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The Importance of the Excitatory Amino Acid Transporter 3 (EAAT3)

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Abstract

The neuronal excitatory amino acid transporter 3 (EAAT3) is fairly ubiquitously expressed in the brain, though it does not necessarily maintain the same function everywhere. It is important in maintaining low local concentrations of glutamate, where its predominant post-synaptic localization can buffer nearby glutamate receptors and modulate excitatory neurotransmission and synaptic plasticity. It is also the main neuronal cysteine uptake system acting as the rate-limiting factor for the synthesis of glutathione, a potent antioxidant, in EAAT3 expressing neurons, while on GABAergic neurons, it is important in supplying glutamate as a precursor for GABA synthesis. Several diseases implicate EAAT3, and modulation of this transporter could prove a useful therapeutic approach. Regulation of EAAT3 could be targeted at several points for functional modulation, including the level of transcription, trafficking and direct pharmacological modulation, and indeed, compounds and experimental treatments have been identified that regulate EAAT3 function at different stages, which together with observations of EAAT3 regulation in patients is giving us insight into the endogenous function of this transporter, as well as the consequences of altered function. This review summarizes work done on elucidating the role and regulation of EAAT3.

Introduction

The neurotransmitter glutamate (Glu) is the main excitatory neurotransmitter in the mammalian brain (Meldrum, 2000, Danbolt, 2001). Because of its ubiquity, it is not surprising that perturbations of the glutamate system have been identified as a key mediator of numerous pathological conditions including schizophrenia, epilepsy, amyotrophic lateral sclerosis (ALS), Huntington's disease and Alzheimer's disease (AD) (Javitt, 2004, Niswender et al., 2005, Corona et al., 2007, Bowie, 2008, Javitt, 2015, Wesseling and Perez-

Conflict of interest

The authors declare no conflict of interest.

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Otano, 2015). Glu itself is also toxic under certain circumstances, such as stroke, traumatic brain injury or in certain neurodegenerative disorders, where an excess of glutamate overstimulates ionotropic glutamate receptors, leading to excitotoxic cell damage and death (Olney, 1969, Gillissen et al., 2002, Mehta et al., 2007). The ubiquity of glutamatergic neurotransmission makes it an important system to understand, but a difficult system to target, pharmacologically, due to off target effects.

Regulation of extracellular Glu is mediated by a class of plasma membrane enzymes called the excitatory amino acid transporters (EAATs). The five subtypes of transporters are, in humans, the products of the genes *SLC1A3*, *SLC1A2*, *SLC1A1*, *SLC1A6* and *SLC1A7*, corresponding to human EAAT1, -2, -3, -4 and -5 proteins, respectively (see table I). The rabbit and rat transport proteins were cloned before the human counterparts and were named GLAST (Storck et al., 1992), GLT-1 (Pines et al., 1992) and EAAC1 (Kanai and Hediger, 1992). The human carriers EAATs1, -2 and -3 (Arriza et al., 1994, Kanai et al., 1994), were subsequently cloned and named in order. Human EAATs -4 and -5 followed (Fairman et al., 1995, Arriza et al., 1997), and the rodent forms of these two have been named similarly. For clarity, we will use the EAAT1 – 5 nomenclature for the proteins of this family from any organism in this review. While the EAATs share ~35–65% amino acid identity, the different subtypes have distinct cellular and developmental expression profiles and some unique pharmacology (Danbolt, 2001, Amara and Fontana, 2002, Jensen et al., 2015), and have different implications for various diseases (Bianchi et al., 2014, Jensen et al., 2015, Šerý et al., 2015).

Structure and Transport Mechanism

The structure of EAATs has been probed biochemically, and a topography of 8–10 transmembrane (TM) helices with intracellular N- and C-termini was predicted (Grunewald and Kanner, 1995, Slotboom et al., 1996, Wahle and Stoffel, 1996, Grunewald et al., 1998, Seal and Amara, 1998, Slotboom et al., 1999, Grunewald and Kanner, 2000, Seal et al., 2000, Grunewald et al., 2002, Yernool et al., 2003). A crystal structure of an aspartate transporter from *Pyrococcus horikoshii*, Glt_{Ph}, which shares ~37 % homology with mammalian transporters (Yernool et al., 2004, Boudker et al., 2007), shows a topography that was generally consistent with the predictions: 8 transmembrane (TM) helices, but with two reentrant loops between TM6 and -7 and between TM7 and -8 (see figure 1). The protein was crystalized as a homo-trimer with each subunit functioning independently, which was also confirmed for the mammalian orthologs (Haugeto et al., 1996, Gendreau et al., 2004, Grewer et al., 2005). Interestingly, there is evidence that hetero-trimeric quaternary structures are possible, as seen with recombinant EAAT3 and EAAT4 expressed in tsA201 cells (Nothmann et al., 2011).

The transporter can be roughly divided into functional sub-domains, with TM1, -2, -4 and -5 serving as a relatively static scaffolding domain, while TM3, -6, -8 and hairpin 1 (HP1) and hairpin 2 (HP2) are part of the transport domain. Different structural conformations of the Glt_{Ph} have been crystalized, and significant insight into the transport mechanism was gained when a cysteine (Cys) crosslinking study yielded what was called the inward facing occluded state (where the substrate-binding site was shifted towards the intracellular side,

but was inaccessible) (Reyes et al., 2009). The transporter functions by a so-called “elevator” mechanism, translocating the substrate binding site of the transport domain $\sim 18\text{\AA}$ through the plane of the plasma membrane, while the scaffolding domain remains relatively static. The movement of flexible loops connecting the scaffolding- and transport domains mediates the relative movement of the two domains. This is the first type of transporter for which this mode of transportation has been observed, though it was recently suggested that VcINDY, a bacterial homolog of the human *SLC13* family of di- and tricarboxylic acid transporters, also share this mechanism (Mulligan et al., 2014).

The EAATs are secondary active transporters, translocating three sodium ions, one proton and counter-transporting one potassium ion for each substrate, which gives the energetic driving force to transport Glu against its electrochemical gradient (figure 2) (Zerangue and Kavanaugh, 1996a). In fact, the EAATs can transport glutamate against a several thousand-fold concentration gradient under basal conditions (Nicholls and Attwell, 1990, Danbolt, 2001). The EAATs can transport (S)-Glu, (S)-Aspartate (Asp) and (R)-Asp, cysteic acid, and serine-O-sulfate. EAAT3 can also efficiently transport (R)-Cys at physiologically relevant conditions. It was recently shown that Cys is transported in the deprotonated form, which interacts with the R447 residue in TM8 of EAAT3 (Watts et al., 2014). This residue is essential in determining the specificity towards negatively charged amino acids since mutation to Cys changes the specificity of the transporter to neutral amino acids (Bendahan et al., 2000). This seemingly contrasts older findings where it was shown that increasing the external pH (deprotonating the free Cys) did not significantly change the Cys uptake currents (Zerangue and Kavanaugh, 1996b). However this was done using electrophysiology in the 1996 study and radiosubstrate uptake in the 2014 study, and the exact pH values used were different (7.5 and 8.5 in 1996 versus 6.9 and 8.5 in 2014), which could explain the apparent discrepancies.

The model of substrate translocation (figure 2) follows a cycle wherein the substrate and co-transported ions are bound to the transport domain in an outward facing, open state. HP2 then repositions itself to close the substrate binding pocket from the extracellular side. The transporter reorganizes to an inward facing conformation where HP1 opens to allow intracellular access the substrate-binding pocket. The substrates exit, a potassium ion binds, HP1 closes and the transporter reorients to an outward facing conformation. Here, HP2 opens, the potassium dissociates, and the cycle can begin anew (Leighton et al., 2006, Boudker et al., 2007, Ewers et al., 2013).

The EAATs also have an anion current that is gated by glutamate, but stoichiometrically uncoupled from transport. Cross-linking the transport and scaffolding domains by introducing Cys residues inhibits uptake while retaining a substrate-dependent increase in uncoupled anion currents (Shabaneh et al., 2014). This current to transport ratio is largest in EAAT4 and EAAT5, which have sometimes been described as glutamate-gated chloride channels rather than transporters (Fairman et al., 1995, Arriza et al., 1997). The binding of substrate is fairly quick (sub-millisecond) while the transport itself is somewhat slower, occurring on a millisecond timescale (Grewer et al., 2000). The mechanism of the uncoupled chloride current has been unclear, but support for a conformation “outside” of the traditional transport cycle was reported (Torres-Salazar et al., 2015). That is, the transporter can change

conformation to a state that is not identical to any part of the transport cycle, but where an aqueous pore opens between the transport- and scaffolding domains to allow anion conductance. An arginine residue, R388, was shown to be important in the transition between the transport- and anion conducting states of EAAT1 (Torres-Salazar et al., 2015). This ion channel property is sensitive to substrate, suggesting that the open probability increases with substrate binding. The substrate sensitivity has been shown to be dependent on a conserved arginine residue in TM7 of the transporter, corresponding to R356 in the human EAAT3. In the human EAAT3, mutating Asp83 has been shown to modify translocation rates and the amount of time the transporter is in an anion conducting state (Hotzy et al., 2012) consistent with increased anion permeability of this mutant carrier. A better understanding of the structure and molecular mechanism of transport is the basis for a more rational design of compounds modulating the EAATs.

Localization

EAAT3 is expressed throughout the mature brain, with higher expression in the cerebral cortex, hippocampus, cerebellum and basal ganglia (Rothstein et al., 1994, Shashidharan et al., 1997). EAAT3 is also expressed outside of the central nervous system (CNS) in the intestine, liver, heart, skeletal muscle, kidneys, placenta, sciatic nerve, dorsal root ganglion and primary afferent fibers terminating in the dorsal spinal horn (Kanai and Hediger, 1992, Velaz-Faircloth et al., 1996, Matthews et al., 1998, Tao et al., 2004, Carozzi et al., 2008). Outside of the brain, EAAT3 appears to be the main Glu and Asp transporter in many cell types. For example, in skeletal muscle cells, it is responsible for maintaining levels of free intracellular Glu (Li et al., 2015); in the kidney, it plays a key role in Glu reabsorption (Hediger, 1999); and in the placenta, it is involved in a fetal-placental Glu/glutamine shuttle, supplying nitrogen for the fetus (Velaz-Faircloth et al., 1996, Matthews et al., 1998). It is also expressed in the peripheral nervous system, where it is found in both neurons and Schwann cells (Tao et al., 2004, Carozzi et al., 2008).

In the CNS, EAAT3 expression is primarily neuronal, with high densities at post-synaptic terminals where a peri-synaptic localization is observed (He et al., 2000). There are reports of EAAT3 expression in various glial cells, including oligodendrocytes (Kugler and Schmitt, 1999, DeSilva et al., 2009) and astrocytes (Conti et al., 1998, Liang et al., 2014); however, two of these studies used cultured cells (DeSilva et al., 2009, Liang et al., 2014) that might not reflect *in vivo* expression, and rigorous controls for the antibodies used in these studies were not presented. In fact, Holmseth and co-workers showed no detectable EAAT3 protein in astrocytes, mature oligodendrocytes or oligodendrocyte precursor cells in the adult rat brain using antibodies verified in KO animals (Holmseth et al., 2012).

Interestingly, since EAAT3 is typically not found at a high density on pre-synaptic glutamatergic terminals, it seems unlikely to be a major player in recycling Glu directly, a role that is filled primarily by astrocytic EAAT2, which converts Glu to glutamine, and shuttles it back to the neuron for re-synthesis into Glu (Albrecht et al., 2010). A distinct sequence on the c-terminus of EAAT3 has been identified that restricts it to dendritic compartments in hippocampal neurons (Cheng et al., 2002).

Significant amounts of EAAT3 has been found in intracellular compartments in C6 glioma cells, neuronal cultures and in the brain *in vivo*, suggesting up to 70–80% intracellular localization (Rothstein et al., 1994, Furuta et al., 1997a, Shashidharan et al., 1997, Conti et al., 1998, He et al., 2000, Sims et al., 2000, Gonzalez et al., 2002, Yang and Kilberg, 2002, Sheldon et al., 2006, Holmseth et al., 2012). Dynamic plasma membrane shuttling and intracellular pools of transporter provide a simple way to rapidly regulate the capacity of these transport systems, by redistributing them to and from the plasma membrane as needed. This is in contrast to kidney EAAT3, which show a predominantly cell surface localization (Holmseth et al., 2012), as well as EAAT1 and EAAT2b, which are primarily plasma membrane localized under conditions studied (Chaudhry et al., 1995, Munir et al., 2000, Holmseth et al., 2012, Underhill et al., 2015). However, the EAAT2a isoform also shows significant intracellular accumulation, and EAAT2b showed internalization upon CaMKII activation in murine astrocytes (Underhill et al., 2015).

Developmental Profiles

During development, the distributions of EAATs change in the CNS. At embryonic days 15 and 18, EAAT3 expression is evident in several brain regions where EAATs 1, -2 and -4 have little to no expression. As expression of the other carriers increases, EAAT3 slightly decreases in most regions, while remaining relatively constant in others, such as layer II of the cortical plate, posterior spinal horn and olfactory bulb. This was thoroughly characterized for EAATs 1, -2, -3 and -4 in the rat central nervous system (CNS), and summarized from embryonic day 15 up to adult rats (Torp et al., 1994, Furuta et al., 1997b).

A role of EAATs has been indicated in the early development of the cortex, where γ -aminobutyric acid (GABA) and Glu receptors have been implicated in the migration of immature cortical neurons (Behar et al., 1999, Hirai et al., 1999, Behar et al., 2000) governed by the function of Glu and GABA transporters (Dvorzhak et al., 2012, Unichenko et al., 2013). The relatively high level of EAAT3 expression, compared to the other subtypes, during early stages of cortex development, suggests an important role of EAAT3 (Martinez-Cerdeno and Noctor, 2014).

Function

Glutamate Clearance

EAAT2 is responsible for the vast majority of glutamate clearance in the CNS; estimates indicate that more than 90% of total clearance in the brain is EAAT2 mediated, which is not surprising since EAAT2 comprises ~1% of total protein in the CNS (Haugeto et al., 1996, Tanaka et al., 1997, Lehre and Danbolt, 1998). The expression of EAAT3 in the mature brain is estimated at ~100 fold lower than EAAT2 (Holmseth et al., 2012), though studies with antisense knockdown of EAAT3 argue that ~20% of glutamate uptake in striatum and ~40% in the hippocampus of rats is EAAT3 mediated (Rothstein et al., 1996). Further, since EAAT3 is often found near dendritic regions, it seems plausible that modulating EAAT3 uptake can have larger effects on the local Glu concentrations than on the global concentrations, and modulate receptors on the same neuron where the EAAT3 is located. Indeed, altered Glu induced currents have been found for both N-methyl-D-aspartate

(NMDA) receptors (NMDAR) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPA) when disrupting EAAT3 function on the post-synaptic neuron (Scimemi et al., 2009, Jarzylo and Man, 2012, Underhill et al., 2014). The predominantly intracellular localization of EAAT3 previously noted also suggests that the capacity of EAAT3 to buffer local receptors could change rapidly in response to certain stimuli. Thus, though EAAT2 is responsible for the bulk of Glu uptake in the whole brain, and it is unlikely that EAAT3 significantly regulates the overall ambient Glu levels, it is not surprising that changes in EAAT3-mediated Glu uptake would have physiologically relevant effects via nearby receptors.

Synaptic Modulation of Glutamate Receptor Subunits

NMDARs have been shown to respond to Glu spillover, detecting non-locally released transmitter in rat hippocampal slices (Diamond, 2001). This was dependent on NR2B containing NMDARs, where the action of a neuronal EAAT was shown to be important in regulating the spillover activation (Scimemi et al., 2004). It was suggested that during high-frequency stimulation, the activity leading to long term potentiation (LTP), depolarization of the plasma membrane could inhibit neuronal EAAT transport by up to 70%, and strengthen the Glu receptor activation locally, thereby both promoting LTP and conferring spatial specificity of the induced LTP (Diamond, 2001).

Changes in EAAT3 activity have been shown to change the trafficking of AMPARs. Increases in local Glu concentrations by blocking EAAT3 function was shown to activate peri-synaptic NR2B containing NMDARs (Scimemi et al., 2009), which in turn lead to a reduction of GluR1- and GluR2-containing AMPARs by an ubiquitin- and proteasome-dependent mechanism in rat hippocampal and cortical neurons (Jarzylo and Man, 2012). Tetraethylammonium (TEA), a potassium channel blocker, induces LTP in mouse hippocampal slices (Aniksztejn and Ben-Ari, 1991, Gu et al., 2010). Treatment of hippocampal slices with TEA result in GluR1 phosphorylation and trafficking to the plasma membrane in wild type (WT), but not in EAAT3 knockout (KO) mice (Cao et al., 2014a), suggesting a role of EAAT3 in TEA-induced LTP. In WT mice, the PKA inhibitor KT5720 blocked GluR1 trafficking and in EAAT3 KO mice the PKA activator 6-BNz-cAMP induced plasma membrane trafficking of GluR1 (Cao et al., 2014a). Conversely isoflurane was shown to decrease GluR1 trafficking to the plasma membrane in hippocampus of EAAT3 KO mice, but not WT mice by a mechanism involving phosphatase activation (Cao et al., 2014b). This correlated to decreased context-related fear freezing duration, a mechanism that has been shown to be hippocampus-dependent (Kim and Fanselow, 1992), suggesting a role for EAAT3 in regulating AMPAR subunit trafficking in synaptic plasticity in the hippocampus.

GABA Synthesis

De novo synthesis of γ -aminobutyric acid (GABA) is a large part of pre-synaptic GABA homeostasis (Conti et al., 2011), and uptake of Glu into GABAergic neurons is an important mechanism of supplying Glu as a precursor for GABA synthesis (Mathews and Diamond, 2003). Spillover of Glu modulates inhibitory currents in the CA1 region of rat hippocampal slices and is sensitive to the non-selective EAAT inhibitor, threo- β -hydroxyaspartate, but not

the EAAT2 selective inhibitor, dihydrokainic acid (DHK), suggesting a role of an EAAT other than EAAT2 (Stafford et al., 2010). This is in contrast to results from the spinal dorsal horn, where both the semi-selective EAAT1 and EAAT2 inhibitor TFB-TBOA and DHK decreased GABA currents (Jiang et al., 2012). Inhibiting Glu uptake into GABAergic neurons decreases the amount of GABA released by those neurons, and the subsequent post-synaptic inhibitory currents generated by GABA release (Sepkuty et al., 2002, Mathews and Diamond, 2003). Treating rats with EAAT3 antisense RNA resulted in a severe decrease in [³H]-GABA synthesis rate and activity, with an approximately 50% decrease in hippocampal GABA levels (Sepkuty et al., 2002). Excitability was increased in both hippocampal and cortical slices, while mIPSC was decreased in CA1 neurons. In rat hippocampal slices, there is a pool of GABA that can only sustain inhibitory responses under basal conditions while *de novo* GABA synthesis was needed under activated conditions (Dericioglu et al., 2008). Together, this suggests an important role of EAAT3 in regulating synaptic inhibition through GABA synthesis.

Energy Substrate

Glu can enter the mitochondria of certain cells (Frigerio et al., 2008), including neurons (Yudkoff et al., 1994) and astrocytes (Hertz et al., 2007), where it is utilized as a catabolic substrate by transamination of oxaloacetate to Asp, which is exported to the cytoplasm, and α -ketoglutarate, which is incorporated into the Krebs cycle. In the brain, this Glu catabolism is most pronounced in astrocytes, where Jackson and co-workers recently showed that mitochondria mobility in both neurons and astrocytes is affected by neuronal activity, and that astrocytic mitochondria can be retained in proximity to EAAT2 in response to astrocytic Glu uptake (Jackson et al., 2014). Glu catabolism is also present in GABAergic neurons (Hertz et al., 1988), implicating a role for plasma membrane EAAT3 in uptake of Glu for catabolic oxidation in these neurons. Traditionally, Asp/Glu carriers of the maleate/Asp shuttle have been ascribed the function of Glu uptake into mitochondria, however a recent report described EAAT3 co-localizing and immunoprecipitating with $\text{Na}^+/\text{Ca}^{2+}$ exchanger 1 in rat hippocampal and cortical, as well as SH-SY5Y and C6 glioma cell line mitochondria (Magi et al., 2012). Synthesis of ATP in isolated mitochondria from rat hippocampus and cortex was inhibited by application of the EAAT inhibitor, DL-TBOA (Magi et al., 2012, Magi et al., 2013), implicating an EAAT in mitochondrial ATP synthesis. This is in stark contrast to observations by Holmseth and colleagues where an initial antibody labeling of EAAT3 in mitochondria was shown to be due to non-specific labeling, thus concluding that no EAAT3 was present in rat brain mitochondria (Holmseth et al., 2006).

Cysteine and Glutathione

Glutathione (GSH) is important in regulating redox homeostasis in the CNS by reducing reactive oxygen species. The high metabolism of neurons produces a lot of oxidative stress, demanding a high-capacity system for maintaining the redox homeostasis. Intracellular Cys seems to be the rate-limiting factor for GSH synthesis in neurons (Griffith, 1999), and oxidative stress and altered GSH levels have been implicated in several diseases including multiple sclerosis, Wilson disease, Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) where protein redox state has been described in

mechanisms of redox-regulated cell death, (Dringen, 2000, Uttara et al., 2009, Haider et al., 2011, Sauer et al., 2011, McBean et al., 2015).

While the cystine/Glu exchanger (X_c^-) is a major player in glial cells, the presence of X_c^- on neurons is a matter of debate. There is evidence of X_c^- function in cultured immature cortical neurons (Murphy et al., 1990), and discrepancies regarding the expression in whole brain. One study found little to no RNA in the neurons of mature mouse cerebellum or cortex by in situ hybridization (Sato et al., 2002) while another showed evidence of protein in intact human and mouse cortex (Burdo et al., 2006). The expression of X_c^- is highly inducible (Sasaki et al., 2002), and the two studies used different approaches, different anesthesia and only the former specified the gender of the animals used. It should therefore be noted that these studies are not directly comparable. In a murine cellular model of Huntington's disease, plasma membrane EAAT3 was found to be upregulated (Petr et al., 2013), with a concomitant decrease in X_c^- expression later described by the same group (Frederick et al., 2014). This is consistent with an EAAT3 up-regulation in the neuronal Huntington's cell line being a compensatory mechanism for maintaining Cys uptake during the observed X_c^- decrease, and a functional role of both transport systems.

The importance of EAAT3 in cysteine uptake and redox homeostasis of EAAT3 expressing neurons is more clear, regardless of whether it is the sole cysteine transporter in these cells. Cysteine uptake in cultured neurons, which is sensitive to EAAT inhibitors but not to X_c^- inhibitors has been demonstrated (Chen and Swanson, 2003, Himi et al., 2003), and the role of EAAT3 in maintaining the redox state of neurons is highlighted in recent studies with KO animals. Hippocampal neurons (Aoyama et al., 2006, Choi et al., 2014), dopamine neurons (Berman et al., 2011) and retinal ganglion cells (Harada et al., 2007) from EAAT3 KO animals all exhibit increased vulnerability to oxidative stress induced by H_2O_2 , the free-radical generating compound 3-morpholinodisulfonimine or a cerebral ischemia model. Further, aged EAAT3 KO mice show signs of accumulated oxidative stress: ventricular enlargement, cortical thinning and reduction in the size of the hippocampal CA1 and corpus callosum (Aoyama et al., 2006, Cao et al., 2012, Lee et al., 2012). These observations correlate with decreased performance in the Morris water maze, a test that assesses cognitive function and spatial memory (D'Hooge and De Deyn, 2001). The observed phenotypes were abolished by treating the mice with N-acetylcysteine (NAC) (Aoyama et al., 2006, Cao et al., 2012), a cell permeable Cys source that circumvents the need for a Cys uptake mechanism (De Vries and De Flora, 1993).

Interestingly, DA neurons are particularly vulnerable to oxidative damage and in EAAT3 KO animals the substantia nigra pars compacta exhibits a loss of more than 40% of their dopaminergic neurons within 12 months of age (Berman et al., 2011). Loss of substantia nigra dopaminergic neurons is a phenotype seen in Parkinson's disease, where there is also evidence for increased oxidative stress (McBean et al., 2015, van der Brug et al., 2015). Treating EAAT3 KO mice with NAC rescues dopaminergic cells, as well as motor function (Berman et al., 2011). As might be expected, NAC could potentially be a treatment in PD patients (Martinez-Banaclocha, 2012), and at the time of writing, clinical trials for the use of NAC as PD treatment are currently posted (<https://clinicaltrials.gov/ct2/show/NCT01470027>, <https://clinicaltrials.gov/ct2/show/NCT02212678>). While dysregulation of

EAAT3 in Parkinson's disease has not, to the best of our knowledge, been reported, development of drugs that modulate EAAT3 expression and/or function may also be beneficial treatments through a similar mechanism.

EAAT3 in Disease

Epilepsy

The role of EAATs in epilepsy is most often associated with EAAT2 since deletion of this carrier induces spontaneous seizures and premature death in mice (Tanaka et al., 1997). This is most likely due to the decreased clearance of extracellular glutamate by this transporter and direct overstimulation of glutamate receptors. Knockdown of EAAT3 also induces epilepsy (Rothstein et al., 1996), though by a different mechanism. Acute EAAT3 knockdown with cDNA leads to a decreased tonic inhibition, due to the lack of EAAT3-mediated Glu uptake into GABAergic neurons where Glu is used for GABA synthesis and subsequent inhibitory neurotransmission (Sepkuty et al., 2002). EAAT3 expression decreases in kainate and methylazoxymethanol-induced seizures (see table II) (Simantov et al., 1999, Harrington et al., 2007) as well as in human neocortex of epileptic patients (Rakhade and Loeb, 2008), supporting the role of decreased EAAT3 expression in epilepsy. It should be noted that abolished EAAT3 function has not led to epileptic phenotypes with developmental disruptions, as seen with KO mice and humans with non-functional EAAT3 (Peghini et al., 1997, Aoyama et al., 2006, Bailey et al., 2011), perhaps due to compensatory mechanisms often seen in knockout models.

However, a recent study found reduced neuronal death in the CA1 region in pilocarpine-induced status epilepticus of EAAT3 KO mice (Lane et al., 2014). These data were interpreted to indicate that, under the experimental conditions used, EAAT3 reverses transport, since during a seizure ATP levels decline and the sodium driving force collapses (Streck et al., 2006; Kovac et al., 2012), leading to the protective effect of knocking out EAAT3 and implicating its role in seizure kindling. Indeed, EAAT3 expression increases in pilocarpine- and 4-aminopyridine-models of epilepsy (Crino et al., 2002, Ross et al., 2011, Medina-Ceja et al., 2012), which would support a role of EAAT3 in inducing or exacerbating pilocarpine-induced seizures. While the exact function of EAAT3 in epilepsy remains elusive, evidence suggests an involvement, and a better understanding of this may be key in determining potential EAAT3-related strategies of intervention.

Obsessive-Compulsive Disorder

A prevailing hypothesis of obsessive-compulsive disorder (OCD) states that there is an OCD-specific imbalance between what is referred to as the direct and indirect loops of the cortico–striato–thalamo–cortical circuit. This imbalance in OCD elicits a preference of the direct loop, which is a positive feedback loop (where the indirect is a negative feedback loop). While there is evidence for the involvement of both serotonin and dopamine pathways in OCD, selective serotonin reuptake inhibitors being used therapeutically (Ivarsson et al., 2015), and alterations in the dopaminergic system (Bokor and Anderson, 2014), there is also an increasing number of studies implicating GABAergic and glutamatergic systems (Goddard et al., 2008, Nikolaus et al., 2010). Genome-wide screens have identified genes

involved in serotonin, dopamine, GABA and glutamate systems, and it has been suggested that several systems and genes could be involved where each would add cumulatively to the risk (Haber and Heilbronner, 2013). A thorough coverage of the complex interplay between different neurotransmitter systems, environmental factors and other risk factors is outside the scope of this review. For further reading into this, we will refer to some excellent reviews (Murphy et al., 2013, Bokor and Anderson, 2014, Grunblatt et al., 2014, Pauls et al., 2014, Wood and Ahmari, 2015). Instead we will focus on the evidence of a functional role of EAAT3 perturbations in OCD.

The first such study implicating *SLC1A1* in OCD was a genome scan on early-onset OCD patients and immediate family members found several polymorphisms, two of which were found in 56 of 65 sequenced individuals (Veenstra-VanderWeele et al., 2001). While the study found no linkage disequilibrium, two later studies (one by the same group) published findings showing a correlation between OCD and *SLC1A1* (Arnold et al., 2006, Dickel et al., 2006). Since then, several genetic variations in, or in proximity to *SLC1A1* have been found to correlate with OCD, either by single marker or haplotype analysis of the SNPs (Stewart et al., 2007, Liang et al., 2008, Shugart et al., 2009, Samuels et al., 2011, Wu et al., 2013a, Wu et al., 2013b, Gasso et al., 2015).

In 2009, Arnold and co-workers found an increase in thalamic volume in OCD patients with the rs3056 variant (Arnold et al., 2009), while later study found no correlation between OCD genotypes and volume of the orbitofrontal cortex (OFC), anterior cingulate cortex (ACC), thalamus, caudate, putamen, globus pallidus or pituitary (Wu et al., 2013c). The first expression correlation study was done in 2009, where Wendland and co-workers reported decreased mRNA levels of *SLC1A1* in post-mortem human dorsolateral prefrontal cortex, as well as transfected SH-SY5Y and PC12 cell lines for the rs301430 SNP (Wendland et al., 2009), which had previously been associated with OCD (Dickel et al., 2006).

In 2011, Bailey and co-workers were the first to functionally characterize two mutants of EAAT3 found in two patients with dicarboxylic aminoaciduria, a disease associated with EAAT3 dysfunction (Bailey et al., 2011). An R445W mutation in TM8 or deletion of I395 in HP2 of EAAT3 (see figure 1) greatly reduces Glu and Cys transport by the mutant carriers. The R445W mutant has greatly reduced expression in MDCK cells while the I395 deletion expression was undetectable. The proband with the R445W mutation had declined assessment of OCD, though admitted to lifelong traits often found in OCD. The proband with the I395 deletion, a four-year-old girl, had no reported OCD phenotype, though this is not usually diagnosed until later childhood or adolescence. Another SNP giving rise to substitution of the conserved Threonine 164 to alanine has previously been identified in a single family (Wang et al., 2010), and shown to decrease EAAT3 V_{max} and K_m in transfected cells (Veenstra-VanderWeele et al., 2012), though no further characterization of this mutant has been published.

Three alternate isoforms of EAAT3 have been described both in healthy subjects and OCD patients. Two arose from skipping of either exon 2, resulting in deletion of TM2, or exon 11, resulting in deletion of HP2 and the first half of TM8 and one from transcription by a secondary internal promoter, resulting in the deletion of the first 3 TMs (see figure 1)

(Porton et al., 2013), a promoter that has previously been described in mice (Jin et al., 2002). The alternate exon skipping transcripts do not have Glu transport capacity on their own, and can in fact impede activity of the WT EAAT3 activity found in HEK293 cells. A possible explanation could be dysfunctional trimeric structures formed, though the mechanism of this inhibition has not been examined. It was furthermore shown that while these isoforms are expressed in healthy individuals, they were differentially expressed and regulated in human lymphocytes from OCD patients (Porton et al., 2013). It should be noted, however, that OCD patients were medicated with selective serotonin reuptake inhibitors, as well as additional unspecified antidepressant- or adjuvant medications complicating interpretations.

While there are a substantial number of studies correlating genetic variants in, and close to *SCL1A1* to OCD, it is only in recent years that work has emerged showing an altered function of EAAT3 in relation to OCD phenotypes. It is likely that perturbations in the expression or function of EAAT3 can add to the risk of OCD-like behavior, though it is probably a part of a large and complex interwoven system. However, in some cases the effect on EAAT3 could be the tipping point, and in these cases a treatment regimen focused on EAAT3 might prove effective.

Schizophrenia

For decades, alterations in the glutamatergic neurotransmission have been associated with schizophrenia (Hu et al., 2015), where decreased expression of EAAT2 was described first (Ohnuma et al., 1998), and decreased EAAT3 expression in striatum was described 4 years later for both schizophrenic and bipolar patients (McCullumsmith and Meador-Woodruff, 2002). While a study reported no changes in EAAT3 mRNA expression in the thalamus of schizophrenia patients (Smith et al., 2001), it was later shown that there was an increase in expression of JWA, the human ortholog of rat GTRAP3-18, in the thalamus (Huerta et al., 2006). Since GTRAP3-18 is known to regulate EAAT3 by means other than transcription/translation (Liu et al., 2008, Aoyama and Nakaki, 2012) (see below for a discussion of the mechanism of GTRAP1-18), altered EAAT3 function might be a contributing factor in the thalamus as well. In the caudate nucleus of schizophrenia patients, EAAT3 protein was decreased (Nudmamud-Thanoi et al., 2007). The cortex showed an increase in EAAT3 (Rao et al., 2012), where increased JWA was also observed (Bauer et al., 2008). A mutation in phosphatidylinositol-4-phosphate 5-kinase II alpha found only in schizophrenia patients displays a dominant negative effect on EAAT3-mediated Glu currents in oocytes. (Fedorenko et al., 2009). These studies implicate EAAT3 in schizophrenia, where they primarily suggest a regulatory change of the EAAT3 membrane targeting or function.

Recently, a novel EAAT3 isoform transcribed from a promoter upstream of exon 2, truncating the first transmembrane helix, was described in schizophrenia patients in a Palauan family (Afshari et al., 2015). This is not the same secondary promoter as described previously (Jin et al., 2002, Porton et al., 2013), but an isoform only missing the intracellular N-terminal and TM1 (see figure 1). This isoform showed a more cytosolic localization than the WT when GFP-tagged versions were expressed in HEK293. The truncated transporter was unable to sustain transport and interestingly, mutant carriers with psychosis (5 out of 9 carriers) but not carriers that did not display psychotic traits (4 out of 9 carriers) had an

increased expression of GluR1, EAAT4 and NR2B. These studies indicate that dysregulation of EAAT3 expression and/or function may play contributory roles in schizophrenia.

The preferred treatment for schizophrenia includes atypical antipsychotics (AAPs). Interestingly, atypical antipsychotics have been associated with induction of OCD-like behavior in schizophrenia patients (Lykouras et al., 2003). SNPs and a haplotype have been identified in or near *SLC1A1*, which correlate to this AAP-induction of OCD-like behavior in schizophrenia patients (Kwon et al., 2009), one of which was later reproduced and shown to correlate with an SNP in the NMDA receptor subtype, *GRIN2B* (Cai et al., 2013).

Regulation of EAAT3

Modulation of Total Expression

In addition to the diseases discussed above, certain chemicals also induce changes in EAAT3 expression (see table II). Notably, chronic morphine treatment decreases EAAT3 expression through stimulation of opioid receptors in both rats, mice and cell lines (Mao et al., 2002). This was dependent on ubiquitination by NEDD4, an E3 ubiquitin ligase, which in turn was dependent on the phosphatase PTEN (Lim et al., 2005, Yang et al., 2008), though also EAAT3 mRNA decreases (Guo et al., 2015). The expression of EAAT3 was time-dependent, and after 12 hours of withdrawal, the levels returned to baseline (Guo et al., 2015). Morphine tolerance was abolished by blocking NR2B containing NMDARs in rats (Mao et al., 2002) and NR2B, but not NR2A, NR2C or NR2D NMDAR subunit expression was increased by chronic morphine treatment in dorsal root ganglion neurons (Gong et al., 2015). It should be noted, however, that this study reports increased EAAT3 expression by chronic morphine treatment, which is in contrast to previous reports, a fact that is noted, but not further commented on. It has been demonstrated in C6 glioma cells that treatment with the selective μ -opioid receptor agonist DAMGO decreased EAAT3 expression (mimicking morphine exposure), while treatment with the selective δ -opioid receptor agonist, DPDPE, increased the expression of EAAT3. This, together with the observations that more DRG neurons express δ -opioid receptors than μ -opioid receptors (Wang and Wessendorf, 2001) could explain the difference in regulation of EAAT3 expression in the previous studies. Morphine-induced EAAT3 decrease can induce epigenetic modifications in the DNA methylation in SH-SY5Y cells, caused by decreased Cys uptake and subsequent S-adenosylmethionine levels (Trivedi et al., 2014), a mechanism that has also been shown for amyloid- β -induced changes in DNA methylation (Hodgson et al., 2013). This was rescued by inhibiting δ -opioid receptors, consistent with their role in mediating the morphine-dependent decrease in EAAT3 expression.

Volatile anesthetics such as isoflurane increase the expression of EAAT3 in addition to their other roles in regulating EAAT3 discussed under “stimulated trafficking” below (Huang and Zuo, 2003). Since isoflurane is a commonly used anesthetic in laboratory animals, it is something to keep in mind when designing animal studies of EAAT3.

Riluzole is the only FDA approved drug for ALS (Lacomblez et al., 1996). Riluzole increases the total amount of EAAT3, as well as the [3 H]-Glu uptake in C6 glioma cells by a protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K)-dependent mechanism

(Dall'Igna et al., 2013). In rat spinal cord synaptosomes, riluzole increases in [³H]-Glu uptake which was unaffected by inhibiting PKA/PKC, or activating PKC, but could be attenuated by co-application of cholera toxin (Azbill et al., 2000).

Recently it was found that, neuregulin 1, an agonist for the ErbB4 tyrosine kinase receptor increases EAAT3 expression in C6 glioma cells and rat cortical cultures (Yu et al., 2015). A previous screen on mice deficient in another tyrosine kinase receptor, c-ros showed decreased EAAT3 expression, consistent with tyrosine kinase receptor-mediated increase in EAAT3 expression (Cooper et al., 2003). Interestingly, a role for neuregulin 1 and ErbB4 has been suggested in schizophrenia (Stefansson et al., 2002, Silberberg et al., 2006).

These studies hint at the importance of EAAT3 in the effects seen when treating animals or humans with these drugs. They also suggest several approaches for modulating EAAT3 expression. However, a greater understanding of the underlying mechanisms of each of these regulatory effects would greatly aid in this.

Constitutive Trafficking

EAAT3 is present in intracellular compartments, as well as in the plasma membrane. The transporter is constitutively endocytosed via a clathrin and dynamin-dependent mechanism and recycled via a Rab11-dependent mechanism (Gonzalez et al., 2007). Intracellular trafficking is regulated in part by a family of small GTPases called Rab proteins, belonging to the Ras superfamily. They are involved in trafficking and sorting between different intracellular compartments and the plasma membrane. Of importance in this review is Rab1, which mediates trafficking through the COPII coated vesicles, shuttling cargo from the ER to the Golgi, and Rab11, associated primarily with “slow” recycling endosomes, which mediate recycling of internalized plasma membrane proteins as well as some proteins going from the trans-Golgi to the plasma membrane through this pathway. Interestingly, dysfunctional Rab11-mediated EAAT3 recycling has been described in primary cortical cultures from embryos of a mouse model of Huntington's disease (Li et al., 2010).

The intracellular localization of a pool of transporters noted in several studies (Rothstein et al., 1994, Furuta et al., 1997a, Shashidharan et al., 1997, Conti et al., 1998, He et al., 2000, Sims et al., 2000, Gonzalez et al., 2002, Yang and Kilberg, 2002, Sheldon et al., 2006, Holmseth et al., 2012) suggests a means of rapid recruitment to the plasma membrane. The plasma membrane half-life of EAAT3 is ~5–7 minutes (Fournier et al., 2004, Gonzalez et al., 2007) while the half-life of EAAT3 protein itself seems to be ~6 hours (Yang and Kilberg, 2002). The relatively fast constitutive cycling suggests several potential points of regulation of surface expression, by *de novo* protein synthesis, redistribution of an intracellular pool to the plasma membrane or by decreased internalization of surface transporter pool.

Stimulated Trafficking

While endogenous modulation of EAAT3 expression no doubt contributes to development in the CNS, other changes may reflect physiological responses (e.g. synaptic potentiation/learning) or pathophysiology in the mature brain. Changes in localization of the carrier at the

plasma membrane suggest an acute and dynamic means of regulation, and several mediators of altered surface expression of EAAT3 have been reported.

PKC α , PI3K and Akt-Mediated Trafficking—Activation of both PKC α (Davis et al., 1998, Gonzalez et al., 2002) and PI3K (Sims et al., 2000) can increase surface expression of EAAT3 (see figure 3). Foster and coworkers found that treatment with oxotremorine-M, a non-selective muscarinic acetylcholine receptor agonist that can activate PKC, increased the plasma membrane localization of EAAT3, an effect that was partially sensitive to PKC and PI3K inhibitors (Foster et al., 2010). PI3K-mediated EAAT3 increase in surface expression is stimulated by platelet-derived growth factor (PDGF) (Sims et al., 2000). Twelve amino acids at the c-terminus of EAAT3, YVNGGFSVDKSD, are sufficient to confer PDGF-mediated trafficking to EAAT2 while residues YVN of the same sequence were necessary for PDGF-mediated EAAT3 trafficking (Sheldon et al., 2006). Interestingly, a PI3K inhibitor, wortmannin, can attenuate the PKC/PMA-induced increase in surface EAAT3 (Davis et al., 1998).

Several reports have shown the potential neuroprotective effects of treatment with volatile anesthetics. Oxygen-glucose deprivation (OGD)-induced neuronal apoptosis in primary cultured cortical rat neurons is attenuated by halothane and isoflurane treatment (Wise-Faberowski et al., 2001, Kapinya et al., 2002) and halothane, isoflurane, desflurane and sevoflurane reduced infarct volume in rat cerebellar slices after OGD (Wang et al., 2007a). Sevoflurane improved CA1 neuron recovery *in vitro* and *in vivo* after hypoxia and ischemia, which was abolished by blocking PKC in rats (Wang et al., 2007b). The protective effects of sevoflurane on infarct size were also attenuated by inhibition of PI3K by either wortmannin or LY294002, as well as overexpression of carboxy-terminal modulator protein (CTMP), an inhibitor of Akt (also known as protein kinase B; PKB) that is increased concomitant with Akt decrease during ischemia (Chen et al., 2015). This suggests a mechanism of Akt being positively regulated by PI3K, and negatively regulated by CTMP in the neuroprotective effects of volatile anesthetics (see figure 3). Interestingly, isoflurane has been shown to increase EAAT3 trafficking to the plasma membrane in PKC α -dependent manner in C6 glioma cells (Huang and Zuo, 2005) and mouse hippocampus (Cao et al., 2014a) by a mechanism involving phosphorylation of S465 (Huang et al., 2006, Huang et al., 2011), suggesting a possible role for EAAT3 in the neuroprotective properties of volatile anesthetics. However, isoflurane has also been shown to protect against apoptosis in human endothelial cells via a sphingosine kinase-1/ERK/MAPK pathway (Bakar et al., 2012). Whether effects on decreased infarct after ischemia are due to EAAT3 expression or another mechanism, but correlated to EAAT3 remains to be elucidated. Transfecting C6 cells with syntaxin 1A, a Q-SNARE protein, causes internalization of EAAT3 (Yu et al., 2006). SNAP-23, another Q-SNARE, is expressed both in neurons and C6 glioma cells. Expressing a dominant negative version of SNAP-23 in C6 cells decreased the surface levels of EAAT3, which was due to a reduced constitutive recycling of the transporter (Fournier and Robinson, 2006).

Other Mechanisms of EAAT3 Trafficking—Independent of PKC and PI3K, activation of the GPCRs, neurotensin receptor 1 (NTS1) or the endothelin-1 receptor ET_A increases the

EAAT3 surface expression (Najimi et al., 2002, 2005), and stimulation of ionotropic Glu receptors can also modulate EAAT3 localization. Brief, 5 minute NMDA treatment of rat hippocampal neurons results in a decrease in surface expression of EAAT3 (Waxman et al., 2007). However, LTP induced by high-frequency stimulation in rat hippocampal slices increases EAAT3 surface expression through an NMDAR-mediated mechanism (Levenson et al., 2002). Treatment with saturating concentrations of AMPA, leads to a time-dependent change in surface EAAT3 in the cerebellum, initially decreasing, then increasing to a maximum at 15 minutes before returning to baseline at 30 minutes (Cabrera-Pastor et al., 2015). The secondary increase in EAAT3 expression was blocked by the NMDAR inhibitor MK-801. These data suggest that both the duration of glutamate receptor stimulation and different glutamate receptor expression profiles contribute differentially to EAAT3 trafficking.

Recently, it was reported that amphetamine decreases the surface expression of EAAT3 (Underhill et al., 2014). This was dependent on a C-terminal sequence, VNGGF, which has previously been identified as important in targeting the transporter to dendrites in hippocampal neurons (Cheng et al., 2002). This also overlaps with motifs important for internalization (YVNGGF) via interaction with the AP2 complex (D' Amico et al., 2010) and PDGF-stimulated increased surface expression (YVN) (Sheldon et al., 2006). The amphetamine-induced decrease in surface EAAT3 was mediated by RhoA (see figure 3). Amphetamine also increased both AMPAR and NMDAR-mediated evoked excitatory post-synaptic currents in substantia nigra pars compacta slices when stimulating glutamatergic inputs, and this was blocked by a VNGGF peptide in the recording pipette. These observations are consistent with the effects being mediated by increased local Glu concentrations due to decreased post-synaptic EAAT3, suggesting that EAAT3 regulation could be a mechanism involved in the learning and memory aspect of amphetamine addiction (Tzschentke and Schmidt, 2003). Interestingly, it was recently reported that the dopamine transporter follows the same RhoA dependent mechanism of amphetamine-induced endocytosis (Wheeler et al., 2015). This effect is time-dependent due to increased cAMP inactivating RhoA, which could suggest a similar regulation for EAAT3 trafficking.

In 2001, a novel interacting partner of the rat EAAT3 C-terminal was identified by a yeast two-hybrid screen of rat brain cDNA (Lin et al., 2001), and later cloned (Butchbach et al., 2002). This protein, a member of the prenylated Rab acceptor family, was named glutamate transporter EAAC1-associated protein (GTRAP3-18), where the mouse protein is addicsin, and the human protein is JWA (Butchbach et al., 2002, Ikemoto et al., 2002). GTRAP3-18 is an ER associated protein that can delay the ER to Golgi trafficking of EAAT3 by binding Rab1a and reducing the density of cargo protein at COPII vesicles (see figure 3) (Ruggiero et al., 2008, Maier et al., 2009). Further support for this role came from the finding that RTN2B, a member of the reticulon family, could bind both EAAT3 and GTRAP3-18, and enhance the ER to Golgi trafficking of EAAT3 (Liu et al., 2008). Knocking out GTRAP3-18 in mice resulted in mice that were leaner and less explorative, but had no other observed phenotypic alterations compared to WT. Analysis of GTRAP3-18 KO mice revealed increased levels of EAAT3, GSH and protection against oxidative stress, as well as enhanced learning and memory in the Morris water maze test (Aoyama et al., 2012) which is the inverse of findings in EAAT3 KO mice, consistent with a higher functional EAAT3 levels.

Interestingly, chronic morphine treatment increased the expression of adducin (GTRAP3-18) in mice (Ikemoto et al., 2002).

In summary, PKC(α) and PI3K activation constitute one mechanism of regulating the surface pool of EAAT3 that can be activated by various mechanisms converging at these kinases, which may proceed through Akt activation. The PI3K-mediated trafficking is dependent on a motif in the C-terminal of the EAAT3, a motif that overlaps with several trafficking-related motifs. PI3K dependent, but PKC-independent trafficking has been demonstrated (Sims et al., 2000), suggesting that either PKC is upstream of PI3K activation or the two are part of a converging mechanism where at least some PI3K activity is necessary.

Different Q-snares can regulate the constitutive trafficking through either endocytosis or recycling, though the contribution of specific proteins *in vivo* is not fully understood. The constitutive trafficking relies on clathrin for endocytosis. However, there is also a clathrin-independent mechanism, which is linked to RhoA activation by intracellular amphetamine (Underhill et al., 2014). The role of NMDAR activation is not clear-cut. While the initial decrease in EAAT3 observed could be due to calcium activation of CaMKII, an upstream effector of RhoA, the mechanism of the subsequent increase is less clear. Another layer of regulation occurs at the ER where GTRAP3-18 and RTN2B regulate the amount of EAAT3 leaving the ER for further processing in the Golgi. Interestingly, GTRAP3-18 was found on a susceptibility locus for epilepsy (Zara et al., 1998).

Direct Functional Regulation

In addition to the regulation by trafficking, EAAT3 function can also be directly modulated by other proteins. PKC ϵ has been implicated in regulating the function of EAAT3 without changing the surface expression (Gonzalez et al., 2002). Likewise, GTRAP3-18, when first identified, was found to change the K_m of EAAT3 without altering the V_{max} (Lin et al., 2001), while subsequent reports have focused on modulation of subcellular localization (Ruggiero et al., 2008, Liu et al., 2009) suggesting multiple levels of regulation by GTRAP3-18. Besides the protein-induced direct modulation, there is also direct pharmacological modulation of the transporter, though we are thus far lacking selective compounds. As previously mentioned, increased understanding of the structure and mechanism could help in the design and discovery of such compounds.

Summary and Conclusions

Increasing evidence of the importance of EAAT3 in the brain in regard to Cys and Glu uptake for various purposes has come to light in recent years, ranging from regulation of glutamatergic and GABAergic neurotransmission to regulating redox homeostasis in neurons. As more studies link EAAT3 dysregulation to pathological states, we are gaining more and more insight into the role EAAT3 plays both in healthy and diseased brains. Though we are beginning to understand some of the functions EAAT3 governs, as well as some of the mechanisms involved in regulation, there is still much left to be elucidated.

Given the emerging appreciation of the importance of EAAT3, pharmacological modulation of EAAT3 is of interest, both as tools for basic scientific understanding as well as a potential clinical approach. While there are selective inhibitors for EAAT1 (UCPH-101) (Abrahamsen et al., 2013) and EAAT2 (DHK) (Arriza et al., 1994), there are none for EAAT3. Though β -benzo-Asp analogs (Esslinger et al., 2005) and NBI-59159 show a slight preference towards EAAT3 (Coon et al., 2004, Dunlop, 2006), this preference is not true selectivity, making pharmacological methods for selectively targeting EAAT3 difficult, which for acute *in vitro* and *in vivo* modulation is still the most feasible method.

For clinical approaches, the regulation of trafficking of EAAT3 is another way to address the function. However, also here, a greater understanding of the exact regulatory mechanisms *in vivo* is required. Due to the constitutive internalization and recycling of EAAT3, it would be prudent to dissect the effects of any observed changes of surface expressed transporter into modulation of either internalization, recycling or *de novo* synthesis, since modulation of each of these could have differential off target effects.

Though there have been success stories of generating specific drugs from rational design based on endogenous ligands (DHK, an Asp analog inhibits EAAT2 inhibitor with ~100 fold selectivity over the other subtypes (Jensen and Brauner-Osborne, 2004)), the nature of using Glu or Asp as a starting point also has some potential problems. Since there are several transporters, as well as receptors, that share the same substrate, it is likely that many compounds based on the endogenous ligand will be less specific. Specific compounds targeting EAAT3, as well as additional methods of modulating the expression and trafficking are needed to further our understanding of this target, and could have therapeutic applications as well.

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Figure 1.

Top: Crystal structure of outward facing GltPh trimer. Protomer 1 is color coded with the scaffolding domain in blue and the transport domain in red, protomer 2 is color coded in 'rainbow' (colors correspond to the topology view below). Left: Viewed from extracellular space. Right: Viewed from the membrane plane, top is extracellular. Bottom: Topology view of EAATs. Transmembrane helices, as well as hairpin loops are numbered.

Figure 2. State diagram of the transport cycle. The transporter opens hairpin (HP) 2, and substrate (red) enters, together with 3 sodium ions (green) and a proton (grey). HP2 closes, the transport domain moves through the membrane plane, where HP1 opens and the substrate and ions exit. A potassium (blue) ion enters from the intracellular space, HP1 closes and the transporter reorients back to an outward facing state, where HP2 opens and the potassium exits.

Figure 3.

Black →: Movement; Colored →: Positive modulation; Any ⇐: Negative modulation; Green: Experimental evidence for positive modulation (as discussed); Red: Experimental evidence for negative modulation (as discussed); Orange: Experimental evidence for one of several mechanisms resulting in similar net regulation; Yellow: Potential modulation or movement. Constitutive internalization occurs through a dynamin and clathrin-dependent mechanism, and recycling through a Rab11-dependent mechanism. PI3K can alter this by either by increased recycling or decreased internalization. This is also true for PKC, which may be mediated through PI3K. A canonical pathway of PI3K is through activation of PKB/Akt, which can regulate plasma membrane protein trafficking (Lee et al., 2013). Drugs such as riluzole and volatile anesthetics (such as isoflurane, depicted above) can increase EAAT3 surface expression through a PKC-sensitive mechanism.

Two Q-snares was shown to regulate EAAT3 surface expression; syntaxin 1A could increase endocytosis (Yu et al., 2006), where SNAP-23 could increase recycling (Fournier and Robinson, 2006).

NMDAR can regulate EAAT3 surface expression, and the direction seems to be time-dependent with an initial decrease, followed by an increase in plasma membrane EAAT3. RhoA is a downstream target of intracellular amphetamine. Both mechanisms of RhoA activation lead to a rapid decrease the surface expression of EAAT3.

GTRAP3-18 and RTN2B differentially regulate the EAAT3 ER to Golgi trafficking through the COPII-coated transport vesicles. GTRAP3-18 binds Rab1a and reduces the amount of EAAT3 in the vesicle. RTN2B binds both GTRAP3-18 and EAAT3 and increases EAAT3 trafficking through ER to Golgi trafficking.

PTEN is a regulator of NEDD4 (Hsia et al., 2014), which decreases EAAT3 expression by increasing ubiquitination and proteasomal degradation. It is also a negative modulator of Akt, though this action has not been specifically investigated in relation to EAAT3 trafficking.

GPCRs such as neurotensin receptor 1 (NTS1) or the endothelin-1 receptor ET_A can increase the surface EAAT3. Activation of the δ-opioid receptor increases the expression,

while activation of the μ -opioid receptor decreases expression. For the μ -opioid receptor, it was both through decreasing mRNA and increasing ubiquitin-dependent degradation. Activation of the ErbB4 receptor by neuregulin increased the expression of EAAT3. Numerous treatments and pathological conditions can change, or have been associated with changes in cellular expression of EAAT3, through molecular mechanisms that are often not fully understood. They have been listed in the purple insert.

Abbreviations: Adapter complex 2 (AP2); Amphetamine (AMPH); Carboxy-terminal modulator protein (CTMP); Dicarboxylic aminoaciduria (DCA); Dopamine transporter (DAT); Excitatory amino acid transporter 3 (EAAT3); G-protein-coupled receptor (GPCR); Glutamate transporter EAAC1-associated protein (GTRAP3-18); N-methyl-D-aspartate receptor (NMDAR); Neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4); Obsessive-compulsive disorder (OCD); Platelet-derived growth factor receptor (PDGFR); Phosphatase and tensin homolog (PTEN); Syntaxin 1A (STX1A); Reticulon 2B (RTN2B).

Table I**EAAT Nomenclature**

Nomenclature and original description of transporter subtypes.

EAAT Nomenclature	Gene Name	Original cloning name (species)
EAAT1	SLC1A3	GLAST (glu-asp transporter, (Storck et al., 1992))
EAAT2	SLC1A2	GLT-1 (glu transporter, (Pines et al., 1992))
EAAT3	SLC1A1	EAAC1 (excitatory amino acid carrier 1, (Kanai and Hediger, 1992))
EAAT4	SLC2A6	EAAT4 (Fairman et al., 1995)
EAAT5	SLC1A7	EAAT5 (Arriza et al., 1997)

Table II

Observed Changes in EAAT3 Expression

Overview of changes in EAAT3 expression observed under different conditions.

Mutation/treatment	Expression up/down	Species	Region/Cell type	Reference
mRNA				
Bipolar disorder	Decreased	Human	Ventral striatum	(McCullumsmith and Meador-Woodruff, 2002)
Epilepsy	Increased	Human, rat	Dentate granule	(Crino et al., 2002)
Ischemia/Hypoxia	Decreased	Rat	CA1, CA3, dentate gyrus	(Montori et al., 2010)
Ischemia/Hypoxia	Decreased	Gerbil	CA1	(Fujita et al., 1999)
Isoflurane	Increased	Rat	C6 glioma cells	(Huang and Zuo, 2003)
Schizophrenia	Decreased	Human	Striatum	(McCullumsmith and Meador-Woodruff, 2002)
Epilepsy	Increased	Rat	Hippocampus (cultures)	(Ross et al., 2011)
OCAD associated SNP, rs3087879	Decreased	Human	Lymphoblastoid and dorsolateral prefrontal cortex	(Wendland et al., 2009)
mRNA and protein				
Epilepsy	Decreased	Rat	Hippocampus	(Simantov et al., 1999, Harrington et al., 2007)
Epilepsy	Decreased	Rat	Hippocampus	(Simantov et al., 1999)
Hypoxia	Increased	Rat	PC12 cells	(Kobayashi and Millhorn, 2001)
Schizophrenia	Increased	Human	Anterior Cingulate Cortex	(Bauer et al., 2008)
Schizophrenia	Increased	Human	Frontal Cortex	(Rao et al., 2012)
Protein				
Alzheimer's disease (3xTg-AD mouse model)	Decreased	Mouse	Hippocampus (but not cortex)	(Cassano et al., 2012)
Alzheimer's disease (A β PP23 mouse model)	Increased	Mouse	Hippocampus (but not cortex)	(Schallier et al., 2011)
Diabetes	Decreased	Rat	Hippocampus	(Song et al., 2015)
Dicarboxylic aminoaciduria SNP, R445W and I395	Decreased	Human		(Bailey et al., 2011)
Epilepsy	Increased	Human	Dentate granule	(Crino et al., 2002)
Epilepsy, Pilocarpine induced	Increased	Rat	Dentate granule	(Crino et al., 2002)
Ischemia/Hypoxia	Decreased	Gerbil	Hippocampus	(Raghavendra Rao et al., 2000)
Ischemia/Hypoxia	Decreased	Pig	Striatum	(Martin et al., 1997)
Ischemia/Hypoxia	Increased	Rat	CA1	(Gottlieb et al., 2000)

Mutation/treatment	Expression up/down	Species	Region/Cell type	Reference
Morphine, chronic	Decreased	Mouse, rat	Spinal cord, C6 glioma cells	(Ikemoto et al., 2002, Mao et al., 2002, Yang et al., 2008, Dall'Igna et al., 2013, Wu et al., 2013d, Guo et al., 2015)
Morphine, chronic	Increased	Rat	Dorsal root ganglion	(Gong et al., 2015)
Neuregulin (tyrosine kinase)	Increased	Rat	C6 glioma cells	(Yu et al., 2015)
OCD associated SNP, R445W and I395	Decreased	Human		(Bailey et al., 2011)
Riluzole	Increased	Rat	C6 glioma cells	(Dall'Igna et al., 2013)
Huntington's disease, STHdhQ111/Q111	Decreased	Mouse	Striatal neural precursor cell line	(Petr et al., 2013)

* see "results note".