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Emerging Evidence for a Direct Link between EAAT-Associated Anion Channels and Neurological Disorders

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Review of Parinejad et al.

Excitatory amino acid transporters (EAATs) are membrane proteins that control excitatory synaptic transmission in the brain (Bergles et al., 1999; Danbolt, 2001; Robinson and Jackson, 2016). As classical secondary active transporters, which use the energy stored within ion gradients to drive substrate translocation, EAATs transport glutamate against its concentration gradient into neurons and glia. To accomplish this, they couple the influx of each glutamate molecule to the influx of two or three sodium ions and one proton and the efflux of one potassium ion (Danbolt, 2001). Dysfunctional glutamate clearance can lead to increased extracellular glutamate concentrations, causing prolonged activation of glutamate receptors and increases in intracellular calcium, resulting in neuronal excitotoxicity. Thus, deficient glutamate uptake is linked to several neurological disorders, including epilepsy, amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease (Danbolt, 2001; Nakagawa and Kaneko, 2013).

In addition to clearing glutamate, EAATs mediate an anion-selective conductance (Fairman et al., 1995), which arises from the diffusion of permeable anions through a glutamate-activated pore within the transporter (Fahlke et al., 2016). Emerging evidence suggests that this EAAT-mediated anion conductance plays a significant role in glutamatergic synaptic transmission. In retinal cells, EAATs act as presynaptic sensors that dampen glutamate release by hyperpolarizing the presynaptic terminal through activation of EAAT-mediated anion currents (Picaud et al., 1995; Veruki et al., 2006; Wersinger et al., 2006).

Several studies have suggested that disruption in EAAT-mediated anion permeation are linked to neurological conditions. For example, a missense mutation leading to arginine substitution for proline (P290R) in EAAT1 (a glial isoform) was identified in a patient with episodic ataxia Type 6, a condition characterized by progressive impairment of coordination and balance and sporadic episodes of hemiplegia, symptoms shown by the patient. This mutation reduced surface expression of EAAT1 and glutamate uptake *in vitro*, but because there are currently no techniques for assessing EAAT1 activity directly in patients, the authors were unable to link the mutation to disrupted glutamate uptake in the patient (Jen et al., 2005).

More recently, Stacey et al. (2010) examined a *Drosophila* knock-out ($EAAT1^{KO}$) and found that the typical full-body peristaltic contractions observed in wild-type larvae were significantly reduced in the $EAAT1^{KO}$, suggesting that EAAT1 is essential for peristaltic motility. Interestingly, despite showing reduced peristaltic contractions, $EAAT1^{KO}$ larvae did not show episodes of paralysis (Stacey et al., 2010). These observations suggest that the paralysis that Jen et al. (2005) linked to the P290R mutation may not solely stem from decreased EAAT1-mediated glutamate uptake, which is absent in the knock-out.

An alternative possibility is that disruption of an EAAT function other than glutamate transport might be responsible for episodes of paralysis. Indeed, Winter et al. (2012) investigated the biophysical properties of anion conduction in the $hEAAT1^{P290R}$ mutant, and concluded that reduced glutamate uptake was accompanied by an increase in the anion channel open probability. Using site-directed mutagenesis to probe structure–function relationships, several groups have characterized mutations that disrupt substrate transport and favor open channel states, suggesting a dynamic equilibrium between the two processes (Borre et al., 2002; Machtens et al., 2015; Torres-Salazar et al., 2015). The

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conclusions by Winter et al. (2012) harmonize with this idea.

In a recent publication in *The Journal of Neuroscience*, Parinejad et al. (2016) tested this hypothesis by creating a *Drosophila* model of ataxia expressing the EAAT1 P to R mutation (dEAAT1^{P243R}). The authors overexpressed the mutant dEAAT1^{P243R} or the homologous human EAAT1^{P290R} (hEAAT1^{P290R}). Unlike overexpressing dEAAT1^{WT} (Stacey et al., 2010), overexpressing dEAAT1^{P243R} or hEAAT1^{P290R} in the null mutant background did not rescue normal larval peristaltic behavior, suggesting that the mutation renders EAAT1 nonfunctional *in vivo*. When the mutant dEAAT1^{P243R} was overexpressed in wild-type larvae, moreover, the animals exhibited episodes of complete paralysis during which they were unresponsive to mechanical stimulation. Notably, the episodes of immobility in the dEAAT1^{P243R} mutants were significantly more frequent and more prolonged than in control groups, consistent with the idea that expressing the chloride channel gain-of-function mutation generates ataxia. Furthermore, when the dEAAT1^{P243R}-expressing larvae were mobile, they attained maximum speeds comparable with those of the control groups, suggesting that the neuronal circuitry responsible for peristalsis was intact. Additional findings revealed that, unlike complete knock-out of EAAT1, the dEAAT1^{P243R} mutation significantly reduced astrocyte infiltration of the CNS neuropil. Because episodes of paralysis and reduced astrocyte infiltration were observed in dEAAT1^{P243R} mutants but not in the EAAT1^{KO}, the authors concluded that increased anion permeation, rather than reduced glutamate transport, was responsible for the significant differences in phenotypes between the two model systems (Parinejad et al., 2016).

The authors corroborated this conclusion by overexpressing the K^+-Cl^- cotransporter (KCC), which is responsible for chloride extrusion from the cell. Interestingly, overexpressing the *Drosophila* KCC (KccB) had effects similar to those overexpressing dEAAT1^{P243R} or hEAAT1^{P290R}, namely, decreased astrocyte infiltration of the CNS neuropil and episodic paralysis. These results support the hypothesis that the anion channel gain of function is the main contributor to the observed episodic ataxia.

To further support this conclusion, Parinejad et al. (2016) overexpressed the *Drosophila* Na^+-K^+-Cl^- cotransporter (Ncc69), which mediates chloride uptake, and found effects opposite to those of KccB overexpression. Ncc69 overexpression caused a 20% increase in neuropil infiltration by astrocytes and significantly increased locomotor activity compared with wild-type. Even more compellingly, simultaneous expression of Ncc69 cotransporters with dEAAT1^{P243R} rescued both neuropil infiltration and locomotor performance, preventing episodes of paralysis. These results provide convincing evidence that the enhanced chloride channel activity resulting from the dEAAT1^{P243R} mutation is responsible for episodic paralysis in the mutant *Drosophila*. This is further supported by the observation of episodic paralysis in EAAT1^{P243R} mutants, but not in EAAT1^{KO} larvae. The authors speculate that, because the mutation would eventually alter chloride osmotic balance, this could lead to astrocyte volume reduction and consequent withdrawal of their infiltrative processes. This is an appealing idea, considering the significant reduction in neuropil infiltration observed in the larvae expressing the mutant transporter.

Although the data provide strong support for the hypothesis that disruption of chloride permeation by the dEAAT1^{P243R} mutation is responsible for the observed phenotypes, these findings could be further explored to fully characterize the relationship between this mutant transporter and the observed morphological and behavioral alterations. For example, there is no direct measurement of astrocyte intracellular chloride concentration ($[Cl^-]_i$) and/or astrocyte membrane potential. If the episodes of paralysis are caused solely by the reduced $[Cl^-]_i$ resulting from excessive chloride efflux, changes in $[Cl^-]_i$ should be detectable using available chloride biosensors (Watts et al., 2012) or chloride-sensitive microelectrodes (Kettenmann et al., 1987). Measurement of astrocytes' membrane potential could also indicate changes in $[Cl^-]_i$. This is important because significant reductions in intracellular chloride would not only affect astrocyte morphology, but also modify the voltage dependence of potassium channels (Bekar et al., 2005), which contribute to the hyperpolarized resting membrane potential and other cellular processes in astrocytes (Olsen, 2012).

Future work should also assess the mechanism by which overexpression of Ncc69 rescues the dEAAT1^{P243R} phenotype. Whereas Ncc69 moves chloride against its concentration gradient through a coupled active transport mechanism (Leiserson et al., 2011), EAATs facilitate chloride movement by simple diffusion down its concentration gradient through an anion-selective channel pore (Fahlke et al., 2016). Although EAAT-associated anion channels are categorized as slow ion channels with an estimated conductance of ~1 pS, they are at least an order of magnitude faster than transporters, such as Ncc69 (Fahlke et al., 2016). It is therefore somewhat surprising that the much slower Ncc69 chloride influx could compensate for the significantly more rapid efflux associated with the dEAAT1^{P243R} chloride channel. A much higher expression of Ncc69 compared with dEAAT1^{P243R} would explain this discrepancy. However, we consider other alternative explanations. Notably, expression of endogenous EAAT1, which was significantly reduced when dEAAT1^{P243R} was overexpressed, was restored when Ncc69 was coexpressed with dEAAT1^{P243R}. The endogenous EAAT1 would be expected to compete with dEAAT1^{P243R} and influence its recruitment to the plasma membrane. A reduction in membrane expression of dEAAT1^{P243R} caused by increased endogenous EAAT1 might therefore explain the recovery of the normal phenotype. Another possible explanation for Ncc69's ability to rescue the dEAAT1^{P243R} phenotype is that an unidentified "third party" molecule is at play. Such a molecule might be affected by the local increase in extracellular chloride concentration and trigger episodic ataxia. The addition of Ncc69 would prevent the presumed local extracellular chloride levels from reaching the minimal threshold necessary to activate such a molecule, thus preventing the episodic ataxia phenotype. These are intriguing questions that, when addressed in future studies, will help explain the capability of Ncc69 to compensate for the increased chloride efflux induced by the dEAAT1^{P243R} mutation.

In conclusion, the studies by Parinejad et al. (2016) show that a single amino acid substitution in the glial isoform of the glutamate transporter, EAAT1, results in increased chloride permeation, which causes neural circuit dysfunction and ultimately produces episodic paralysis in *Drosophila* larvae. Future studies to identify changes in both extracellular and intracellular chloride concentrations will further illuminate how the EAAT1-mediated anion channel is involved in behavioral phenotypes. This work also illustrates the importance of EAAT1-associated anion conductance for normal astrocytic function. Moreover, it offers strong evidence that this EAAT function

correlates with neuropathology, setting the stage for new studies that will help elucidate its role in the normal operation of the brain and in other neuropathological processes.

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