Trafficking to target membrane	Disease phenotype	Mode of inheritance	OMIM#
SLC6A6	TauT	A78E, G399V	Loss-of-function	Unaltered	Childhood retinal degeneration with or without cardiomyopathy	AR	
SLC6A8	CRT1	>80 point mutations reported	Loss-of-function	Reduced in many	Cerebral creatine deficiency syndrome 1	XLR	300352
SLC6A17	NTT4	G162R, P633R	?	Unaltered for G162R, altered for P633R	Intellectual disability	AR	616269
SLC6A18	B^0AT3	G79S, Y319X, P478L, G496R	?	Reduced (for G79S,Y319X, G496R), unaltered (for P478L)	Digenic iminoglycinuria (DIG)/hyperglycinuria (HG) when *SLC36*A2 mutations are co-inherited	AD, AR, DR, CHet	242600 (DIG) 138500 (HG)
SLC6A19	B^0AT1	>20 mutations	?	?	Hartnup Disorder	AR	234500
		IVS7–4G → A	?	?	Iminoglycinuria (IG)/ hyperglycinuria (HG) when *SLC36*A2 mutations are co-inherited	AD, AR, DR	242600 (DIG) 138500 (HG)
SLC6A20	XTRP3	T199M	Reduced	Unaltered	Iminoglycinuria (IG)/ hyperglycinuria (HG) when *SLC36*A2 mutations are co-inherited	AD, AR, DR	242600 (DIG) 138500 (HG)

AR: Autosomal recessive; AD: Autosomal dominant; CHet: Compound heterozygotes; XLR: X-linked recessive; DR: Digenic recessive.

Table 5

Mutations in human *SLC17, SLC18,* OCT and PMAT transporters.

Gene	Protein name	Missense coding variants	Transport phenotype	Trafficking to target membrane	Disease phenotype	Mode of inheritance	OMIM#
SLC17A5	Sialin	>30 mutations	Loss-of-function	Reduced, increased, unaltered or altered	Salla disease, intermediate severe Salla disease or Infantile Sialic Acid Storage Disorder	AR/CHet	604369 (SD) 269920 (ISSD)
SLC17A7	VGLUT1	L516M, P551S	?	?	Schizophrenia	?	
*SLC17*A8	VGLUT3	A211V	Loss-of-function	Reduced and altered	Non-syndromic sensorineural deafness	AD	605583
		I78V, M206Nfs*4, A374S	?	?			
*SLC17*A9	VNUT	R9C, R311N	?	?	Disseminated superficial actinic porokeratosis	AD	616063
SLC18A2	VMAT2	P387L, P316A	Loss-of-function	Unaltered protein levels, trafficking studies not undertaken	Brain monoamine vesicular transport disease	AR	618049
		P237H	?	?			
SLC18A3	VAChT	G360R	Loss-of-function	Protein undetected	Congential myasthenic syndrome	AR	617239
		V52F, G186A, C372*, D398H	?	?			
SLC22A3	OCT3	M370I	Loss-of-function	Unaltered	Obsessive Compulsive Disorder	AD	
*SLC29*A4	PMAT	A138T, D326E	Loss-of-function	Unaltered	Autism spectrum disorder	AD	

AR: Autosomal recessive; AD: Autosomal dominant; CHet: Compound heterozygotes.