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Distribution of excitatory amino acid transporters (EAATs) in cerebellar granule cell cultures: an immunocytochemical study

Kristen Reed

The University of Montana

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Distribution of Excitatory Amino Acid Transporters (EAATs) in Cerebellar Granule Cell Cultures; An Immunocytochemical Study

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B.S., Microbiology 1999
University of Montana

Submitted in partial fulfillment of the requirements for the
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Department of Pharmaceutical Sciences
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Chairman, Board of Examiners
Dean of the Graduate School

12-19-01
Date
GLAST, GLT-1, EAAC1, and EAAT4 are high-affinity, sodium-dependent glutamate transporters identified in the mammalian central nervous system (CNS). In this study, the expression patterns of these transporter subtypes were characterized in two types of primary cell cultures: cerebellar granule neurons (CGN) and mixed CGN/glial cultures. Both culture types have been used extensively in studies of glutamate-mediated excitotoxicity. Cells were isolated from 7-day-old rat cerebella and grown for 10 days. Obtaining >90% pure CGN cultures was achieved by adding cytosine arabinoside to halt the proliferation of glial cells, whereas the mixed CGN/glial cell cultures were obtained by permitting glial cell proliferation. Subtype-specific anti-peptide antibodies were used to identify the glutamate transporters. Antibodies against neuronal cell markers (anti-neuronal nuclei (NeuN) and anti-neurofilament (NF)) and against an astrocyte marker (anti-glial fibrillary acidic protein (GFAP)), were used to determine cell type specific expression of the transporters. The results indicate that both CGN and mixed CGN/glial cell cultures contain cells that are immunopositive for the glutamate transporters GLAST, GLT-1, EAAC1, and EAAT4. While GLAST and GLT-1 are generally designated as “glial glutamate transporters”, we report here that GLAST and GLT-1 immunostaining is predominantly found on cells that are immunopositive for the neuronal cell markers, NF and NeuN. The previously reported “neuronal glutamate transporter”, EAAC1, localizes to NF and NeuN positive cells only in these culture systems. However, the previously described “neuronal glutamate transporter”, EAAT4, localizes predominantly to GFAP positive cells. These findings demonstrate that expression patterns in vitro do not necessarily mimic previously reported in vivo glutamate transporter expression patterns. Additionally, in the mixed CGN/glial cell cultures, we demonstrate that both GLAST and EAAT4 appear to be expressed by a greater number of GFAP positive astrocytes than in the CGN cultures. GLT-1 and EAAC1 expression appears consistent between both culture systems. These findings represent an important foundation for future studies involving glutamate transporters, particularly as related to regulation of expression, functional studies, developmental expression, and glutamate-mediated excitotoxicity.
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INTRODUCTION

L-Glutamate

L-Glutamate (L-GLU) has attracted considerable attention in recent years because of its role as both the primary excitatory amino acid (EAA) neurotransmitter in the mammalian central nervous system (CNS) and as a potent neurotoxin. Beginning with the demonstration of the convulsive properties of L-GLU and the direct activation of CNS neurons by this EAA, considerable effort has been directed toward establishing the role of L-GLU in fast excitatory neurotransmission (Curtis et al., 1959; Takahashi et al., 1997; Bridges, 2001). Numerous techniques including immunocytochemistry, pharmacology, and electrophysiology collectively demonstrate that L-GLU exhibits the classic characteristics of a neurotransmitter. Thus, L-GLU is sequestered in synaptic vesicles, released into the synaptic cleft in a Ca^{++} dependent manner, activates receptors for signal propagation, and is rapidly transported into neurons and neighboring glial cells for the purposes of signal termination and transmitter recycling (Kanai et al., 1994a). In addition to its role in standard fast synaptic communication, L-glutamate-mediated neurotransmission plays a role in higher order signal processing (e.g., learning, memory, developmental plasticity), as well as in neuropathology (e.g., Alzheimer’s disease, stroke) (Choi, 1988; Lipton and Rosenberg, 1994; Billups et al., 1998).

The diversity of actions of L-GLU is to a great extent attributable to a sophisticated receptor system. Multiple receptor classes for L-GLU, have been identified and cloned, including both ionotropic and metabotropic receptor families. Ionotropic (ion channel-coupled) L-GLU receptors are named after selective agonists and include: N-methyl-D-aspartate (NMDA), kainic acid (KA), and α-amino-3-hydroxy-5-methyl-4-
isoazolepropionic acid (AMPA) receptors. These receptors are coupled to both voltage-dependent and voltage-independent ion channels that gate currents carried by Na\(^+\), K\(^+\), and sometimes Ca\(^{++}\). Unlike the ionotropic KA and AMPA receptors, which are principally thought to mediate fast excitatory signaling, the ionotropic NMDA receptor and the metabotropic receptors are involved in the higher order processing required in development, plasticity, learning, and memory (Monaghan et al., 1989). The metabotropic L-GLU receptors (mGluRs) are coupled by guanine nucleotide binding proteins (G proteins) to intracellular second messenger systems. To date, the mGluR family comprises eight receptor subtypes that are divided into three groups based on coupling to second messengers, sequence similarity, and ligand selectivity (Conn and Pin, 1997). Group I includes mGluR1 and mGluR5, which are coupled to polyphosphoinositide (PPI) hydrolysis. MGlRs from groups II (mGluR2 and mGluR3) and III (mGluR4, mGluR6, mGluR7, and mGluR8) are negatively coupled to adenylate cyclase activity. In addition, a mGluR receptor coupled to phospholipase D has been identified in the hippocampus (Monaghan et al., 1989).

Although L-GLU receptors typically participate in normal physiological signaling, overactivation of some receptor subtypes can mediate neuronal degeneration. The ability of L-GLU to damage neurons is specifically referred to as excitotoxicity, a term coined by J.W. Olney (Olney, 1982). L-Glutamate receptor overactivation occurs when an agonist, such as glutamate or kainate, is present at the synaptic cleft in elevated concentrations. L-Glutamate receptor-mediated neurotoxicity is comprised of at least two components: osmotic-mediated damage and Ca\(^{++}\)-mediated damage (Kiedrowski, 1999). Osmotic-mediated neurotoxicity occurs when excessive levels of L-GLU produce
a large influx of Na\(^+\) through ionotropic receptors followed by membrane depolarization and the passive influx of Cl\(^-\) and H\(_2\)O. The excessive influx of ions subsequently leads to membrane swelling and cell lysis (Dunlop et al., 1999). This type of neurotoxicity is typically acute, occurring within minutes of excitotoxic onset. Conversely, calcium-mediated neurotoxicity is primarily caused by overactivation of NMDA receptors by L-GLU and is marked by gradual cellular disintegration. In normal physiological signaling, activation of select ionotropic receptors causes membrane depolarization, accompanied by cation influx (especially Ca\(^{++}\)). This intracellular Ca\(^{++}\) then acts as a second messenger that can modify numerous properties within the postsynaptic cell. Chronic overactivation of the NMDA receptor, however, leads to a rapid and persistent increase in cytoplasmic free Ca\(^{++}\) (Choi, 1995). This leads to the overactivation of many Ca\(^{++}\)-regulated enzymes (e.g., proteases, lipases, cyclooxygenases) as well as to the induction of a number of pathological schemes, such as free radical production (Choi, 1987; Castilho et al., 1998). Both osmotic- and Ca\(^{++}\)-mediated neuronal cell deaths have been reported to occur by necrosis or apoptosis, or a combination of both types of cell death (Dunlop et al., 1999). L-Glutamate mediated excitotoxicity involving either osmotic-mediated or Ca\(^{++}\)-mediated cell death, or both, has been implicated in acute neuropathologies (e.g., ischemia and spinal cord trauma), as well as chronic neuropathologies (Huntington’s disease, Amyotrophic Lateral Sclerosis (ALS), and Alzheimer’s disease) (Rothstein et al., 1993; Olney et al., 1997; Roettger and Amara, 1999; Meldrum, 2000; Rowland, 2000).
Presynaptic Neuron

Glutamine → Glutamate

Glutamine Synthetase

Glutamate → Glu-, 3Na+, H+ → Metabotropic Receptors

K+ → EAAT

Glial Cell

Postsynaptic Neuron

Glu → Glu-, 3Na+, H+ → Ionotropic Receptors

K+ → EAAT

Glu → ATP, ADP, Na+
L-Glutamate Transporters (Excitatory Amino Acid Transporters, EAATs)

The potential for L-GLU to participate in either normal cellular signaling or pathology suggests that extracellular L-GLU concentrations must be carefully controlled (Olney et al., 1986; Choi et al., 1987; Headley, 1990). L-Glutamate transporters are believed to be essential to this regulation. While L-GLU can act as a substrate of numerous cellular uptake systems, the specific L-GLU transporters that are thought to play a primary role in CNS excitatory neurotransmission are referred to as the high-affinity, sodium-dependent transporters. Efficient removal of L-GLU from the extracellular space by high-affinity, Na⁺-dependent, excitatory amino acid transporters (EAATs) is postulated to contribute to signal termination, transmitter recycling, and maintenance of L-GLU concentrations below those which are excitotoxic (Cotman et al., 1995; Billups et al., 1998).

The presence of high-affinity transporter systems for L-GLU in CNS tissue was first described by Logan and Snyder in 1971 (Logan W.J., 1971). Initially characterized as both high- and low-affinity uptake components for the accumulation of radiolabelled amino acid into rat brain synaptosomes, it was subsequently suggested that the high-affinity component may represent a mechanism for the removal of synaptically released L-GLU, thereby inactivating L-GLU neurotransmitter activity. In contrast, the low-affinity uptake component, described as responsible for metabolic trafficking, did not appear to function in the process of neurotransmission (Dunlop et al., 1999). Subsequent studies have demonstrated that Na⁺-dependent, high-affinity L-GLU transport occurs in a sodium-, potassium-, and energy dependent manner, relying on transmembrane ion
gradients from the Na\textsuperscript{+}/K\textsuperscript{+} pump (Meldrum, 2000). Research has also shown that the ion gradients used to drive the uptake of L-GLU are necessary to maintain high intracellular concentrations of L-GLU. Under normal conditions, L-GLU concentrations in the extracellular space range between 0.6 μM and 10 μM, while, estimated intracellular L-GLU concentrations in neurons and glial cells are 5-20mM and 0.1-5mM, respectively (Kuhar and Zarbin, 1978). Thus, EAATs function to maintain intracellular L-GLU concentrations at 10,000 times the extracellular concentrations (Nicholls, 1990), a process which can be achieved in milliseconds (Diamond and Jahr, 1997; Grewer et al., 2000). Consequently, loss of ion gradient control can lead to an efflux of L-GLU through the transporters, increased concentrations of extracellular L-GLU, and an increased likelihood of excitotoxic pathology (Takahashi et al., 1997; Koch et al., 1999).

Similar to the diversity of the EAA receptor system, evidence indicates high-affinity EAA transport is not mediated by a single transporter system. Existence of multiple subtypes of EAATs emerged when kinetic and pharmacological properties of Na\textsuperscript{+}-dependent L-GLU uptake were compared in different cell types or brain regions (Balcar et al., 1987; Kanai, 1992; Bridges et al., 1999; Fairman and Amara, 1999; Koch et al., 1999; Brauner-Osborne et al., 2000). L-Glutamate uptake inhibitors such as dihydrokainic acid (DHK), L-α-amino adipic acid (L-α-AA), and L-trans-2,4-pyrrolidine dicarboxylate (2,4-PDC) exhibit a variety of potencies dependent on both regional specificity and cell types (Bridges et al., 1991). The existence of distinct L-GLU transporters was confirmed by nearly simultaneous isolation of three cDNAs from non-human species, each encoding a distinct transporter subtype: glutamate/aspartate transporter (GLAST) (Storck et al., 1992), glutamate transporter-1 (GLT-1) (Pines et al.,
1992), and excitatory amino acid carrier 1 (EAAC1) (Kanai, 1992). The human homologues of these were subsequently cloned and named EAAT1, EAAT2, and EAAT3 (Arriza et al., 1994). Cloning of the plasma membrane glutamate transporters revealed that they are members of a unique family of molecules separate from the other neurotransmitter transporters such as GABA, glycine, noradrenaline, dopamine, and 5-hydroxytryptamine. L-Glutamate is recognized as the predominant endogenous substrate for these transporters, although D- and L- aspartate, as well as a number of sulfur containing EAA analogues, can also be translocated by these transporters (Dunlop et al., 1999). In addition to EAAT1 (GLAST), EAAT2 (GLT-1), EAAT3 (EAAC1), two more transporters have been cloned, EAAT4, and EAAT5 (Arriza et al., 1994; Rothstein et al., 1994; Fairman, 1995; Gegelashvili and Schousboe, 1998), bringing the total to five. Although each EAAT subtype has a distinct pharmacology and distribution within the CNS, they exhibit sequence homology of 40-65% within species. When individual subtypes are compared across species, they demonstrate 90% homology. Both neurons and glia are capable of mediating high-affinity, sodium-dependent L-GLU transport through the expression of various EAATs (Gadea and Lopez-Colme, 2001). An essential step towards understanding the contribution of EAATs to L-GLU-mediated physiology and pathology is the elucidation of specific roles and functions of each transporter subtype.

**Role of EAATs in Synaptic Transmission**

Although the EAATs are thought to participate in signal termination, transmitter recycling, and maintenance of L-GLU concentrations below those, which are excitotoxic, the relative contribution of each transporter subtype to each of these roles has not been
firmedy established. Neurortransmitter recycling occurs by the well-described glutamate-glutamine cycle. L-Glutamate is released from synaptic vesicles in the presynaptic neuron to evoke an excitatory signal in a neighboring neuron. High-affinity, L-GLU transporters on surrounding astrocytes, then preferentially take up L-GLU, where it is rapidly converted to glutamine by the glial-specific enzyme, glutamine synthetase (Alvarez-Maubecin et al., 2000). Astrocytes then release glutamine and it is subsequently taken up by neurons and utilized for synthesis of new L-GLU (Waagepetersen et al., 2000). This cycle allows for replenishment of L-GLU in neurons, as these cells lack an anaplerotic pathway for net synthesis of this neurotransmitter. Thus, transporter expression in both neurons and astrocytes is critical to normal function of the EAA system.

The role of the EAAT system in maintaining L-GLU concentrations below those that are excitotoxic has become clearer through the utilization of transport inhibitors. Co-administration of L-GLU with a Na\(^{+}\)-dependent uptake inhibitor, β-threo-hydroxy aspartate, produces greater \textit{in vivo} pathology than administration of L-GLU alone (Seal and Amara, 1999). These studies suggest that decreased transporter activity may increase extracellular L-GLU concentrations, and increase the likelihood of L-GLU-mediated excitotoxicity. An inverse relationship between excitotoxic damage and EAAT capacity is also suggested by studies showing that greater concentrations of L-GLU are required to produce lesions \textit{in vivo} than EAA agonists that are non-transportable substrates (Seal and Amara, 1999). Abnormalities in L-GLU transport are thought to be associated with specific neuropathologies. Studies have shown that EAAT2 expression is decreased in the motor cortex and spinal cord of patients with the sporadic form of ALS, leading to
elevations of L-GLU in the cerebral spinal fluid (Ye et al., 1999). Additionally, evidence implicates the dysregulation of activity and/or expression of the EAATs in the pathogenesis of several acute neurological diseases, including brain ischemia, brain or spinal cord trauma, and epilepsy (Dunlop et al., 1999), as well as in slowly progressing neurodegenerative disorders such as Huntington’s chorea and Alzheimer’s disease (Rothstein et al., 1996; Takahashi et al., 1997; Trotti et al., 1998).

The EAATs are also thought to play an essential role in signal termination. Studies using electrophysiological methods and transport inhibitors demonstrate altered postsynaptic signaling in subsets of glutamatergic synapses (Diamond and Jahr, 2000). Subsequent investigations showed that the transport inhibitors, β-THA and L-trans-2,4-PDC, slow AMPA receptor-mediated postsynaptic currents in climbing and parallel fiber synapses of cerebellar Purkinje neurons, suggesting they affect the speed of clearance of transmitter. However, these inhibitors showed no measurable effect in modulating postsynaptic response kinetics in other glutamatergic synapses in the cerebellum and hippocampus (Sarantis et al., 1993; Bridges et al., 1999). Thus, the development of novel selective inhibitors may further elucidate whether the distinct EAAT subtypes can influence the strength of excitatory signaling by modulating concentration and/or time course of L-GLU at individual synapses (Bridges et al., 1999). Additional roles for the EAATs in excitatory signaling have been suggested: 1) transport may limit desensitization of postsynaptic receptors, 2) uptake may limit the diffusion of L-GLU thus preventing “spill-over” and unwanted activation of L-GLU receptors, and 3) transport may play a role in maintaining a high specificity of synaptic transmission by
limiting the extent of cross talk between neighboring synapses (Rothstein et al., 1996; Dunlop et al., 1999).

**Distribution of L-Glutamate Transporters**

Cloning of the EAATs in the early 1990's enabled the production of sequence-specific molecular probes (cDNA and oligo probes, and antipeptide antibodies) to elucidate the distribution of individual transporters and corresponding mRNA (Gegelashvili and Schousboe, 1998). These probes have demonstrated that GLAST/EAAT1 is abundant in the CNS, as well as in peripheral tissues, such as heart, muscle, placenta, lung, liver, testes, and retina in several species (Furness and Lehre, 1997; Gegelashvili and Schousboe, 1998; Seal and Amara, 1999). GLT-1/EAAT2 mRNA localizes to the CNS of human and rodent, with low levels of expression in placenta. EAAC1/EAAT3 mRNA is abundant in the CNS, although it has also been detected in kidney, heart, muscle, lung, placenta, and liver (Gegelashvili and Schousboe, 1998). While GLAST/EAAT1 and EAAC1/EAAT3 demonstrate significant distribution in peripheral tissues, GLT-1/EAAT2 is thought to be more specific to the CNS (Rothstein et al., 1994). The more recently cloned transporter, EAAT4 (Fairman, 1995) is found predominantly in the CNS with some expression observed in the placenta (Gegelashvili and Schousboe, 1998) while, the most recently identified transporter, EAAT5 (Arriza et al., 1997) localizes to the retina (Danbolt et al., 1998; Eliasof et al., 1998; Vandenberg, 1998).

Regional distribution studies within the CNS demonstrate that the cerebellum is specifically enriched with GLAST/EAAT1, with lower expression levels in the forebrain and the olfactory bulb (Rothstein et al., 1994). High expression levels of GLT-1/EAAT2
mRNA are detected in hippocampus, cerebral cortex, thalamus, and striatum, with lower expression observed in brain stem, spinal cord, cerebellum, and olfactory bulb (Rothstein et al., 1994). Localization studies of EAAC1/EAAAT3 demonstrate high expression levels in cerebral cortex, caudate-putamen, hippocampus, and olfactory bulb, with lower levels in spinal cord and cerebellum (Rothstein et al., 1994). High levels of EAAT4 are localized in the cerebellum, particularly in the Purkinje cell layer, with low levels of expression in other forebrain regions including hippocampus and cortex (Furuta et al., 1997).

Considerable research has been devoted to the cellular localization of L-GLU transporters both in vivo and in vitro (Gegelashvili and Schousboe, 1998; Seal and Amara, 1999). It is now generally accepted that GLAST/EAAT1 is the most abundant transporter in the cerebellum and is expressed in both glial cells and in neurons. Initial cloning experiments revealed GLAST/EAAT1 protein expression predominantly localized to astrocytes (Arriza et al., 1994). However, reports have since shown that GLAST/EAAT1 mRNA and protein localizes to various neuronal cell types including cerebellar granule neurons, rat cochlear hair cells, and neuronal cell lines (Rothstein et al., 1994). GLT-1/EAAT2 primarily localizes to astrocytes, thus GLT-1/EAAT2's designation as the “glial glutamate transporter”. Initial studies reported GLT-1/EAAT2 protein exclusively in rat astrocytes (Danbolt et al., 1998) however, GLT-1/EAAT2 mRNA has been found in vitro in cultures of rat astrocytes, microglia and oligodendrocytes. Additionally, GLT-1/EAAT2 shows some, although limited neuronal expression, GLT-1 mRNA had been found in a subset of hippocampal neurons (Torp et al., 1994). Despite EAAC1/EAAAT3 being commonly referred to as the “neuronal
glutamate transporter”, EAAC1/EAAT3 protein has been found in the rat C6 glioma cell line and several human tumors of glial origin (Kanai, 1992; Kanai et al., 1994a; Rothstein et al., 1994; Vandenberg, 1998). Within the CNS, EAAT4 is expressed predominantly in Purkinje cell layer neurons (Vandenberg, 1998). Finally, EAAT5 localizes to the retina with specific cellular localization yet to be determined (Arriza et al., 1994).

Studies examining the subcellular distribution of the EAATs have provided great insight into the functional significance of different subtypes of high affinity L-GLU transporters. GLAST/EAAT1 and GLT-1/EAAT2 proteins are situated in the plasma membrane of neurons and glial cells, with highest transporter density in the thin processes where transporters colocalize and likely couple functionally with glutamine synthetase (Derouiche and Rauen, 1995; Gegelashvili and Schousboe, 1998). Interestingly, GLT-1/EAAT2 is not expressed uniformly in astrocyte membranes. More GLT-1/EAAT2 protein is present in those portions of glial cell membranes that face nerve terminals, axons, and dendritic spines (Takahashi et al., 1997). These findings are consistent with the hypothesis that glial glutamate transporter uptake functions to maintain L-GLU concentration below neurotoxic levels (Takahashi et al., 1997). Neuronal L-GLU uptake is conventionally thought to occur in presynaptic terminals, where it terminates the synaptic action of L-GLU and recycles the transmitter into synaptic vesicles for re-use (Yamada et al., 1996). However, EAAC1/EAAT3 expression has been found throughout the soma and dendritic tree of Purkinje cells (Rothstein et al., 1994) and further studies have demonstrated EAAC1/EAAT3 immunoreactivity in presynaptic boutons of GABAergic neurons (Gegelashvili and Schousboe, 1998). Tissue
distribution, CNS distribution, cellular distribution, and developmental expression patterns of the L-GLU transporter subtypes are summarized in Table 1.

A differential function for GLAST/EAAT1 and GLT-1/EAAT2, compared to EAAC1/EAAT3 is suggested by experiments in which molecular biological methods were used to prevent transporter expression (Rothstein et al., 1996). Deleting GLAST/EAAT1 and GLT-1/EAAT2 led to a general rise of extracellular L-GLU concentrations, which induced neuronal cell death (Rothstein et al., 1994). In contrast, loss of EAAC1/EAAT3 was reported to not produce a rise in extracellular L-GLU concentrations (Gegelashvili and Schousboe, 1998). Further studies are needed to clarify not only the roles of these three transporters but also the roles of EAAT4 and EAAT5.
Table 1. Distribution of Glutamate Transporters

<table>
<thead>
<tr>
<th>TRANSPORTER SUBTYPE</th>
<th>MAMMALIAN TISSUE DISTRIBUTION</th>
<th>REGIONAL DISTRIBUTION WITHIN THE CNS</th>
<th>CELLULAR DISTRIBUTION</th>
<th>DEVELOPMENTAL EXPRESSION PATTERNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLAST1/EAAT1</td>
<td>CNS Skeletal Muscle Liver Lung Placenta</td>
<td>Cerebellum Forebrain Olfactory Bulb</td>
<td>Neurons Astrocytes - Bergmann Glia</td>
<td>Increases during development</td>
</tr>
<tr>
<td>GLT-1/EAAT2</td>
<td>CNS Placenta Pineal Gland Retina</td>
<td>Cerebral Cortex Hippocampus Thalamus Spinal Cord Cerebellum</td>
<td>Neurons - limited Astrocytes</td>
<td>Increases during development</td>
</tr>
<tr>
<td>EAAC1/EAAT3</td>
<td>CNS Intestine Kidney Liver Heart Placenta</td>
<td>Cerebellum Hippocampus Olfactory Bulb Spinal Cord Caudate Putamen</td>
<td>Neurons</td>
<td>Decreases during development</td>
</tr>
<tr>
<td>EAAT4</td>
<td>CNS Placenta</td>
<td>Cerebellum Hippocampus Cerebral Cortex</td>
<td>Neurons - Granule - Purkinje maybe Astrocytes</td>
<td>Increases during development</td>
</tr>
<tr>
<td>EAAT5</td>
<td>Retina</td>
<td>??</td>
<td>??</td>
<td>??</td>
</tr>
</tbody>
</table>

1 (Storck et al., 1992)
2 (Arriza et al., 1994)
3 (Bar-Peled et al., 1997)
4 (Pines et al., 1992)
5 (Kanai, 1992)
6 (Fairman, 1995)
7 (Eliasof et al., 1998)
Regulation of Transporter Expression

Recent evidence that EAAT expression is a developmentally regulated process has emerged from studies utilizing mouse and rat CNS and spinal cord tissue (Sutherland et al., 1996; Furuta et al., 1997). Prenatally, GLAST expression predominates, with very low levels of GLT-1 in evidence (Bar-Peled et al., 1997). Postnatally, both GLAST and GLT-1 increase dramatically during periods corresponding to synaptogenesis. Similar changes in EAAT 1-4 expression have recently been described in human brain during development (Bar-Peled et al., 1997).

Primary dissociated CNS cultures remain a useful tool for the investigation of EAAT expression and neurotransmitter uptake and metabolism by different cell types during development (Reynolds and Herschkowitz, 1987). However, in vitro culture systems must be used with some caution. Various in vitro culture systems exhibit different patterns of EAAT expression from those found in vivo, indicating growth conditions may influence EAAT expression. For example, cultured astrocytes do not exhibit the same high levels of expression of the GLT-1/EAAT2 subtype, characteristic of adult brain (Gegelashvili et al., 1997). Manipulating astrocyte cultures has also been shown to alter EAAT expression patterns. Exposure to neuron-conditioned medium (Gegelashvili and Schousboe, 1997a), co-culture with neurons (Swanson et al., 1997), and exposure to dibutyryl cAMP (Schlag et al., 1998) all result in a dramatic upregulation of EAAT2 expression in astrocytes. Studies demonstrating changes in EAAT expression raise the possibility of identifying factors, which may increase or decrease EAAT expression both in vivo and in vitro, providing insight into transporter abnormalities and alterations during disease states and also as potential therapeutic targets.
Specific Aims

The purpose of the present work is to obtain fundamental information about EAAT distribution in primary cell cultures as a tool for evaluating the roles of the EAATs, particularly as related to excitotoxicity. Before a clear understanding of transporter function can be attained, it is necessary to determine EAAT distribution patterns and expression levels. Therefore, this project will define the cellular distribution of the excitatory amino acid transporters, GLAST, GLT-1, EAAC1, and EAAT4, in cerebellar granule neuron (CGN) cultures. Additionally, this project will examine changes in EAAT expression associated with the presence of glial cells. The glial cell-depleted population is greater than 90% homogenous, containing predominantly CGNs (Schousboe et al., 1985; Schousboe et al., 1989). In contrast, the glial cell-enriched populations (mixed neuron/glial cell cultures) are heterogeneous, containing not only CGNs, but also astrocytes and a few oligodendrocytes (Campbell and Shank, 1978). Cerebellar granule neurons were chosen for the following reasons: 1) CGNs are glutamatergic neurons (Drejer et al., 1983a), 2) CGNs can be grown in the presence or absence of astrocytes, unlike other primary neuronal cultures, and 3) they are an often used model in L-GLU-mediated excitotoxicity studies. The specific aims of this study can be summarized by the following questions:

1) What is the cellular distribution of GLAST, GLT-1, EAAC1, and EAAT4 in primary cultures of rat cerebellar granule neurons?

2) Does the presence of glial cells alter the expression patterns of GLAST, GLT-1, EAAC1, and EAAT4 in primary cultures of rat cerebellar granule neurons?
These studies will improve the current understanding of the distribution of the glutamate transporters in primary rat CGN cultures. This investigation will also provide further understanding of the role that glial cells play in influencing EAAT expression patterns in neurons. Finally, this study will provide a needed data base for future studies on L-GLU transporters, particularly as related to regulation of expression, functional studies, developmental expression, and glutamate excitotoxicity. Future studies will aim to better understand the influence of astrocytes on neuronal EAAT expression and to utilize molecular methods to gain insight into how transporter expression patterns change with altered conditions, as can happen in pathological situations.
MATERIALS AND METHODS

Tissue Culture

Primary cultures of rat cerebellar granule neurons (CGNs) and mixed CGN/glial cell cultures were prepared essentially as previously described (Schousboe et al., 1989). All animal procedures were in strict accordance with NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Montana AAALAC Animal Care Committee. Briefly, cerebellar tissue was aseptically removed from 7-day-old rat pups obtained from timed pregnant Sprague Dawley rats (Simonsen Laboratories, Gilroy, CA). The day of birth was designated as postnatal day 0 (P0). Both culture types were prepared by a similar protocol, except that glial proliferation in CGN cultures was suppressed by adding cytosine arabinoside (20μM) 24 hours after plating. This procedure kept the number of non-neuronal cells below 5% (Schousboe et al., 1989). Cerebellar tissue was dissected free from the meninges, transferred to a beaker containing 10ml Krebs buffer (120mM NaCl, 5mM KCl, 1.2mM KH₂PO₄, 1.2mM MgSO₄, 25mM NaHCO₃, 13mM glucose, and 0.3% bovine serum albumin, pH 7.4, equilibrated with atmospheric air/CO₂), and minced with sterile dissecting scissors. Minced tissue was transferred to a sterile 50ml tube, triturated with a Pasteur pipette, and centrifuged (3 min 200 x g) at room temperature. Cells were dissociated by trypsinization (Type IX, Sigma, 0.025% (w/v) in Krebs buffer, 15 min., 37° C). Immediately after the incubation, 20ml of Krebs buffer containing 0.004% (w/v) DNase I (Sigma) and 0.03% (w/v) soybean trypsin inhibitor (Type I-S, Sigma) was added to halt proteolytic activity. The mixture was centrifuged for 5 min at 200 x g; subsequently, the supernatant was removed and discarded. The pellet was resuspended in the presence of DNase I (0.004% (w/v) in
Krebs buffer) and soybean trypsin inhibitor (0.003% (w/v) in Krebs buffer) followed by trituration. The cell suspension was left in the hood to allow sedimentation to occur. The supernatant, containing dissociated cells, was resuspended in 3ml of Eagle’s Minimum Essential Medium (EMEM, GibcoBrl). Media was supplemented to contain 24.5mM KCl, 30mM glucose, 7μM para-aminobenzoic acid, 100 mU/liter insulin, 10% fetal calf serum, (GibcoBrl), and 1 % antibiotic-antimycotic (GibcoBrl), then sterilized by filtration (.22μM, Corning), and equilibrated with atmospheric air/CO\textsubscript{2} at 37° C (modified EMEM). The suspended cells were centrifuged for 5 min at 200 x g, the supernatant was discarded, and the pellet was resuspended in 10ml culture media (modified EMEM). Twenty-four hours prior to cell seeding, tissue culture plates (12-well, 3.8cm\textsuperscript{2}, Costar) were prepared by inserting a round glass coverslips (18mm, VWR Scientific) in each well, coating the coverslips with poly-L-lysine (0.1M, Sigma), and allowing plates to sit in the tissue culture hood before rinsing and cell seeding. Cells were then seeded (1.0 x 10\textsuperscript{6} cells/well) on the poly-L-lysine coated glass coverslips in 2 ml of culture media and allowed to grow for 10 days.

**Culture Maintenance and Immunocytochemistry Pretreatment**

Twenty-four hours after seeding, 12-well plates were treated, where indicated, with 20μM cytosine arabinoside (Sigma, St. Louis, MO) to halt proliferation of non-neuronal cell types. Tissue culture media was changed every third day, and the cells were fixed at 10 days *in vitro* (10 DIV) with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 20 minutes. Fixative was aspirated, replaced with storage
buffer (0.1M PBS, pH 7.4), and cells were stored at 4°C until processed. All immunostaining took place with in 5 days of fixation.

**Antibodies**

Four different subtype-specific antipeptide antibodies to glutamate transporters were used in this study. These rabbit polyclonal antibodies recognize distinct proteins for GLAST (543 amino acids; C-terminal), GLT-1 (573 amino acids; C-terminal), EAAC1 (523 amino acids; C-terminal), and EAAT4 (561 amino acids; C-terminal) (Alpha Diagnostics). Monoclonal antibodies to neurofilament (RMO270; NF; courtesy of Dr. Virginia Lee, Philadelphia, PA) and neuronal nuclei (NeuN, Chemicon, Temecula, CA) were used as markers for neuronal cytoskeleton and neuron specific nuclear protein, respectively. A monoclonal antibody against glial fibrillary acidic protein (GFAP, Chemicon) was used as a marker for astrocytes.

**Single Label Immunocytochemistry**

Plates were removed from storage, rinsed twice with 0.1M PBS, and blocked with either 5% normal horse sera (NHS, Vector Laboratories, Burlingame, CA) or 5% normal goat sera (NGS, Vector Laboratories) in 0.1M PBS for 10 min. Cells were then permeabilized with 1% Triton X-100 in 0.1M PBS for 10 min. A second 20-minute block with 5% NHS or 5% NGS, respectively, followed the permeabilization step. Cells were then immunostained using primary antibodies against one of the following: NF (1:100), NeuN (1:100), GFAP (1:100), GLAST (1:100), GLT-1 (1:100), EAAC1 (1:100), or EAAT4 (1:100). Both primary and secondary antibodies were diluted in 0.1M Tris
buffer solution containing 0.01% sodium Azide and 0.1% Bovine serum albumin (TAB). Primary antibody was added to the appropriate well, and incubated for 2 hours at 37°C. Primary antibodies were detected with the appropriate secondary antibody, FITC-conjugated horse anti-mouse (1:100; Vector Laboratories) or Cy3-conjugated goat anti-rabbit (1:800; Jackson ImmunoResearch, West Grove, PA), for 1 hour at room temperature. Cells were rinsed twice with 0.1M PBS, once with deionized water, coverslipped with Vectashield (Vector Laboratories), and stored at 4°C in the dark. For no-primary antibody controls, wells were treated in the same manner as experimental wells except control cells were incubated with secondary antibody alone.

**Double Label Immunocytochemistry**

Plates were removed from storage, rinsed twice with 0.1M PBS, then blocked with both 5% NHS and 5% NGS in 0.1M PBS for 10 min. Cells were then permeabilized with 1% Triton X-100 in 0.1M PBS for 10 min. A second 20-minute block with both 5% NHS and 5% NGS followed the permeabilization step. Cells were then immunostained using primary monoclonal antibody against either NF (1:100) or NeuN (1:100) or GFAP (1:100) diluted in TAB, and added to the appropriate well. Cells were incubated in primary antibody for 2 hours at 37°C. Primary monoclonal antibodies were detected with secondary antibody, FITC-conjugated horse anti-mouse (1:100; Vector Laboratories), for 1 hour at room temperature. Cells were rinsed twice with 0.1M PBS. Cells were then immunostained using primary polyclonal antibody GLAST (1:100), GLT-1 (1:100), EAAC1 (1:100), or EAAT4 (1:100) diluted in TAB, and added to the appropriate well. Cells were incubated in primary antibody for 2 hours at 37°C. Primary polyclonal
antibody was detected with secondary antibody, Cy3-conjugated goat anti-rabbit (1:800; Jackson ImmunoResearch) for 1 hour at room temperature. Cells were rinsed twice with 0.1M PBS, once with deionized water, coverslipped with Vectashield, and stored at 4°C in the dark. For no-primary antibody controls, wells were treated the same way as experimental wells except control cells were incubated with both secondary antibodies alone.

**Immunocytochemical Analysis**

All observations are based on a minimum N = 5, where N is the number of different tissue culture preparations. Within each preparation, at least 2 different culture plates were immunostained for the same transporters. Additionally, staining was done in duplicate by staining adjacent wells within each plate.

Microscope slides were viewed with a Nikon E-800 fluorescent microscope at magnifications ranging from 4-40X. Representative photographs were taken using Kodak Color Chrome Elite slide film (ASA 200) in a Nikon FX-35A camera. Slides were scanned into a Macintosh G4 computer using a Nikon Coolscan 4000 slide scanner and Nikon Scan 3.1 image analysis software. Adobe Photoshop 5.5 image analysis software was used to merge images into double labels. Additionally, some microscope slides were viewed with a Bio-Rad Radiance 2000 MP laser scanning confocal microscope. Representative photographs were taken using a Bio-Rad radiance 2000 MP multi-photon imaging system and viewed on a Dell Power Edge 1300 computer using LaserSharp 2000 software.
RESULTS

Cell Culture Description – Cell Type Determination

Cerebellar granule neuron (CGN) cultures and mixed CGN/glial cell cultures prepared from 7-day old rats were characterized by immunocytochemistry. Immunoreactivity for neurofilament (NF) and neuronal nuclei (NeuN) were used to identify neurons, while astrocytes were identified by immunoreactivity for glial fibrillary acidic protein (GFAP). Cerebellar granule neuron cultures contain small granule neurons (6-9 um) which typically grow into large aggregates containing numerous cell bodies (Fig. 2C, arrows) with neuronal processes extending from the clusters of cell bodies (Fig. 2A, arrows) (Schousboe et al., 1989). Although, replication of non-neuronal cells was halted by the addition of cytosine arabinoside in these cultures, a small number of astrocytes can remain. These astrocytes were rarely observed, however Fig. 2E (arrows) shows an example of astrocytes present in CGN cultures. Mixed CGN/glial cell cultures, in which cytosine arabinoside was not added, contain many neurons, as well as astrocytes (Fig. 2F, arrows). Although many astrocytes are present in this culture type, the neurons still grow as characteristic clusters of cell bodies (Fig. 2D, arrows) with numerous processes projecting outward (Fig. 2B, arrows). In both culture types, neuronal clusters, as well as astrocytes appeared to be viable and healthy (based on trypan blue exclusion; results not shown) with intact cell bodies and many long processes.

Cell Culture Description – L-Glutamate Transporter Expression

To investigate whether the two culture types (CGN cultures and mixed CGN/glial) contain the glutamate transporters, GLAST, GLT-1, EAAC1, and EAAT4,
subtype specific antibodies are used to label the transporters. Both culture types contain various cells that are immunopositive for the glutamate transporters, GLAST, GLT-1, EAAC1, and EAAT4.

Both CGN cultures and mixed CGN/glial cell cultures are positively immunoreactive for GLAST. Punctate, intense, labeling of GLAST is observed on filaments in both culture types, presumably neuronal filaments as judged by morphology (Fig. 3A and 4A, arrows). Positive GLAST immunoreactivity in clusters of cell bodies is also observed in both culture types (Fig. 3A and 4A, arrows). Clusters of cell bodies seen in CGN cultures (Fig. 3A, arrows) and in mixed CGN/glial cell cultures (Fig. 4A, arrows) are moderately labeled for GLAST. These clusters of cells are characteristic of cerebellar granule neurons grown in culture, as described in previous studies (Schousboe et al., 1985; Schousboe et al., 1989). Additionally, the size of these cells is consistent with being granule neurons (Schousboe et al., 1985; Schousboe et al., 1989) as well as the staining pattern (positive NeuN and NF immunoreactivity).

Immunostaining patterns for GLT-1 differs slightly between CGN cultures and mixed CGN/glial cell cultures. Although, there are many neuronal filaments in both culture types, the number of GLT-1 positive filaments slightly increases in CGN cultures (Fig. 3B, arrows) compared to the mixed CGN/glial cell cultures (Fig. 4B, arrows). From the staining patterns, it was quite apparent that NF staining was similar between these two culture types. Thus, the changes in GLT-1 immunoreactivity between cultures may not be attributable to changes in the number of neurofilaments, but rather to differences in GLT-1 immunoreactivity on these filaments. Moderate labeling of the clusters of cell bodies is similar in both culture systems (Fig. 3B and 4B, arrows).
Intense labeling of EAAC1 is observed in clusters of cell bodies in both CGN (Fig. 3C, arrows) and mixed CGN/glial cell cultures (Fig. 4C, arrows). From the staining, we could see that there was not a differential EAAC1 pattern of expression between these two culture types.

Immunostaining of EAAT4 in both types of cultures exhibit different morphological patterns than those observed for all other EAATs on filaments. While filaments that are immunostained for GLAST, GLT-1, and EAAC1 are linear and typically connected clusters of cells, the EAAT4 positive filaments display a stellate appearance and are morphologically similar to astrocytic filaments (Fig. 3D and 4D). In addition, the number of EAAT4 positive cells varies between the two culture types. In CGN cultures, EAAT4 is expressed on a limited number of cells (Fig. 3D), while in mixed CGN/glial cell cultures (Fig. 4D), EAAT4 is expressed on numerous cells, as is expected if these filaments are indeed astrocytic filaments. Immunoreactivity of EAAT4 on clusters of cell bodies is observed within both culture types (Fig. 3D and 4D). These cells display characteristic clumping typical of CGNs grown in vitro. Localization of the L-GLU transporter subtypes in both CGN cultures and mixed CGN/glial cell cultures is summarized in Table 2.
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Fig. 2. Cerebellar granule neuron (CGN) cultures and mixed CGN/glial cell cultures are immunostained for neurofilament (NF), neuronal nuclei (NeuN), and glial fibrillary acidic protein (GFAP) (green) (10 DIV). A) CGN cultures immunostained for NF. B) Mixed CGN/glial cell cultures immunostained for NF. C) CGN cultures immunostained for NeuN. D) Mixed CGN/glial cell cultures immunostained for NeuN. E) CGN cultures immunostained for GFAP. F) Mixed CGN/glial cell cultures immunostained for GFAP. Immunostaining demonstrates that both types of primary cell cultures contain neurons with numerous filaments extending from clusters of cell bodies. Although GFAP positive astrocytes are not generally observed in CGN cultures, Fig. 2E is representative of those astrocytes present in CGN cultures. Numerous astrocytic filaments are observed in the mixed CGN/glial cell cultures. Mag Bar=50 μm
Fig. 3. Cerebellar granule neuron (CGN) cultures immunostained for the excitatory amino acid transporters (red), GLAST, GLT-1, EAAC1, and EAAT4 (10 DIV).

A) CGN cultures immunostained for GLAST. B) CGN cultures immunostained for GLT-1. C) CGN cultures immunostained for EAAC1. D) CGN cultures immunostained for EAAT4. Immunostaining exhibits the presence of the four glutamate transporters in this culture system. While the four transporters are present, they clearly have distinct expression patterns within this primary cell culture type. GLAST is mainly expressed on straight filaments, GLT-1 is found in both filaments and clusters of cell bodies. EAAC1 is predominantly found on clusters of cell bodies, and EAAT4 localizes to fibers with a stellate morphology. Mag Bar=50 μm
Fig. 4. Mixed cerebellar granule neuron/glial cell cultures (mixed CGN/glial) immunostained for the excitatory amino acid transporters (red), GLAST, GLT-1, EAAC1, and EAAT4 (10 DIV). A) Mixed CGN/glial cell cultures immunostained for GLAST. B) Mixed CGN/glial cell cultures immunostained for GLT-1. C) Mixed CGN/glial cell cultures immunostained for EAAC1. D) Mixed CGN/glial cell cultures immunostained for EAAT4. While the four transporters are present, they have distinct patterns of expression within this primary cell culture type. GLAST is mainly expressed on filaments, with moderate expression on clusters of cell bodies. GLT-1 is found in both filaments and clusters of cell bodies. EAAC1 is found on clusters of cell bodies with limited expression in filaments. EAAT4 localizes to numerous fibers with a stellate morphology. Mag Bar=50 μm
Double Labeling of CGN Cultures and Mixed CGN/Glial Cell Cultures

To confirm transporter location on distinct cell types, dual-label experiments were used to probe the cultures for cell type (neurons, NF or NeuN and astrocytes, GFAP) and transporter subtype (GLAST, GLT-1, EAAC1, EAAT4) expression. Utilization of the neuronal markers, NF and NeuN, allow for further delineation of transporter localization on either neuronal processes or neuronal cell bodies.

Identification of GLAST Positive Cells

Double labeling CGN cultures and mixed CGN/glial cell cultures with GLAST (red) and either NF, NeuN, or GFAP, (green) demonstrate that GLAST is expressed by both neurons and astrocytes. Neuronal processes are positively immunoreactive for GLAST in both CGN cultures (Fig. 5A, arrows) and in mixed CGN/glial cell cultures (Fig. 5D, arrows). Neuronal cell bodies also express GLAST in both culture types (Fig. 5B and 5E, arrows). In general, there are few astrocytes present in CGN cultures, yet weak expression of GLAST is seen on a few of the remaining astrocytic filaments (Fig. 5C). While numerous astrocytic filaments appear to be GLAST positive in the mixed cultures (Fig. 5F).

Identification of GLT-1 Positive Cells

Double labeling CGN cultures and mixed CGN/glial cell cultures with GLT-1 (red) and either NF, NeuN, or GFAP (green) demonstrates that GLT-1 is expressed by neurons, but not astrocytes. GLT-1 expression is evident on neuronal filaments in both cultures (Fig. 6A and 6D). Neuronal filaments that express GLT-1 are more numerous in
the CGN cultures than in the mixed CGN/glial cell cultures (Fig. 6A and 6D) and again this is not attributable to fewer filaments present in the mixed CGN/glial cell cultures. Intense GLT-1 expression is exhibited on neuronal cell bodies in both culture types (Fig. 6B and 6E). GLT-1 expression by astrocytes is not observed in either culture type (Fig. 6C and 6F), although neuronal filaments expressing GLT-1 do lie in close proximity to astrocytic filaments.

**Identification of EAAC1 Positive Cells**

Double labeling cell cultures with EAAC1 (red) and either NF, NeuN, or GFAP (green) demonstrates that EAAC1 is only expressed in neurons. While little to no expression of EAAC1 is observed on the neuronal filaments in both culture types (Fig. 7A and 7D), intense EAAC1 expression is evident on neuronal cell bodies (Fig. 7B and 7E). EAAC1 does not co-localize with GFAP positive astrocytes in either culture type (Fig. 7C and 7F).

**Identification of EAAT4 Positive Cells**

Double labeling CGN cultures and mixed CGN/glial cell cultures with EAAT4 (red) and either NF, NeuN, or GFAP (green) demonstrates that EAAT4 is predominantly expressed in astrocytes, although some expression is observed in neurons. EAAT4 expression is not present on neuronal filaments (Fig. 8A and 8D) but localizes to neuronal cell bodies (Fig. 8B and 8E). EAAT4 expression is mainly observed in astrocytes as shown by occasional co-localization with GFAP positive filaments in CGN
cultures (Fig. 8C) and numerous GFAP positive astrocytes in mixed CGN/glial cell cultures (Fig. 8F).

Confocal laser scanning microscopy with a highly sensitive fluorescence signal amplification system was used to visualize the distribution of L-GLU transporter immunoreactivity more clearly. This technique is used to confirm cell type specific, GLAST, GLT-1, EAAC1 and EAAT4 transporter localization. These results show that GLAST, GLT-1, EAAC1 and EAAT4 are expressed by granule neurons (Fig. 9). Neither GLT-1 (Fig. 10B) nor EAAC1 (Fig. 10C) display astrocytic expression, however, both GLAST (Fig. 10A) and EAAT4 (Fig. 10D) are localized to cerebellar astrocytes present in both CGN cultures and mixed CGN/glial cell cultures.
Fig. 5. Cerebellar granule neuron (CGN) cultures and mixed CGN/glial cell cultures are dual-labeled for the glutamate transporter, GLAST (red), and one of the cell-type markers, neuronal markers, NeuN or NF, or astrocyte marker, GFAP (green) (10 DIV). A) CGN cultures immunostained for GLAST and NF. B) CGN cultures immunostained for GLAST and NeuN. C) CGN cultures immunostained for GLAST and GFAP. D) Mixed CGN/glial cultures immunostained for GLAST and NF. E) Mixed CGN/glial cultures immunostained for GLAST and NeuN. F) Mixed CGN/glial cultures immunostained for GLAST and GFAP. Staining indicates that GLAST expression patterns do not vary widely between these two culture types. In both cultures GLAST is localized to neurons, both on the neuronal filaments and on the clusters of cell bodies. GLAST is also found on astrocytes in both culture types. Mag Bar=50 μm
Fig. 6. Cerebellar granule neuron (CGN) cultures and mixed CGN/glial cell cultures double labeled for the glutamate transporter, GLT-1 (red), and one of the cell-type markers, neuronal markers, NeuN or NF, or astrocyte marker, GFAP (green) (10 DIV). A) CGN cultures immunostained for GLT-1 and NF. B) CGN cultures immunostained for GLT-1 and NeuN. C) CGN cultures immunostained for GLT-1 and GFAP. D) Mixed CGN/glial cell cultures immunostained for GLT-1 and NF. E) Mixed CGN/glial cell cultures immunostained for GLT-1 and NeuN. F) Mixed CGN/glial cell cultures immunostained for GLT-1 and GFAP. Immunostaining shows that neurons do express GLT-1. Immunoreactivity on neuronal clusters is similar in both culture types; however, more neuronal filaments in the CGN cultures are labeled for GLT-1 than the neuronal filaments in mixed CGN/glial cell cultures. Astrocytes do not appear to express GLT-1 in either culture type, although GFAP positive filaments are sometimes found in close proximity to GLT positive filaments. Mag Bar=50 μm
Fig. 7. Cerebellar granule neuron (CGN) cultures and mixed CGN/glial cell cultures dual-labeled for the glutamate transporter, EAAC1 (red), and one of the cell-type markers, neuronal markers, NeuN or NF, or astrocyte marker, GFAP (green) (10 DIV). A) CGN cultures immunostained for EAAC1 and NF. B) CGN cultures immunostained for EAAC1 and NeuN. C) CGN cultures immunostained for EAAC1 and GFAP. D) Mixed CGN/glial cell cultures immunostained for EAAC1 and NF. E) Mixed CGN/glial cell cultures immunostained for EAAC1 and NeuN. F) Mixed CGN/glial cell cultures immunostained for EAAC1 and GFAP. Clusters of neuronal cell bodies are strongly immunopositive for EAAC1, while neuronal filaments only exhibit weak, if any, expression of EAAC1 in both culture types. Co-localization of EAAC1 and GFAP is not observed, suggesting astrocytes in these culture types do not express EAAC1. Mag Bar=50 μm
Fig. 8. Cerebellar granule neuron (CGN) cultures and mixed CGN/glial cell cultures are dual-labeled for the glutamate transporter, EAAT4 (red), and one of the cell-type markers, neuronal markers, NeuN or NF, or astrocyte marker, GFAP (green) (10 DIV). A) CGN cultures immunostained for EAAT4 and NF. B) CGN cultures immunostained for EAAT4 and NeuN. C) CGN cultures immunostained for EAAT4 and GFAP. D) Mixed CGN/glial cell cultures immunostained for EAAT4 and NF. E) Mixed CGN/glial cell cultures immunostained for EAAT4 and NeuN. F) Mixed CGN/glial cell cultures immunostained for EAAT4 and GFAP. Neuronal filaments are not immunopositive for EAAT4, although clusters of neuronal cell bodies do moderately express EAAT4 in both culture systems. The few remaining astrocytes present in CGN cultures as well as the numerous astrocytes present in the mixed CGN/glial cell cultures are intensely immunopositive for EAAT4. Mag Bar=50 μm
Fig. 9. Confocal images of cerebellar granule neuron (CGN) cultures double labeled for NeuN (green) and one of the glutamate transporters (red), GLAST, or GLT-1, or EAAC1, or EAAT4 (10 DIV). A) CGN cultures immunostained for NeuN and GLAST. B) CGN cultures immunostained for NeuN and GLT-1. C) CGN cultures immunostained for NeuN and EAAC1. D) CGN cultures immunostained for NeuN and EAAT4. The four glutamate transporters, GLAST, GLT-1, EAAC1, and EAAT4 do colocalize with NeuN, confirming that granule neurons express these transporters. Mag Bar=50 μm
Fig. 10. Laser scanning confocal microscopy images of cerebellar granule neuron (CGN) cultures double labeled for GFAP (green) and one of the glutamate transporters, GLAST, or GLT-1, or EAAC1, or EAAT4 (red) (10 DIV). A) CGN cultures immunostained for GFAP and GLAST. B) CGN cultures immunostained for GFAP and GLT-1. C) CGN cultures immunostained for GFAP and EAAC1. D) CGN cultures immunostained for GFAP and EAAT4. GLAST and EAAT4 demonstrate co-localization with GFAP, while no co-localization was observed with GLT-1 or EAAC1 and GFAP. Mag Bar=50 μm
DISCUSSION

The existence of multiple excitatory amino acid transporter (EAAT) subtypes raises the possibility that EAATs play a variety of roles in the central nervous system (CNS). Studying the expression patterns of L-GLU transporter subtypes is an important approach for elucidating the potential for distinct EAAT contributions to excitatory signaling, metabolic regulation, or excitotoxic protection. Major advances in the field came with the cloning of Na\(^{+}\)-dependent excitatory amino acid transporter subtypes, GLAST (Storck et al., 1992), GLT-1 (Pines et al., 1992), EAAC1 (Kanai, 1992), and EAAT4 (Fairman, 1995). Molecular probes were then produced which allowed for identification of the tissue distribution of these transporter subtypes. The initial studies examining the localization of EAATs involved mRNA studies utilizing adult mammalian tissue preparations. More recently, localization studies using antibodies generated against the various transporter subtypes determined localization via immunocytochemical and immunohistochemical methods, again in mature mammalian CNS systems. These in vitro and in vivo studies provide insight into the multiple tasks that the transporters execute in their regulation of extracellular L-GLU concentrations. Additionally, these studies set the accepted standards for cellular localization: GLAST and GLT-1 being described as 'glial glutamate transporters', EAAC1 as the 'neuronal glutamate transporter', and EAAT4 as being restricted to cerebellar Purkinje neurons. However, more recent results have revealed that these presumed transporter distribution patterns are not absolute. Studies have continued to utilize subtype specific antibodies to further elucidate cellular and subcellular localization of the EAATs in a variety of in vitro and in vivo preparations throughout each of the stages of development.
Determining transporter localization and subtype specific roles in excitatory transmission and excitotoxicity provides the potential for the development of agents with which to regulate transporter-mediated processes. Numerous investigations focusing on transmission, transport, and L-GLU mediated excitotoxicity have utilized cerebellar granule neuron cultures as a model experimental system. These cultures represent a glutamatergic neuronal system and provide a highly controlled environment for experimentation. Additionally, these cultures are useful because neurons and glia are easily distinguished at the microscopic level and because CGNs can be cultured in the absence of glia. The present study characterizes the cellular localization of the Na⁺-dependent, high affinity L-GLU transporters, GLAST, GLT-1, EAAC1, and EAAT4, in primary cerebellar granule neurons (CGNs) and mixed CGN/glial cell cultures prepared from 7-day-old rat cerebella. Our results demonstrate that GLAST, GLT-1, EAAC1, and EAAT4 are found in granule neurons and that GLAST and EAAT4 are also present on astrocytes provides important data towards defining the functional importance of these transporters.

**Cellular Distribution of EAATs**

In the present *in vitro* study, GLAST predominantly localizes to granule neurons in both culture types. Additionally, GLAST localizes to some, but not all, GFAP positive astrocytes. Our demonstration of GLAST localization to neurons, contrasts with initial reports demonstrating that GLAST is restricted to mature glial cells, particularly Bergmann glia in the cerebellar cortex (Storck et al., 1992; Schmitt et al., 1997). More recent studies, however, demonstrate that this presumed astroglial transporter is also
expressed in neuronal cells. These studies report that GLAST is localized to neurons in mature cerebellar tissue slices (Rothstein et al., 1994), as well as in human CNS tissue (Arriza et al., 1994). Similar findings have also been reported for cultures of rat hippocampal neurons (Mennerick, 1998). Therefore, our finding that both cerebellar granule neurons and astrocytes express GLAST is consistent with the more recent conclusions that GLAST may function as either a neuronal or astrocytic L-GLU transporter.

Similarly, our results provide evidence that neurons also express the L-GLU transporter, GLT-1. We demonstrate GLT-1 expression is localized to cerebellar granule neurons in CGN cultures and mixed CGN/glial cell cultures. More specifically, we show that the majority of staining is present on neuronal processes and cell bodies. Again, our findings contrast with initial cloning experiments that showed GLT-1 mRNA and protein were largely restricted to astrocytes in adult rat preparations (Pines et al., 1992; Kondo et al., 1995; Danbolt et al., 1998). However, in addition to the present study, more recent reports have also demonstrated that GLT-1 is expressed by neurons (Mennerick, 1998). GLT-1 expression has been described in glial cell-depleted cultures of cortical neurons and in certain neurons of the retina (Wang et al., 1994). Additionally, GLT-1 mRNA has been detected in neurons from the hippocampus (Torp et al., 1994) and from several other regions (Schmitt et al., 1997). Thus, our results provided further evidence that CNS expression of the L-GLU transporter GLT-1 is not restricted to astrocytes as originally hypothesized.

Our results demonstrate that EAAC1 is localized to granule neurons, grown both in the presence and absence of astrocytes, and that EAAC1 is not localized to GFAP
positive astrocytes. These findings are in agreement with initial cloning studies that reported observing only neuronal EAAC1 localization (Kanai, 1992; Vandenberg, 1998), not only in rat (Velaz-Faircloth et al., 1996), but also in humans (Arriza et al., 1994). Our immunocytochemical data are consistent with the specific expression of EAAC1 in neurons, since double labeling using an antibody against GFAP verified that astrocytes, at least in 10-day-old cultures, were not contributing to the observed transporter immunoreactivity.

We found EAAT4 protein localized predominantly to astrocytes in both CGN cultures and mixed CGN/glial cell cultures. In some instances however, neuronal cell bodies, but not neuronal processes, exhibit EAAT4 expression. Initial studies demonstrated that EAAT4 localizes to cerebellum, specifically Purkinje neurons (Fairman, 1995). However, our results suggest that EAAT4 is not strictly localized to Purkinje neurons, but is also present on granule neurons in addition to cerebellar astrocytes. To date, this is the first report demonstrating that EAAT4 can be expressed by astrocytes. It must be noted, however, that commercial antibodies have only been recently made available against this transporter, so future studies may find additional examples which show that astrocytes in other brain regions do in fact express EAAT4.

As demonstrated by this project, as well as by many others, there has been considerable variation among studies aimed at determining the cellular distribution of the various EAAT subtypes. There are, however, significant variables that may account for differences in the results from these studies. Variations in transporter expression patterns during development have been reported, as well as differences related to in vitro culturing conditions (Gegelashvili and Schousboe, 1997a). Developmental expression patterns of
L-GLU transporter subtypes have been the subject of several reports (Sutherland et al., 1996; Bar-Peled et al., 1997; Yamada et al., 1997). L-Glutamate is thought to participate in several processes that occur during neuronal development, such as cell migration, cell differentiation, and neuronal and astroglial proliferation (Bar-Peled et al., 1997). In this respect, L-GLU transporters may play a role in the developmental processes by regulating L-GLU levels. Currently, it is known that transporter expression patterns change throughout CNS maturation. GLAST protein and mRNA are found at low levels in the postnatal cerebellum and increase to reach adult expression levels near postnatal day 26 (Furuta et al., 1997). Additional studies suggested that GLAST expression may be both neuronal and glial in early development, switching to a strict astroglial expression pattern in the mature brain (Bar-Peled et al., 1997). Our investigation provides further evidence demonstrating GLAST expression in the cerebellum at an early developmental stage in both neurons and astrocytes. Like GLAST, GLT-1 levels also increase during development. GLT-1 levels are virtually undetectable at birth, gradually increasing in the first month of development, reaching peak levels near postnatal day 26 (Kondo et al., 1995; Furuta et al., 1997; Swanson et al., 1997). This may explain why we did not see GLT-1 expression in astrocytes in our experiments. Interestingly, neuronal EAAC1 expression decreases throughout development. EAAC1 expression is highest early in development with peak levels observed near postnatal day 5 (Rothstein et al., 1994; Yamada et al., 1996). We demonstrate strong expression of EAAC1 by granule neurons in our cultures, consistent with previously described developmental expression patterns. The presence of EAAT4 mRNA in neurons has been shown to increase dramatically during maturation, especially during the period associated with synaptogenesis in rat
(Furuta et al., 1997). However, EAAT4 protein expression in Purkinje cells may occur much later in development, being detectable primarily in the mature cerebellum (Yamada et al., 1996). Our results show that cell types, other than just Purkinje neurons may express EAAT4 especially early in development.

Several hypotheses have been developed regarding alterations in transporter expression patterns observed during development. Potentially, cells may have an uncommitted L-GLU transporter phenotype, with cell-type specificity for L-GLU transporter phenotype occurring with maturity (Yamada et al., 1996). Another possibility is that neurons may express a greater diversity of transporter subtypes during development (Yamada et al., 1996). Such a possibility is consistent with the hypothesis that L-GLU transport by neurons predominates over astroglial transport of L-GLU during development (Furuta et al., 1997). While these hypotheses have yet to be proven, our results demonstrating neuronal expression of GLAST, GLT-1, EAAC1, and EAAT4 appear consistent. Additionally, our results are consistent with the idea that a different group of transporters may be responsible for the clearance of extracellular L-GLU early in development than in the mature brain (Bar-Peled et al., 1997). The diversity of EAAT subtypes observed during the developmental process suggests multi-functional roles for L-GLU transporters. Before synapses have completely formed, L-GLU transporters may play additional roles beyond maintaining low concentrations of L-GLU in the extracellular space.

Several reports have demonstrated that in vitro culture systems exhibit patterns of L-GLU transporter expression which differ from those observed in vivo, suggesting that culture conditions may influence L-GLU transporter activity and expression [Kondo,
1995 #27; Swanson, 1997 #56]. Interestingly, our results did not show a recognizable change in neuronal transporter expression when the cells were grown in the presence or absence of astrocytes. Others have shown changes in expression, particularly with GLT-1, although, these studies were not performed in cerebellar granule neuron cultures. Specifically, studies have demonstrated that GLT-1 expression is severely downregulated in cultured cortical astrocytes when compared to in vivo findings (Gegelashvili et al., 1996; Gegelashvili and Schousboe, 1998). Further evidence suggesting changes in transporter subtype expression due to growth conditions is provided by studies that show transporter sensitivity to uptake inhibitors differ when compared between in vitro and in vivo experiments (Gegelashvili and Schousboe, 1998). Molecular expression and activity of L-GLU transporters are also amenable to differential regulation by a number of bioactive substances (Gegelashvili et al., 1996). For example, a deficiency of certain metabolites in the culturing media may be involved in regulation of transporter expression (Gegelashvili et al., 1996). Interestingly, it has been reported that certain neuronal growth factors (e.g., nerve growth factor, fibroblast growth factor, brain-derived neurotrophic factor) may be essential for the expression of GLT-1 in astrocytes (Drejer et al., 1983b; Gegelashvili et al., 1997; Swanson et al., 1997; Figiel and Engele, 2000). Therefore, L-GLU transporter expression patterns observed in this investigation, may be a reflection of the ex vivo environment of the cultured cells. Specifically, the expression of GLAST by cerebellar granule neurons, the lack of astrocytic GLT-1 expression, and expression of EAAT4 by both cerebellar granule neurons and astrocytes, may be attributable to the conditions in which these cells were grown.
Functional Significance

As stated before, it is currently thought that L-GLU transporters may play a role in transmitter recycling, signal termination, and excitotoxic protection. In this respect, determining the cellular and subcellular distribution of the transporters should provide valuable insight into subtype specific roles in these processes. For example, it can be presumed that transporters must be localized to the synaptic cleft region to participate in signal termination. In contrast, localization to areas outside of the synaptic cleft may be indicative of roles other than signal termination. Indeed, cell body or axonal localization may indicate that the transporters are playing a metabolic role in these cells, such as protein synthesis or precursor acquisition for neurotransmitter synthesis. Based on studies performed to date, neuronal expression of EAATs is postulated to function in excitotoxic protection and in metabolism. Unfortunately, in this investigation the image resolution could not provide information as to the subcellular localization of the transporters. Therefore, we could not determine whether the various transporters demonstrated distinct subcellular patterns of expression, showing either cell body localization or synaptic cleft localization.

GLAST localization to the synaptic cleft would be consistent with the presumed role in clearance of neurotransmitter for signal termination, however, GLAST localization to areas outside of the synaptic cleft would suggest that GLAST plays a more significant role in precursor uptake. Kinetic studies have shown that GLAST has a higher Km (77 μM) for L-GLU than both GLT-1 (2 μm) and EAAC1 (17 μm) (Gegelashvili and Schousboe, 1997b). This information supports the idea that GLAST
functions as a lower affinity reserve uptake system backing up the higher affinity systems in CNS tissue (Kanai et al., 1994a). L-Glutamate uptake through GLAST has been suggested to play a role in metabolism due the localization of GLAST to peripheral tissue as well as CNS tissue. Therefore, in addition to terminating the action of synaptically released L-GLU at central synapses, the localization of GLAST to glutamatergic neurons may suggest additional functions in the regulation of other aspects of L-GLU signaling.

It is generally well accepted that L-GLU transporters play a physiological role in astrocytes. While the predominant astrocytic L-GLU transporter GLT-1 plays a major role in the glutamate-glutamine shuttle, our findings demonstrating astrocytic GLAST localization suggests that this EAAT may also play a role in the glutamate-glutamine shuttle. Astrocytic GLAST may allow for L-GLU uptake leading to the subsequent metabolic conversion to glutamine by the action of glutamine synthetase, an enzyme that is highly concentrated in astrocytes. Neighboring neurons are then able to use the released glutamine as a precursor for L-GLU synthesis. Thus, astrocytic expression of GLAST may provide additional glutamate-transporting capacity (Kanai et al., 1994b) that may serve to protect neurons from excitotoxicity.

Gegelashvili et al., 1997 reported that the ‘glial L-GLU transporter’ GLT-1, has the highest affinity for L-GLU when compared to other EAATs (Gegelashvili and Schousboe, 1997b). It is also known that glial cells are responsible for clearing the majority of synaptically released L-GLU. Although, L-GLU uptake sites have also been shown to exist on neurons (Gegelashvili and Schousboe, 1998). Interestingly, Mennerick et al. (1998) showed that neuronal GLT-1 participates in the clearance of synaptically released L-GLU (Mennerick, 1998). Therefore, cerebellar granule neuron expression of
GLT-1 may represent an alternate mechanism for clearance of synaptically released L-GLU in the cerebellum. A subcellular distribution pattern for GLT-1 is necessary to establish whether GLT-1 plays a role in excitotoxic protection or signal termination in granule neuron cultures. Our demonstration of GLT-1 localization to neuronal cell bodies in CGN cultures may be indicative of an alternate role in neurotransmission.

Neuronal expression of EAAC1 has previously been documented (Fairman, 1995). To date, glial expression of EAAC1 has only been shown in tumor cells, specifically rat C6 glioma cell lines and several human tumors of glial origin (Kanai, 1992; Kanai et al., 1994a; Rothstein et al., 1994; Vandenberg, 1998), and not in primary cultures or tissue sections. Neuronal expression of this high affinity L-GLU transporter (Km 17μm) suggests that neuronal uptake of L-GLU plays an important role in excitatory amino acid neurotransmission. We demonstrate EAAC1 localization predominantly to neuronal cell bodies and not neuronal filaments or astrocytes. This finding suggests that EAAC1 probably does not play a major role in signal termination, but may rather function in a capacity more closely linked to metabolism and excitotoxic protection in these culture systems. However, not all of the information known regarding EAAC1 is consistent with this role for EAAC1 in granule neurons. EAAC1 mRNA is found in GABAergic neurons isolated from rat preparations (Rothstein et al., 1994; Gegelashvili and Schousboe, 1998). These data raise the possibility that EAAC1 plays a role transporting the necessary precursors for GABA synthesis, instead of or in addition to, a role in L-GLU clearance. Localization of EAAC1 to peripheral tissue as well as CNS tissue is consistent with this idea.
EAAT4 expression on cerebellar granule neurons as well as astrocytes within our culture system provides little insight as to the physiological role of EAAT4. EAAT4 localization on granule neuron cell bodies suggests this transporter does not play a prominent role in signal termination. However, our demonstration of EAAT4 localization on glial cells lying in close proximity to clustered neurons suggests astrocytic EAAT4 may play a role in signal termination, excitotoxic protection, and/or the glutamate-glutamine cycle. A more complete subcellular distribution of this transporter in both neurons and glia is necessary to elucidate the functional significance of this transporter and develop specific therapies for the treatment of various disease states.

**L-Glutamate Transporters as Therapeutic Targets**

Selective pharmacological agents are required to further elucidate the specific roles of L-GLU transporters in the pathogenesis of neurological disorders. Because L-GLU transporters are widely distributed throughout the CNS, they may prove to be valuable as potential molecular targets for both neuropsychiatric and neurodegenerative disorders (Lipton and Rosenberg, 1994; Dunlop et al., 1999). Determining subtype specific pharmacology through the use of transport inhibitors may lead to a better understanding of the respective roles that transporters play in normal and pathological states (Rothstein et al., 1996; Bergles and Jahr, 1997). Targeting these transporters for the development of drugs was initially considered to be counterintuitive because total inhibition of L-GLU transport was assumed to have neurotoxic effects. However, it is now becoming apparent that not all L-GLU transporters contribute equally to signal termination, transmitter recycling, and excitotoxicity. Therefore, partial inhibition of a
subset of L-GLU transporters may be therapeutically useful in the treatment of neurological disorders. For example, partial inhibition of L-GLU transport may beneficial in schizophrenia, a condition in which reduced glutamatergic output is thought to contribute to pathogenesis (Robinson, 1998). In the case of epilepsy, excessive L-GLU is released and a strategy to enhance the activity of L-GLU transporters would be desirable (Olney et al., 1986; Akbar et al., 1998).

Developing L-GLU transport inhibitors may also be effective in the treatment of pathogenic conditions where reverse transport is implicated. Reverse transport occurs when L-GLU is released by the cell through the reversal of L-GLU uptake and the movement of L-GLU down its concentration gradient into the extracellular space leading to excitotoxicity (Billups et al., 1998). Reverse transport may occur in cases of ischemia where lack of oxygen and depleted energy supply cause transmembrane ion gradients to run down (Billups et al., 1998). Therefore, compounds that selectively block reverse transport may be useful in limiting reverse L-GLU transport associated with ischemia.

Cerebellar granule neuron cultures are widely utilized in studies of transporter function that rely on pharmacological inhibitors (Campbell and Shank, 1978; Balcar et al., 1987; Griffins et al., 1989; Lysko et al., 1994). Dihydrokainate (DHK) has been previously identified as a selective inhibitor of GLT-1 (Monaghan et al., 1989; Bridges et al., 1999; Koch et al., 1999; Brauner-Osborne et al., 2000). Results obtained from our investigation suggest that GLT-1 localizes to neurons in CGN cultures and mixed CGN/glial cell cultures unlike some other experimental systems where GLT-1 is found on astrocytes and not neurons. When observing transport inhibition by DHK in other culture systems, it may have been assumed that DHK was blocking uptake in to glia not
neurons. Therefore, results obtained in our investigation may change the interpretation of previous transport inhibitor studies performed in CGN culture system. Additionally, our results demonstrating cell type transporter expression in CGN cultures will be essential to properly interpret future results obtained from pharmacological transport inhibitor studies using this *in vitro* preparation.

**Future Studies**

Because CGN cultures are a widely utilized system for studying glutamatergic neurotransmission, it is essential to precisely define specific cell-type transporter expression patterns to proceed with future studies utilizing this system. CGN culture systems may provide valuable insight into the regulation of transporter expression (e.g., growth factor regulation and developmental regulation) and the design of pharmacological agents with which to regulate transporter function (e.g., pharmaceutical inhibitors and methods to increase transporter function such as gene therapy). Thus, our description of transporter expression patterns in this culture system is essential to gain further understanding into the specific roles that L-GLU transporters play in normal physiology and pathology.

An essential step in determining functional roles of the transporter subtypes is to further define the subcellular distribution patterns of these transporters. Immunocytochemical methods represent one potential method for determining subcellular distribution. Investigations could utilize antibodies against synaptic proteins (proteins that specifically localize to the synaptic areas of neurons) to see if they co-localize with L-GLU transporter subtypes. It is important to know which transporters
have synaptic localization as this knowledge may allow for speculations regarding the specific roles that transporters play in glutamatergic neurotransmission.

Future studies are also needed to develop therapies targeted toward specific L-GLU transporter subtypes. Researchers have previously explored L-GLU receptor antagonists, metabolic inhibitors, and release inhibitors to decrease the toxic effects to neurons by L-GLU with varying degrees of success. The task has proven to be very difficult due to differences in function and distribution that each transporter exhibits depending on the nature of the uptake systems (brain slice, cell cultures, synaptosomes, and other membrane preparations). Because L-GLU transporters represent a potential regulatory mechanism to control the balance between physiological signaling and excitotoxic pathology, modulation of L-GLU transporter expression may prove to be a strong target for novel drug discovery (Takahashi et al., 1997; During and Asthenden, 1998; Dunlop et al., 1999).

Future studies using molecular methods to transfec
t the various EAATs into neurons or glia in a cell culture environment, as well as animal models of disease, may have therapeutic value in diseases like Amyotrophic Lateral Sclerosis (ALS) and epilepsy (Hurko and Walsh, 2000). Evidence indicates that chronic suppression of L-GLU transport in ALS may be a significant factor in the pathophysiology of this disease (Rothstein et al., 1992). Lin et. al showed that the presence of aberrantly spliced transcripts from the GLT-1 gene are responsible for the reduction of functional GLT-1 protein and activity observed in ALS brains (Lin, 1998). Therefore, gene therapy aimed at enhancing expression of the GLT-1 transporter in ALS patients is a therapeutic goal.
In order to utilize CGN cultures to design future gene therapy experiments, it is essential to understand which transporter subtypes should be expressed and which cell type to attempt to increase expression. For example, if the goal were to increase expression of GLT-1 into neurons, it would be valuable to determine whether or not neurons can express GLT-1 at some time in their developmental process. Data obtained from our investigation shows that cerebellar granule neurons do have the capacity to express GLT-1. Thus, transflecting neurons with GLT-1 may be a reasonable strategy for gene therapy.

With cell-type specific expression patterns of the L-GLU transporters in CGN cultures now known, results obtained from future studies utilizing these cultures may be more precisely interpreted. Utilizing this *in vitro* culture system may prove to be a valuable tool gaining a more precise understanding of transporter function. Additionally, this *in vitro* culture system may be a useful tool for development of specific therapies targeted towards the L-GLU transporters.
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