

Review

## Glutamate transporters: animal models to neurologic disease

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**Glutamate is the primary excitatory amino acid neurotransmitter in the central nervous system and its activity is carefully modulated in the synaptic cleft by glutamate transporters. A number of glutamate transporters have been identified in the central nervous system and each has a unique physiologic property and distribution. Glutamate transporter dysfunction may either be an initiating event or part of a cascade leading to cellular dysfunction and ultimately cell death. Animal models of glutamate transporter dysfunction have revealed a significant role for these proteins in pathologic conditions such as neurodegenerative diseases, epilepsy, stroke, and central nervous system tumors. Recent work has focused on glutamate transporter biology in human diseases with an emphasis on how manipulation of these transporter proteins may lead to therapeutic interventions in neurologic disease.**

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Glutamate is the primary excitatory neurotransmitter in the central nervous system. Under normal conditions, glutamate is released into the synaptic cleft and binds to glutamate receptors resulting in the propagation of an action potential. The modulation of this synaptic activity occurs both by modulation of glutamate receptors and by the removal of glutamate from the synaptic cleft by glutamate transporters. The importance of glutamate in normal central nervous system synaptic function has been well described and the observation that increased amounts of glutamate in the synaptic cleft leads to neurotoxicity has been well established (Choi et al., 1987). An important part of the regulation of extracellular glutamate relies on the function of sodium-dependent glutamate transporters present perisynaptically on astrocytes and, to a lesser degree, neurons. Despite the importance of glutamate transporters in maintaining synaptic function, the question of whether glutamate transporter dysfunction, leading to a rise in extracellular glutamate, plays a role in the development or propagation of neurologic disease has only more recently been addressed.

This review will discuss some experimental animal models of neurologic diseases including ALS, Alzheimer's disease, Parkinson's disease, stroke, epilepsy, and others in which alterations in glutamate transporter biology have offered insights into the pathophysiology of human neurologic diseases. A discussion of how the manipulation of these glutamate transporters using gene expression strategies has provided us with powerful tools to understanding their biology follows. How are glutamate transporters affected in human neurologic diseases? We will discuss important findings using human tissue to analyze the role of glutamate transporters in potentially propagating cell death from glutamate neurotoxicity and discuss some of the advantages and shortcomings of human studies.

### Glutamate transporter subtypes

Five plasma membrane glutamate transporter subtypes have been identified thus far. In human tissues, they are called excitatory amino acid transporters (EAAT) 1–5. In mammalian systems, the nomenclature is different. GLAST (EAAT1) (Shashidharan and Plaitakis, 1993; Storck et al., 1992) is primarily an astroglial transporter and the principal transporter protein present during CNS development (Furuta et al., 1997b). Its concentrations in adult tissue are particularly high in the Bergmann glia of the cerebellum with less expression in the brain and spinal cord. GLT1 (EAAT2) (Shashidharan et al., 1994) is an astroglial transporter expressed postnatally and is responsible for up to 90% of all glutamate transport in adult tissue (Danbolt et al., 1992; Tanaka et al., 1997). GLT1b (EAAT2b) is a naturally occurring splice variant of EAAT2, which has 11 fewer C-terminal amino acids, a unique 22 amino C-terminal domain, and is located not only in astrocytes but in neurons as well (Chen et al., 2002; Schmitt et al., 2002; Utsunomiya-Tate et al., 1997). EAAC1 (EAAT3) (Kanai and Hediger, 1992) is a neuronal glutamate transporter with high densities on postsynaptic membranes. It is present most notably in the hippocampus, cerebellum, and basal ganglia (Furuta et al., 1997a). EAAT4 is a glutamate transporter largely limited to the Purkinje cells of the cerebellum (Barpeled et al., 1997; Fairman et al., 1995; Furuta et al., 1997a). EAAT5 is found primarily in the retina on photoreceptors and bipolar cells (Arriza et al., 1997; Pow and Barnett, 2000). Recently, a class of glutamate transporters (VGLUT1-3) has been identified that concentrates glutamate into presynaptic vesicles (Bellocchio et al., 1998; Takamori et al., 2000, 2002). The vesicular glutamate transporters have different physio-

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Table 1  
Glutamate transporter subtypes

Glutamate transporter subtype	Human homologue	Cell type	Anatomic localization	References
GLAST	EAAT1	Astrocytes	Cerebellum > cortex, spinal cord	Shashidharan and Plaitakis (1993), Storck et al. (1992)
GLT1	EAAT2	Astrocytes	Throughout brain and spinal cord	Danbolt et al. (1992), Shashidharan et al. (1994), Tanaka et al. (1997)
GLT1b	EAAT2b	Astrocytes and neurons	Throughout brain and spinal cord	Chen et al. (2002), Schmitt et al. (2002), Utsunomiya-Tate et al. (1997)
EAAC1	EAAT3	Neurons	Hippocampus, cerebellum, and striatum	Furuta et al. (1997a,b), Kanai and Hediger (1992)
EAAT4	EAAT4	Purkinje cells	Cerebellum	Barpeled et al. (1997), Fairman et al. (1995), Furuta et al. (1997a,b)
EAAT5	EAAT5	Photoreceptors and bipolar cells	Retina	Arriza et al. (1997), Pow and Barnett (2000)
Vglut		Neurons	Presynaptic terminals	Bellocchio et al. (1998), Takamori et al. (2000, 2002)

logic roles than the membrane transporters and will not be the focus of this review (Table 1).

### Potential sites for glutamate transporter dysfunction

Abnormalities in glutamate transporter expression as a result of altered transcription or splicing, increased turnover of the transporter, altered trafficking of glutamate transporters, abnormal phosphorylation or cleavage of the protein, and reduced transport capacity are all potential sites where glutamate transporter dysfunction can occur (Fig. 1).

At the transcriptional level, an important finding was the recent identification of the human EAAT2 promoter (Su et al., 2003). Careful analysis using EAAT2 promoter–reporter assays demonstrated that epidermal growth factor (EGF), transforming growth factor (TGF- $\alpha$ ), and dibutyryl cAMP all resulted in a transcriptional increase in EAAT2 expression. TNF- $\alpha$  was found to inhibit EAAT2 transcription (Su et al., 2003). All of the factors identified play important roles in cellular responses, and study of the promoter will help to elaborate pathways involved in transcriptional regulation of the EAAT2 protein. Promoter-based assays may now be constructed to offer insights into glutamate transporter regulation both in disease states and normal physiologic conditions. In addition, the identification of the promoter may also provide fruitful information on pharmacologic agents, which may up-regulate EAAT2 transcription and offer neuroprotective strategies.

In addition to transcriptional activation, alterations in EAAT2 RNA splicing can affect protein levels. For example, abnormal splicing of glutamate transporter mRNA results in truncated mRNA species. This was reported by Lin et al. (1998) in ALS motor cortex. They demonstrated that while mRNA levels of the GLT1 transporter in ALS patients were equal to that of controls, altered EAAT2 splice products were identified and lead to the production of truncated EAAT2 protein in ALS patients (Lin et al., 1998). While subsequent studies have also identified these splicing variants in control tissues (Flowers et al., 2001; Meyer et al., 1999; Nagai et al., 1998), the large number of aberrant species in ALS deserves particular attention. The mechanism by which these truncated proteins cause alterations in glutamate transport may be because they have a reduced capacity for glutamate transport or potentially by acting in a

dominant fashion by sequestering normal full-length protein within the cell (Lin et al., 1998).

The EAATs are membrane-bound proteins that normally are compartmentalized to selected regions of neuronal and astroglial membranes. The normal targeting and activation of the EAATs requires glutamate transporter-associated proteins (or interacting proteins), which regulate their cellular and membrane trafficking. Glutamate transporter-associated protein (GTRAP 3-18), an endogenous protein localized to the cytoplasm and membrane of numerous CNS cell types, interacts with the C-terminal portion of EAAC1 and appears to reduce EAAC1's affinity for glutamate (Lin et al., 2001). Two other glutamate transporter-associated proteins, GTRAP 41 and 48, interact with the C-terminal portion of EAAT4. These proteins appear to stabilize or anchor EAAT4 to the cell membrane making it less likely to be internalized or degraded (Jackson et al., 2001). Therefore, in contrast to GTRAP 3-18, GTRAPs 41 and 48 resulted in an increase in glutamate transport capacity. Interactions between the glutamate transporter N-terminal portion of GLT1 and the cytoskeletal membrane of astrocytes was also demonstrated to occur with the LIM protein Ajuba (Marie et al., 2002)—although the functional role for this interaction is as yet unknown. Although abnormalities in glutamate transporter-associated proteins have not been directly linked to any neurologic diseases, understanding their biology may have important implications in future studies for both neural dysfunction and normal synaptic transmission.

Other potential mechanisms by which glutamate transporters can be damaged include redox modulation of reactive amino acids. Oxidation of certain intracellular amino acids of GLT1, by oxygen-free radicals, or peroxynitrite could alter functional transport. This may result in increased extracellular glutamate in the synaptic cleft resulting in further activation of glutamate receptors and a cascade of glutamate neurotoxicity. In support of this hypothesis, Trotti et al. (1999) demonstrated that mutant superoxide dismutase (SOD1), a gene product associated with familial amyotrophic lateral sclerosis (ALS), inactivated the glutamate transporter GLT1 when expressed in oocytes. This effect was blocked by the antioxidant Mn(III)TBAP, suggesting a possible role of free radical development and glutamate transporter dysfunction in ALS (Trotti et al., 1999). The same group has also suggested that abnormal caspase cleavage of the transporter protein could occur (Trotti, unpublished observation). Most of the transporters have potential caspase cleavage

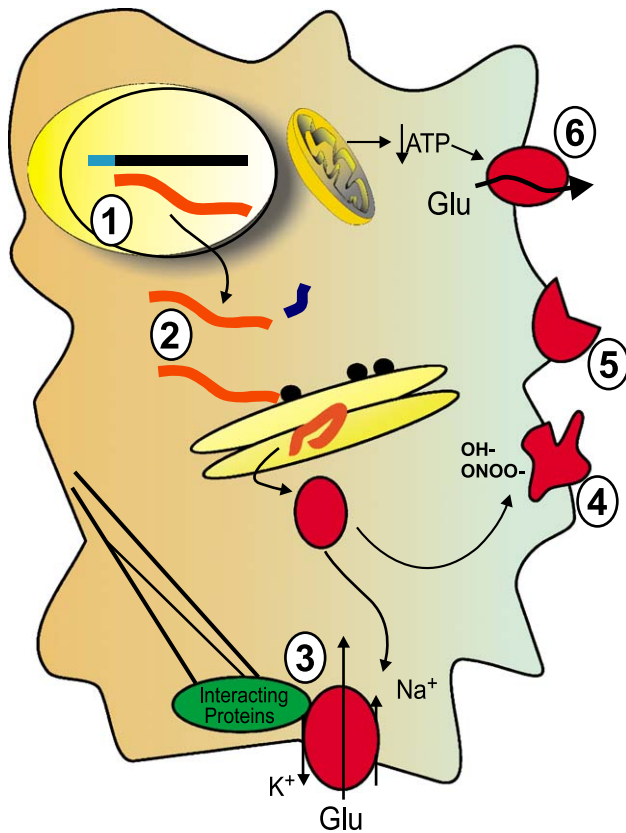


Fig. 1. Sites of potential glutamate transporter regulation or dysfunction. (1) Transcription. The recently identified GLUT1 promoter shows transcriptional regulation by factors such as TGF, dibutyryl cAMP, and others. (2) Posttranscriptional modifications or splicing occurs in both functional transporter splice variants (GLT1b) and aberrant, potentially toxic aberrant species (ALS). (3) Glutamate transporter-interacting proteins anchor glutamate transporters to cytoskeletal proteins. (4) Reactive oxygen species (SOD1/ALS) may result in glutamate transporter dysfunction and increased extracellular glutamate. (5) Aberrant or truncated proteins (ALS) result in glutamate transporter dysfunction or sequestering of normal glutamate transporter protein. (6) Reduced ATP reserves (hypoxic or ischemia) result in reversal of the Na-dependent transporter and reversal of glutamate transport into the extracellular space, exacerbating accumulation of extracellular glutamate.

motifs in their intracellular carboxy terminal domains. Preliminary studies suggest that truncation of the protein at these sites leads to diminished transporter activity—similar to that seen with the truncated splice variants reported by Lin et al. (1998).

Another possible mechanism for glutamate transporter protein dysfunction involves ATP depletion resulting in a loss of the ATPase activity driving the Na-dependent glutamate transporters. In models of hypoxic injury, studies have demonstrated an actual reversal of glutamate transport resulting in an accumulation of extracellular glutamate. Rossi et al. (2000) used a hippocampal slice model of ischemia to demonstrate reversal of glutamate transporter activity and accumulation of extracellular glutamate. By patch clamping CA1 pyramidal neurons and measuring the anoxic depolarizing current (a function of glutamate transporter reversal), they demonstrated that the use of dihydrokainate (DHK), a selective inhibitor of the astroglial glutamate trans-

porter GLT1, did not have any effect on transporter reversal and postulated that the rise in extracellular glutamate during ischemia is a result of neuronal glutamate transporter reversal (Rossi et al., 2000). This group recently extended this hypothesis using hippocampal slices from the GLUT1 knockout mouse in which the time to occurrence of the anoxic depolarizing current, its amplitude, and plateau were indistinguishable from wild-type mice (Hamann et al., 2002).

### Glutamate transporter null (knockout) mice

An important tool in understanding the role of glutamate transporters in disease, as well as in normal synaptic biology, is the study of glutamate transporter knockout mice. Both antisense knockdown and the GLUT1 null mouse provided insight into the important contribution of GLUT1 to total glutamate transport in the CNS. GLUT1 null mice retain <10% of total glutamate transport in the cortex confirming that GLUT1 is responsible for the bulk of extracellular glutamate clearance in the CNS. These mice also offer insights into disease pathogenesis. Subacute loss of GLUT-1, by antisense methods, leads to hind limb paralysis, while GLUT1 null mice develop hippocampal pathology, seizures, and most die by several weeks of age (Tanaka et al., 1997).

Knockout of the neuronal glutamate transporter EAAC1 leads to the development of dicarboxylic aminoaciduria. This is due to the loss of normal glutamate and aspartate uptake from the renal tubules by EAAC1 (normally expressed in kidney). Interestingly, no neuronal loss was observed in regions normally rich in EAAC1 expression (hippocampus, cerebellum, and cortex). No motor incoordination was observed in rotorod testing and learning seemed unimpaired in water maze testing. The only significant behavioral observation was a decrease in spontaneous motor activity as measured by open-field monitoring chamber (Peghini et al., 1997). In comparison, knockdown of EAAC1 in adult rats leads to an epilepsy due in part to the disruption of GABA metabolism (Mathews and Diamond, 2003; Sepkuty et al., 2002).

Knockout of the GLAST transporter, which is particularly rich in the cerebellum, did not produce an overt ataxic phenotype in null mice as one might expect. Furthermore, cerebellar anatomy was not grossly altered and electrophysiologic study in the cerebellum did not implicate a major role for GLAST in the synaptic clearance of glutamate from Purkinje cells. However, when examined in more detail using rotorod analyses of gait and coordination, GLAST null mice did not perform well at high revolutions of a rotating rod. At low speeds, however, they performed equal to that of wild-type controls. Additionally, using a cold probe to induce traumatic injury, the edema volume was greater in the cerebellum of GLAST null mice than in wild-type controls. In a finding consistent with the distribution of GLAST in the CNS, edema volumes from traumatic injury were greater in the cerebellum than cerebral cortex of the null mice (Watase et al., 1998). In EAAT4 null mice, no overtly abnormal phenotype has been observed and they do not appear to be different from wild-type controls (personal observations).

Despite the abundance of the glutamate transporter subtypes in the CNS, their regional specificity, and reported important role in clearance of extracellular glutamate, knockout of GLAST, EAAC1, and EAAT4 do not produce significantly abnormal CNS pathology—results that are at odds, in part, with adult antisense knock-

Table 2  
Glutamate transporter null mice

Glutamate transporter null mice	Physiology	Pathology	Phenotype	References
GLAST (EAAT1)	No changes in cerebellar physiology	None	Motor incoordination and increased susceptibility to cerebellar cold-induced injury	Watase et al. (1998)
GLT1 (EAAT2)	<5% of total glutamate transport remaining	Hippocampal neuron loss	Seizures, 50% mortality by 6 weeks	Tanaka et al. (1997)
EAAC1 (EAAT3)	Not studied	None	Dicarboxylic aminoaciduria	Peghini et al. (1997)
EAAT4 (EAAT4)	Not studied	No Purkinje cell loss	None	

down experiments (Rothstein et al., 1996). One reason may be that although the other glutamate transporters do not appear to compensate by increases in protein expression, the absence of these transporters during development may result in alterations of

synaptic biology including the glutamate receptor subtypes. This may explain the apparent differences in behavioral phenotypes associated with the gene null mice versus the use of antisense knockdown experiments. The development of conditional knock-

Table 3  
Glutamate transporters in animal models of neurologic disease

Disease	Experimental model	Transporter	Phenotype	References
ALS	SOD1 mutant G85R	GLT1 protein reduced	Progressive hindlimb paralysis and death	Bruijn et al. (1997)
	SOD1 mutant rat G93A	GLT1 protein reduced	Progressive hindlimb paralysis and death	Howland et al. (2002)
	GLT1 antisense knockdown in rat	GLT1 protein reduced	Progressive motor neuron loss and hindlimb paralysis	Rothstein et al. (1996)
Alzheimer's disease	Mutant APP overexpression in transgenic mice	EAAT1 and EAAT2 protein reduced with normal mRNA levels	Amyloid plaque formation and behavioral changes	Maslah et al. (2000)
	Cultured rat astrocytes with amyloid-beta peptide	Glutamate transporter reversal		Noda et al. (1999)
Parkinson's disease	Nigrostriatal denervation	No change unless L-dopa added then 37% increase in GLT1 protein expression		Lievens et al. (2001a,b)
	Denervation of striatal input	Reduction in GLT1 and GLAST		Ginsberg et al. (1995), Levy et al. (1995)
Huntington's disease	Expression of mutant huntingtin (R6/2)	GLT1 mRNA and protein levels reduced		Behrens et al. (2002), Lievens et al. (2001a,b)
Epilepsy	GLT1 knockout mouse	GLT1 protein reduced	Seizures and death at 6 weeks of age	Tanaka et al. (1997)
	GLAST knockout	GLAST protein reduced	Increased susceptibility to PTZ-induced seizures	Watase et al. (1998)
	EAAC1 antisense knockdown	Reduced EAAC1 and reduced GABA	Staring episodes with epileptiform activity by EEG	Sepkuty et al. (2002)
Stroke	7-day-old rat cortex and hippocampal hypoxia ischemia	GLAST, GLT1, EAAC1 reduced at 12 h		Fukamachi et al. (2001)
	Hippocampal slice cultures from GLT1 knockout mice	Glutamate uptake reduced but no reversal of transport related to GLT1		Hamann et al. (2002)
	MCA occlusion in GLT1 heterozygous animals	Brain edema increased at 1 and 24 h following reperfusion		Namura et al. (2002)
	Rat MCA occlusion with antisense oligonucleotides to GLT1 and GLAST	GLAST and GLT1 reduced by AS oligo	45% increase in stroke volume, worsened neurostatus and increased mortality	Rao et al. (2001a,b)
	Rat MCA occlusion	Decreased GLT1 and EAAC1	Contralateral hemiparesis	Rao (2001a)
Rat MCA occlusion	GLAST increased	Contralateral hemiparesis	Tao et al. (2001)	
Hypoxic neonatal pig	GLT1 and EAAC1 reduced at 24 h in striatum		Martin et al. (1997)	
Glioma	Glioma cell lines	GLAST mislocalized to nucleus		Ye et al. (1999)

out mice for the glutamate transporter subtypes may provide additional insights into their physiologic and phenotypic effects as a result of sudden loss of glutamate transporters in an adult animal (Table 2).

The use of the in vitro culture models and animal models described above provides evidence that glutamate transporter dysfunction may occur at many points along the glutamate transporter pathway. The relationship of these pathways to neurologic disease is examined in more detail below (Table 3).

### Amyotrophic lateral sclerosis (ALS)

A significant breakthrough in understanding potential pathogenic mechanisms in ALS came with the identification of mutations in the SOD1 gene (Rosen et al., 1993; Siddique et al., 1991), which has been identified in 5–10% of familial cases of ALS. This represents 1–2% of all ALS cases. Transgenic mice made from the overexpression of this mutant protein develop a slowly progressive paralysis characteristic of ALS (Bruijn et al., 1997; Gurney et al., 1994), with associated loss of brainstem and spinal lower motor neurons. In all SOD1 mouse transgenic models, including the G85R, G37R, and G93A mutants, a large reduction in the GLT1 glutamate transporter was observed in end-stage mutant SOD1 mice when compared with controls (Bruijn et al., 1997)—thus linking observations of the sporadic disease to the familial form of the disease.

Recently, this observation has been extended in a transgenic mutant SOD1 (G93A) rat where a >90% reduction in GLT1 protein was observed in a mutant SOD1 G93A rat model. Immunohistochemically, the loss of GLT1 in the ventral horns appeared focal. This loss was also evident before the onset of hindlimb paralysis characteristic of the model (Howland et al., 2002). In a similar transgenic rat model of the mutant SOD1 phenotype, CSF glutamate levels were found to be unchanged from control animals (Nagai et al., 2001). Why does a loss of GLT1 not lead to elevations in measurable CSF glutamate? In fact, it's unlikely that a small focal loss of the transporter protein would alter large regions of extracellular glutamate—given the high concentration of transporter proteins throughout the CNS. Thus, a focal loss of protein may cause or contribute to severe focal neural injury—but have little effects on more distant tissue.

A possible link between mutant SOD1 and reduced function of GLT1 was identified by Trotti et al. (1999). Using recordings from oocytes, he demonstrated that GLT1 was a target of mutant SOD1 mediated oxidation (Trotti et al., 1999) and that its glutamate transporter capacity was reduced.

Using an antisense oligonucleotide method for knocking down the glutamate transporter GLT1, Rothstein et al. (1996) demonstrated that CSF administration of GLT1 antisense oligonucleotide results in a reduction of GLT1 protein expression. Phenotypically, this leads to progressive hindlimb paralysis and motor neuron degeneration (Rothstein et al., 1996). Interestingly, in the GLT1 knockout mouse, no motor neuron loss is observed. Instead, approximately 50% of these animals die of refractory seizures during the first 6 weeks of life (Tanaka et al., 1997). One explanation for the lack of a motor neuron disease phenotype in the GLT1 knockout mice is that these mice die before onset of motor neuron degeneration, or compensation from other glutamate transporters, such as GLAST, may play a role in preventing pathology in other CNS regions.

In human ALS tissue, multiple lines of evidence suggest that glutamate may have a neurotoxic role in ALS. Initial studies demonstrated that cerebrospinal fluid glutamate levels may be elevated in patients with sporadic ALS (Rothstein et al., 1990). More recently, this was confirmed in a study of a large number of ALS patients (Spreux-Varoquaux et al., 2002). A potential mechanism for the elevated CSF glutamate in ALS patients was subsequently provided by tissue studies of glutamate transporters. These studies revealed that up to 60–70% of patients with sporadic ALS have a 30–90% loss of the EAAT2 protein, in both motor cortex and spinal cord (Rothstein et al., 1995). The loss of EAAT2 appears to be specific to these regions in most but not all patients. This loss of EAAT2 protein cannot be attributed to cell death since there is no significant astroglial loss in ALS.

While mounting evidence suggests that glutamate transporter dysfunction is not the primary insult leading to the development of motor neuron disease, the data suggest that glutamatergic pathways and transporters are at least important in the propagation of motor neuron loss. Important future investigation will be the study of how other mechanisms believed to play a role in ALS, that is, free radical production, neurofilament disorganization, and mitochondrial dysfunction, may affect glutamate transporter function.

### Alzheimer's disease (AD)

There are data in human Alzheimer's disease cases to suggest alterations in the function, anatomical location, and expression of glutamate transporters in Alzheimer's disease models as well as in human tissue. Animal models of AD have lent insight into the potential alterations in glutamate transporter expression and biology. Recent studies have shown that amyloid precursor protein (APP), which plays a central role in Alzheimer's disease, protects against excitotoxic neuronal injuries by regulating the function of the glial glutamate transporters through protein kinase A and protein kinase C dependent pathways (Masliah et al., 1998).

In support of this hypothesis, transgenic mice expressing a mutant form of the human APP from the Thy-1 promoter showed a significant decrease in  $V_{max}$  and  $K_D$  for aspartate uptake when compared to nontransgenic controls. This decrease in glutamate transporter activity was associated with decreased protein expression of EAAT1 and EAAT2 but did not affect mRNA levels. These results suggest that expression of mutant forms of APP disturbs astroglial transport of excitatory amino acids at the posttranscriptional level leading in turn to increased susceptibility to glutamate toxicity (Masliah et al., 2000).

An interesting alternative hypothesis for the possible contribution of glutamate transporters to neuronal dysfunction in AD comes from an in vitro model. The treatment of astrocyte cultures with A $\beta$  resulted in the increased clearance of extracellular glutamate and the decrease in glutamatergic synaptic function in cocultured neurons. These findings could not be attributed to cell death from A $\beta$  toxicity but may be related to the redistribution of GLAST to the astrocyte membrane. These changes then may contribute to synaptic dysfunction rather than cell death (Ikegaya et al., 2002).

In support of glutamate transporter reversal as a possible mechanism in the propagation of glutamate neurotoxicity, Noda et al. (1999), using cultured rat microglia, demonstrated that at the pathological sites where extracellular K<sup>+</sup> concentration may in-

crease, the activation of microglia by amyloid-beta peptide causes an increase in extracellular glutamate concentration via reversal of glutamate transport. This approach was unique in its focus on microglia rather than astrocytes in glutamate transporter dysfunction particularly because of the relationship between activated microglia in AD pathology. The authors speculate that this mechanism may contribute to the pathogenesis of neuronal dysfunction and death in Alzheimer's disease (Noda et al., 1999).

Scott et al. (2002) demonstrated the altered expression of EAAT1, typically an astroglial glutamate transporter in human Alzheimer's tissue. In cases showing Alzheimer-type neuropathology, EAAT1 was expressed in neurons, primarily a subset of pyramidal cells, and in dystrophic neurites. This aberrant expression was closely associated with tau deposition and neurofibrillary changes. The inclusion of cases that did not have Alzheimer-type pathology (i.e., controls, cases with pure Lewy body disease, and a dementia case with infarct pathology) confirmed that these changes in EAAT1 expression were specific to cases showing neurofibrillary pathology. This is interesting not only because the expression of EAAT1 was present in tau-positive cells, but also that EAAT1 was expressed in neurons at all since EAAT1 is almost exclusively expressed in astroglia (Scott et al., 2002). This may be the result of aberrant processing of EAAT1 transcripts under pathologic conditions.

In a related study, similar findings with EAAT2 were observed. EAAT2-immunoreactive neurons were observed throughout the cortex, striatum, hypothalamus, and reticular formation in Alzheimer's brain tissue (Thai, 2002). These EAAT2-immunoreactive neurons also displayed cytoskeletal abnormalities and tau deposits suggesting neuronal pathology. Importantly, none of the control cases without AD-related pathology showed EAAT2-immunoreactive neurons. The misexpression of these proteins may lead to important clues to their relationship with AD since previous studies looking at the levels of expression of EAAT1 and EAAT2 were not found to be significantly different between AD and control cases (Beckstrom et al., 1999). Whether the altered expression of glutamate transporters is a secondary phenomenon related to a "sick cell," a compensatory response to high extracellular glutamate, or whether it plays a more central role in the propagation of AD is not well understood.

Abnormal expression of glutamate transporters has also been reported to occur in human AD brains. In a postmortem analysis of AD brains, Li et al. (1997) showed that while mRNA expression of EAAT2 was not different between control and AD brains, the protein levels of EAAT2 were reduced in AD frontal cortex. Interestingly, EAAT2 immunoreactivity was inversely correlated with APP770 mRNA levels, suggesting a relationship between APP processing of amyloidogenic fragments and glutamate transporter misregulation. A predilection for selective EAAT2 involvement was also observed with EAAT1 and EAAT3 expression patterns unchanged between the two groups (Li et al., 1997).

Functional alterations in glutamate transport in human AD tissue have also been observed. Compared to control brains, AD brains displayed a 34% decrease in levels of D-[3H]aspartate binding, a 30% decrease in L-[3H]aspartate binding, and a 48% loss of synaptophysin immunoreactivity. Increased levels of brain spectrin degradation products correlated with a decrease in levels of D-[3H] and L-[3H]aspartate binding and decreased levels of synaptophysin immunoreactivity. Levels of L-[3H]aspartate binding correlated with levels of synaptophysin immunoreactivity.

These results suggest that decreased glutamate transporter activity in AD is associated with increased excitotoxicity and neurodegeneration, supporting the possibility that abnormal functioning of this system might contribute to the synaptic damage present in AD (Masliah et al., 1996).

While each of the studies cited above focuses on a particular aspect of glutamate transporter dysfunction in Alzheimer's disease, the data as a whole provide interesting findings with respect to glutamate transporter biology because they suggest that multiple pathways in glutamate transporter trafficking and physiology may be involved in Alzheimer's disease including modulation of transporter function, glutamate transport reversal, and posttranscriptional modification of transporter proteins. Whether these alterations in any way contribute to synaptic dysfunction or neuronal injury in AD is not clear—since the majority of studies are descriptive. Functional manipulations of glutamate transporters, for example, under expression, in AD transgenic models has not yet been carried out. Of potential significance is the observation that EAAT1 and EAAT2, primarily astroglial transporters, may be "mislocalized" to neurons under the pathologic conditions seen in AD. This theme of misexpression and mislocalization is also reflected in an ischemic model and glioma discussed below.

### Parkinson's disease

Evidence for a direct role for glutamate transporter dysfunction in the development of Parkinson's disease is less well established than in other models. Increased glutamatergic drive to basal ganglia output nuclei is considered a likely contributor to the pathogenesis of Parkinson's disease. One possibility for the increased excitatory tone may be related to impairment in glutamate transport. To study this hypothesis *in vitro*, MPTP, a toxin that is frequently used as a model for inducing parkinsonism in animal models, was applied to astrocytes in culture. The application of MPTP to these cultures resulted in a 39% reduction in transport and removal of MPTP resulted in recovery of transporter activity (Hazell et al., 1997).

Perhaps more importantly, studies of motor circuits relevant to Parkinson's disease and other movement disorders have provided important clues about transporter protein regulation. In experimental models involving denervation of the glutamatergic input to the striatum, regulation of excitatory amino acid transporter expression has been varied. Ginsberg et al. (1995) employed unilateral aspiration of the cerebral cortex corpus collosum pathway to disrupt striatal glutamatergic innervation. They demonstrated approximately 50% reduction in GLT1 protein expression and approximately 40% reduction in GLAST activity with sparing of the neuronal glutamate transporter EAAC1 (Ginsberg et al., 1995). A similar observation was observed following cortical lesions resulted in a down-regulation of the GLT1 and GLAST by approximately 20–30%. This was accompanied by a reduction in total glutamate transport in the striatum (Levy et al., 1995).

In contrast, however, bilateral cortical thermocoagulation resulted in an increase in GLT1 mRNA (45–101%) and protein expression in the striatum but a 35% decrease in total glutamate transport (Lievens et al., 2000). The other glutamate transporter GLAST was not studied in this model, and it is possible that the overall reduction in glutamate transport in this study reflected a

loss of GLAST. Furthermore, because different experimental paradigms were used in inducing striatal denervation (aspiration by Ginsberg et al., 1995, and Levy et al., 1995, versus thermo-coagulation by Lievens et al., 2000), the changes in transporter expression may have been altered.

In attempting to address whether glutamate transporter expression could be up-regulated, Lievens et al. (2000) extended their studies of nigrostriatal denervation to include the treatment of lesioned animals with levodopa. Treated animals had a 36% increase in striatal GLT1 mRNA levels and an increase in GLT1 protein levels. This effect appeared to be selective as GLAST and EAAC1 levels were unchanged following lesioning and subsequent levodopa treatment. These models offer some initial insights into the possible alterations of glutamate transporter expression through the manipulation of the nigrostriatal system (Lievens et al., 2001a).

### Huntington's disease

In a transgenic model of Huntington's disease, the polyglutamine repeat expansion results in a movement disorder with neuronal pathology. A reduction in the mRNA levels of the GLT1 in the striatum and cortex of these mice and a decrease in glutamate uptake were observed. These changes, however, occurred after the onset of the movement disorder and the appearance of polyglutamine aggregate pathology. Because down-regulation of GLT1 in denervated regions would normally be expected, as described above in experimental models of denervation, the authors were careful to note that the changes in GLT1 expression occurred before any neurodegeneration, thus potentially implicating GLT1 in part of a cascade of neuronal death (Lievens et al., 2001b).

Similar findings using the R6/2 transgenic expressing an N-terminal fragment of mutant huntingtin (R6/2) were also observed. Biochemical studies demonstrated an age-dependent down-regulation of the GLT-1 mRNA and protein appearing as early as 6 weeks of age, resulting in a progressive reduction of transporter function. While these changes occurred before the onset of disease, extracellular glutamate levels measured by microdialysis were not observed. These authors did observe an increase in extracellular glutamine levels and alterations to glutamine synthetase immunoreactivity, which they believe suggested a perturbation of the glutamate–glutamine cycle in this model (Behrens et al., 2002).

The study of glutamate transporters in human Huntington's disease is limited. In a small study of three human Huntington's disease brains analyzed postmortem, GLT1 mRNA was reduced in the neostriatum and the degree of reduction correlated with disease severity. The losses were found to be particularly prominent in the putamen and less so in the caudate. As has been observed in other neurodegenerative disorders, an astrogliosis with GLT1 expression in those cells was also observed. Other studies have failed to find significant changes in glutamate transport in Huntington's brain (Rothstein et al., 1992). More conclusive implications for the role of glutamate transporters in human Huntington's disease remain to be elucidated (Arzberger et al., 1997).

### Epilepsy

Given that glutamate is the primary neurotransmitter in the CNS and activation of glutamate receptors appears to be involved in the

generation of seizure activity in some animal models (Meldrum, 1994), it is an attractive hypothesis to suggest that alterations of glutamate transporter expression and function may play a role in epileptogenesis.

Direct evidence of the potential importance of glutamate transporters comes from the GLT1 knockout mouse. The absence of this astroglial transporter results in only 5% residual glutamate transport in GLT1 homozygous null mice. Histologic and physiologic changes were observed in the hippocampus with neuron loss that resulted in seizure activity and subsequent early mortality (50% die by 6 weeks of age) in these mice (Tanaka et al., 1997). However, the exact mechanisms for epilepsy in these mice have not been thoroughly studied and was not seen in subacute loss of the GLT-1 protein in antisense studies.

In GLAST knockout mice, more severe stages of PTZ-induced seizure activity were observed when compared with wild-type mice (Watanabe et al., 1999). Increases in GLT1 and EAAC1 glutamate transporter were observed in the frontal cortex but not in the hippocampus. This may be a compensatory effect in an attempt to modulate extracellular glutamate. However, changes in glutamate receptor expression were also observed with an increased expression of Glu-R1 in the hippocampus coupled with decreased cortical expression of Glu-R2 and increased NMDA-R1 and -2A, -2B expression. These additional alterations may account for seizure susceptibility in these animals (Ueda et al., 2002).

The role of glutamate transporters in epilepsy may not be related directly to synaptic glutamate but rather as a result of the role of glutamate in the metabolism in another important inhibitory neurotransmitter—GABA. GABA synthesis is in part dependent on glutamate recycling from extracellular stores. Sepkuty et al. (2002) demonstrated that there was a 50% loss of hippocampal GABA levels associated with knockdown of EAAC1, and newly synthesized GABA from extracellular glutamate was significantly impaired by reduction of EAAC1 expression. Reduced expression of EAAC1 by antisense treatment led to behavioral abnormalities, including staring-freezing episodes and electrographic (EEG) seizures (Sepkuty et al., 2002).

Alterations in EAAC1 expression in the hippocampi of kindled mice with little or no changes in GLT1 in this region have been reported. However, the response of EAAC1 to seizure activity has been variable—perhaps in part to the different models utilized. Simantov et al. (1999) noted a down-regulation of the neuronal transporter EAAT3 in restricted hippocampal regions. Using a kainate model, Furuta et al. (2003) also observed clear alterations in the expression and cellular distribution of EAAC1—with increases of the protein in the Golgi complex and less on plasma membranes. Conversely, others (Miller et al., 1997) observed that kindling induced an increase in EAAC1 levels in piriform cortex or amygdala and hippocampus once the animals had reached a specified severity. Similarly, in a rat model of pilocarpine-induced temporal lobe epilepsy, the expression of EAAT3 or EAAC1 mRNA was increased threefold in single dissociated hippocampal dentate granule cells from rats with pilocarpine-induced temporal lobe epilepsy (TLE) when compared with age-matched controls (Crino et al., 2002).

Are similar processes occurring in human epilepsy? Microdialysis studies of human epileptogenic hippocampus revealed elevated levels of glutamate following epileptic activity. This was an important first step in suggesting that alterations in glutamate metabolism, either from increased release of glutamate, decreased

glutamate transport, or a combination of both, may be playing a role in epileptiform activity (During and Spencer, 1993).

In patients undergoing temporal lobectomy for temporal lobe epilepsy, no significant changes in either mRNA or protein levels of the glutamate transporters EAAT1 and EAAT2 were observed in the medial temporal cortex and hippocampus (Tessler et al., 1999). Interestingly, Crino et al. (2002) also noted that astroglial EAAT2 levels were not changed in human temporal lobe epilepsy but that increases in the neuronal glutamate transporter EAAT3 mRNA and protein were observed in these tissues. Anatomic analysis of glutamate transporter immunoreactivity in the CA1-3 regions of the human hippocampus in temporal lobe epilepsy tissue demonstrated regionally specific changes in EAAT1-3. Notably, Mathern et al. (1999) noted that surviving hippocampal neurons were still surrounded by EAAT2-expressing cells and EAAT3 immunoreactivity was increased near cell bodies.

Proper et al. (2002) further divided cases of temporal lobe epilepsy into hippocampal sclerosis and nonsclerotic tissue and studied differences in glutamate transporter expression. EAAT3 immunoreactivity in individual neurons was increased in both hippocampal sclerosis and nonsclerotic tissue (Proper et al., 2002). What do these changes in glutamate transporter expression collectively suggest? One possibility is that the increase in the EAAT3 expression in these tissues may be a compensatory neuronal response to excess glutamate in the synaptic cleft. However, some caution in interpreting results in human studies should be maintained since differences in postmortem and/or processing delays in human tissues and severity of disease may account for differences observed in different human studies.

The study of glutamate transporters in animal models of human epilepsy is of particular importance since the role of glutamate in ictal activity is well established and because a prominent feature of the GLT1 null mouse is epilepsy. Furthermore, the potential of obtaining fresh human tissue during temporal lobectomy for studying glutamate transporter biology makes it unique and potentially more reliable since generally the study of other disorders (ALS, Parkinson's disease, and Alzheimer's disease) relies on the use of human postmortem tissue.

### Stroke and ischemia

A number of well-described animal models of ischemia have been applied to the study of glutamate transporter expression. However, the endpoints and number of different models have yielded variable and often conflicting characterizations of the changes in glutamate transporters to ischemia, making interpretation of their findings difficult. Extensive and well-controlled analyses of human tissues have not been performed, which is almost certainly because brain tissue for glutamate transporter analysis is rarely obtained in stroke cases. Additionally, given the acuity of ischemic events, glutamate transporter expression and function may change over minutes and hours making analysis of the temporal relationships between glutamate transporter dysfunction in ischemia impractical.

Fukamachi et al. (2001) measured the protein concentrations of GLAST, GLT1, and EAAC1 in 7-day-old rat cortices and hippocampi at 12, 24, 48, and 72 h following a hypoxic–ischemic insult. They noted that all transporters at the ischemic core were reduced at 12 h following injury with subsequent recovery of GLAST and GLT1 in the hippocampus. Increased EAAC1 expression was

noted in the perikaryon. This study demonstrated a complex relationship in the temporal expression of these transporters following injury as well as a regional variation between cortex and hippocampus (Fukamachi et al., 2001).

Tao et al. (2001), in an analysis of GLAST expression in neonatal ischemia, performed unilateral common carotid artery occlusion in PND 7 rats. GLAST appears to be the major astroglial transporter neonatally with GLT1 expression increasing later in development (Furuta et al., 1997b). Using immunohistochemical methods, increases in GLAST immunostaining in ischemic regions were observed 24, 48, and 72 h following occlusion. Interestingly, GLAST immunostaining was also observed in neurons as well as astrocytes in these same regions. Conversely, the intraventricular administration of GLAST antisense oligonucleotides before the induction of ischemia resulted in an exacerbation of neuronal injury. This study has several important implications in demonstrating that glutamate transporters may increase in a compensatory response to injury. Second, glutamate transporters typically associated with astroglia may be expressed in neurons under these conditions and that GLAST may play an important role in ischemia (Martin et al., 1997; Tao et al., 2001).

The observation that a possible compensatory change in the cell-type expression of glutamate transporters in response to ischemic injury was also suggested in an analysis of glutamate transporters in a neonatal piglet model of hypoxic–ischemic injury. Following 30 min of hypoxia, the striatum was analyzed at 24, 48, and 96 h. By immunocytochemistry, glutamate transporter 1 (GLT1) was lost after ischemia in the astroglial compartment but gained in cells appearing as neurons, whereas neuronal excitatory amino acid carrier 1 (EAAC1) dissipated. By immunoblotting, GLT1 and EAAC1 levels were 85% and 45% of control, respectively, at 24 h of recovery. The authors postulate that the postischemic neuronal induction of GLT1 may be an adaptive response to neuronal injury (Martin et al., 1997).

Utilizing the heterozygous GLT1 mouse, which expresses approximately half the GLT1 protein levels as wild-type mice, Namura et al. (2002) examined whether the reduced protein levels of GLT1 would exacerbate ischemia. Infarct volume was not changed following a 1-h MCA occlusion in the heterozygous mice when compared with control wild-type animals. However, brain edema was significantly increased. This effect was maintained 24 h following reperfusion (Namura et al., 2002). One interpretation would be that the amount of GLT1 remaining in heterozygous mice is enough to prevent neurotoxicity in this model. The significance of the increase in brain edema in these mice requires further study.

In an adult rat model of MCA occlusion, a significant argument for the importance of GLT1 in ischemia was demonstrated. Antisense oligonucleotides to lower GLT1 protein expression were infused into the CSF followed by MCA occlusion, which resulted in a 45% increase in infarct volume with a worsened neurologic status and increased mortality. When antisense oligonucleotides against the neuronal transporter EAAC1 were generated and infused into the CSF, no changes were noted (Rao et al., 2001b). These results are particularly important when one considers theories of glutamate toxicity mechanism of ischemic injury. Some have argued that reversal of either the neuronal or the astroglial glutamate transporter could be responsible for the high extracellular concentrations of glutamate observed in microdialysis studies during vascular ischemic injury. Could loss of a transporter population prevent or protect against ischemic injury? Clearly,

the antisense GLT1 results would suggest otherwise. Conversely, could overexpression of glutamate transporters help prevent neural injury due to excessive glutamate levels? Future studies with transporter transgenic over expression mice will help evaluate these therapeutic options.

In an adult rat model of middle cerebral artery occlusion, Rao et al. (2001a) demonstrated that a reduction of both GLT1 and EAAC1 occurred ipsilaterally at 24 and 72 h following the insult. This contrasts with the neonatal rat model (PND7) described in this section in which GLAST expression was increased in these regions (Raghavendra et al., 2001).

Glutamate transporter expression in human hypoxic–ischemic injury has not been extensively studied and reliable conclusions cannot be made. However, in one study of human neonatal hypoxic–ischemic injury, cerebellar expression of glutamate transporters, EAAT1 and EAAT4, was reduced. The authors postulated that these reductions could account for the selective vulnerability of Purkinje cells to hypoxic–ischemic injury because the cerebellum is particularly rich in EAAT1 expression and EAAT4 expression is Purkinje cell specific (Inage et al., 1998).

In a postmortem study of EAAT2 expression in human autopsy cases, a subset of deaths ( $n = 15$ ) due to asphyxia showed only weak EAAT2 expression in cortex, striatum, and hippocampus using a combination of immunohistochemical and immunoblotting techniques. This analysis was severely limited, however, by the significant variations in postmortem delay and it is difficult to draw reliable conclusions from such differences (6 h to 1.5 days) (Ikematsu et al., 2001).

## Glioma

A number of recent studies on the pathophysiology of glioblastoma growth suggest that glutamate may participate in glioma growth and invasion. Several studies implicate glutamate release and transport properties of gliomas in their toxicity. Recent studies of cell lines derived from human gliomas suggest that GLT1 is absent and GLAST is mislocalized to the nucleus. The result is a 100-fold reduction in glutamate transport in these cells. In glioma cell lines, over 50% of glutamate transport was Na(+)-independent and mediated by a cystine–glutamate exchanger [system x(c)(–)]. Extracellular L-cystine dose-dependently induced glutamate release from glioma cells. One intriguing hypothesis is that this glutamate release from cystine–glutamate exchange from gliomas results in an increase extracellular glutamate in surrounding tissue, thus initiating glutamate neurotoxicity in surrounding brain parenchyma. This would allow the potential expansion of the tumor into surrounding parenchyma (Ye et al., 1999). In fact, various glioma lines appear to release glutamate in vivo and manipulation of glutamate release or receptor blockade significantly affects glioma growth and animal survival (Takano et al., 2001). Others have observed that a reduction in the number of GLT1 splice variants may be a property of gliomas (Munch et al., 2001). The presence of EAAT3 (primarily a neuronal transporter) as the principle glutamate transporter in the U373 astrocytoma cell line suggests that these undifferentiated cells have atypical transporter expression not associated with mature cells and may be associated with the high mitotic activity of these cells (Dunlop et al., 1999). These studies are particularly intriguing, implying that aberrant glutamate transporter function may play a role in tumor growth and cell cycling, features which have not been heavily studied in normative gluta-

mate transporter biology. These observations may also provide strategies for the suppression of glioma growth with antiglutamatergic agents in and around the tumor bed to slow the progression of tumor growth and infiltration.

## Other neurologic disorders with implications in glutamate transporter dysfunction

The role of glutamate transporter dysfunction in neurologic disease is only beginning to be understood. While the focus of this review has been on glutamate transporter dysfunction in the neurologic disorders studied most extensively, recent work also suggests that glutamate transporter biology may play a role in HIV dementia (Pappas et al., 1998; Vallat-Decouvelaere et al., 2003; Wang et al., 2003) and traumatic brain injury (McAdoo et al., 2000; Rao et al., 1998). Intoxications with prominent neurologic dysfunction such as ethanol exposure (Schreiber and Freund, 2000), Wernicke's encephalopathy, and hyperammonemia (Hazell et al., 2001, 2003) are also being studied. It is not surprising that glutamate transporters are affected in a host of different, presumably unrelated, disorders given that glutamate is ubiquitous in the central nervous system both as a neurotransmitter and as a metabolite. Of greater interest, however, is whether glutamate transporter dysfunction in these disorders is a primary or significant contributor to pathology or merely a bystander in a cascade of events leading to cell death.

## Conclusion: unanswered questions and future investigation

Central to the understanding of how glutamate transporter dysfunction contributes to disease is the study of how other mechanisms involved in pathogenesis of disease may alter glutamate transporter biology. The pathophysiology of the glutamate transporter null mice suggests that glutamate transporter function (at least in the GLAST, EAAC1, and EAAT4 null mice) is not critical for normal development nor do these models display significant nervous system pathology. However, does the loss of these transporters in the null mice predispose these animals to other insults that would not otherwise produce pathology? The crossing of glutamate transporter null or heterozygous mice with mouse models of other diseases, that is, mutant SOD1 mouse (ALS model), may answer questions as to whether glutamate transporter loss speeds disease progression, exacerbates pathology (in stroke or epilepsy models), or produces new phenotypes not previously observed. If this were the case, the interactions between glutamate neurotoxicity and other pathways involved in cell death could potentially be delineated.

A caveat to the study of glutamate transporter biology in human tissues is the potential importance of postmortem delay in studying brain tissue. Incongruous results between studies of human tissue and animal models should be predicated on the likelihood that glutamate transporter proteins are degraded in the postmortem period. Therefore, an important issue to human tissue study design is the inclusion of age-matched and other disease controls.

An understanding of how glutamate transporter biology is affected in neurologic disease may lead to the future development of strategies for enhancing glutamate transporter expression and/or function in treating these disorders. A significant first step suggesting that this may be a rational approach came from recent data in

which a transgenic GLT1-overexpressing mouse was crossed with the mutant SOD1 mouse (an ALS model). The resulting mice had a delay in grip strength decline and motor neuron loss but, ultimately, not survival (Guo et al., 2003). Of course overexpression models are merely genetic pharmacological experiments. The outcome may depend on the degree of over expression. Increasing numbers of transporter proteins and increased transporter activity could lead to greater (or lesser) amounts of disease alterations. A second, unpublished EAAT2 transgenic mouse has considerably more glutamate transporter activity—with an associated greater degree of neuroprotection (Scholz and Sutherland, 2003).

The delivery of glutamate transporters to at-risk regions of the brain is a strategy that may be employed by the recent advances in stem cell transplantation and viral vector delivery of genes of interest. Stem cells can be easily transduced to carry genes of interest. They offer advantages in that they can integrate into host architecture and continue to provide a source of trophic support over long periods, obviating the need for recurrent administration of therapeutic compounds. The viral vector delivery of glutamate transporters may be of value in delivering genes to specific cell types based on the infectivity of the virus or the gene promoter employed. While both approaches are novel and potentially exciting, some issues surrounding the safety of their use remain unanswered. Other ethical issues surround the use of stem cells derived from embryos, but the emergence of other stem cell types (mesenchymal, umbilical cord) offers promise for therapy in neurologic disease.

The advances in delivery of genes by viral vectors and stem cells do not mean, however, that traditional pharmacologic approaches should be ignored. In a recent screen of current FDA approved drugs in neurodegenerative disease-related models, several potential candidates for increasing glutamate transporter expression were identified. This represents a burgeoning interest in the potential for regulating glutamate transporter biology through conventional pharmacotherapeutics with direct applications to the treatment of neurologic disease.

Given glutamate's ubiquitous presence in the central nervous system and the importance of regulation of this neurotransmitter under normal and pathologic conditions, understanding glutamate transporter biology is ripe for investigation. The incorporation of in vitro cultures, in vivo studies of glutamate transporter null mice and other disease paradigms in animals, and finally human studies will afford a more thorough understanding of the relationship of glutamate transporters to human neurologic disease.

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