Excitatory Amino Acid Transporter Expression and Regulation in Prefrontal Cortex in Schizophrenia

by

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List of Abbreviations

2D-DIGE	Two-dimensional fluorescence difference gel electrophoresis
ABP-L	AMPA receptor-binding protein with 7 PDZ domains
ACC	Anterior cingulate cortex
AMPA	A-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ARH	ARHGEF11, rho guanine nucleotide exchange factor 11 (interacts with EAAT4)
BHK	Baby hamster kidney
C6	A rat glioma cell line
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
cm	centimeter
CNQX	6-cyano-7-nitroquinoxaline-2,3,-dione
CNTF	Ciliary neurotrophic factor
D2	Type 2 dopamine receptor
DLPFC	Dorsolateral prefrontal cortex
EAAC1	Excitatory amino acid transporter 1 (rodent EAAT3)
EAAT	Excitatory amino acid transporter
EAAT1A	Exon 3 skipping variant of excitatory amino acid transporter 1
EAAT1Δ9	Exon 9 skipping variant of excitatory amino acid transporter 1
EAAT2B	Intron 10-11 retention variant of excitatory amino acid transporter 2
EAAT2Δ9	Exon 9 skipping variant of excitatory amino acid transporter 2
EndoH	Endoglycosidase H
ER	Endoplasmic reticulum
fMRI	Functional magnetic resonance imaging
GABA	Gama-aminobutyric acid
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GCPII	Carboxypeptidatse II
GFAP	Glial fibrillary acidic protein
GLAST	(rodent EAAT1)
GLT-1	(rodent EAAT2)
GluR	Glutamate receptor
GPS1	G-protein pathway suppressor 1 (Interacts with EAAT2-4)
GRIP	Glutamate receptor interacting protein

GRM3	Glutamate receptor, metabotropic 3
GTRAP3-18	Glutamate transporter associated protein 3-18 (rodent JWA)
GTRAP-41	Glutamate transporter associated protein 41 (rodent KIAA0302)
GTRAP-48	Glutamate transporter associated protein 48 (rodent ARHGEF11)
HBGTII	High affinity glutamate transporter II (5” variant of EAAT2)
HBGTIIA	High affinity glutamate transporter IIA (5” variant of EAAT2)
HBGTIIB	High affinity glutamate transporter IIB (5” variant of EAAT2)
HBGTIIC	High affinity glutamate transporter IIC (5” variant of EAAT2)
HCCS	Holocytochrome c synthetase
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
I7R	Intron 7 retaining variant of excitatory amino acid transporter 2
ISH	*In situ* hybridization
JM4	Jena muenchen 4
JNK	c-Jun NH_2 terminal kinase
KA1	Kainate receptor type 1
KA2	Kainate receptor type 2
kDa	kilodalton
KIAA	KIAA0302
LCA	Lens culinaris agglutinin
LIM	Lin11, Isl-1, and Mec-3
LTP	Long term potentiation
MES	2-(N-morpholino)ethanesulfonic acid
mGluR	Metabotropic glutamate receptor
MK-801	Dizocilpine
mm	millimeter
NAA	N-acetyl-alpha-L-aspartic acid
NAAG	N-acetyl-alpha-L-aspartl-L-glutamate
NCAM	Neural cell adhesion molecule
NHERF1	Na/H exchange regulatory factor-1
NFL	Neurofilament light
nm	nanometer
NMDA	N-methyl-D-aspartic acid
NR1	NMDA receptor subunit 1
NR1C2'	Variably spliced NR1 exon cassette C2’
NR2A	NMDA receptor subunit 2A
NR2B	NMDA receptor subunit 2B
NR2C	NMDA receptor subunit 2C
NR2D	NMDA receptor subunit 2D
NR3A	NMDA receptor subunit 3A
NR3B	NMDA receptor subunit 3B
NSF	N-ethylmaleimide-sensitive factor
PBST	Phosphate buffered saline with 0.1% Tween
PCP	Phencyclidine

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDZ	PSD95, Drosophila disc-large tumor suppressor gene, and ZO-1
PFC	Prefrontal cortes
PI3K	Phosphoinositide 3-kinase
PICK	Protein interacting with C kinase 1
PKC	Protein kinase C
PMI	Postmortem interval
PNGaseF	Peptide-N^4-(N-acetyl-beta-glucosaminyl)asparigine amidase F
PSD93	Post-synaptic density protein 93 kilodaltons
PSD95	Post-synaptic density protein 95 kilodaltons
PVC	Primary visual cortex
PVDF	Polyvinylidene fluoride
QPCR	Quantitative polymerase chain reaction
Rap2	Ras-related AP2 domain-containing transcription factor
RFX1	Regulatory factor X, 1
SAP102	Synapse associated protein 102 kilodaltons
SAP97	Synapse associated protein 97 kilodaltons
Sept2	Septin 2
SDS	Sodium dodecyl sulfate
SLC1A3	Solute carrier family 1 member 3 (the EAAT1 gene)
SNARE	SNAP receptor
TBST	Tris buffered saline with 0.1% Tween
µg	microgram
VCP	Valosin containing protein
VGLUT	Vesicular glutamate transporter
WGA	Wheat germ agglutinin

Abstract

Excitatory Amino Acid Transporter Expression and Regulation in Prefrontal Cortex in Schizophrenia

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The glutamate hypothesis of schizophrenia, which is based primarily on NMDA receptor dysfunction, can be expanded to include additional components of the glutamate synapse including the excitatory amino acid transporters (EAATs) which buffer and transport synaptic glutamate. I studied multiple levels of expression and regulation of the EAATs in postmortem prefrontal cortex from patients with schizophrenia and comparison subjects. I used *in situ* hybridization, Western Blot analysis, enzymatic deglycosylation, and QPCR to study EAAT expression and regulation in the prefrontal cortex

in schizophrenia. I found decreased EAAT1 protein expression, and found several factors that indicate decreased localization of EAATs at the synapse including decreased glycosylation of EAAT1 and EAAT2, increased expression of the ER retention variant EAAT2Δ9, and increased expression of molecules that retain EAATs in the ER, GPS1 and JWA. These data suggest decreased overall EAAT expression, and abnormal localization of EAATs that are expressed. Therefore, there are likely fewer EAATs expressed at the cell surface, and thus a decrease in the capacity for glutamate buffering and transport. These data demonstrate novel sites of disruption in the glutamate synapse in schizophrenia. This work could therefore provide alternative targets for developing new treatments for schizophrenia.

Chapter 1

Introduction

1.1 Schizophrenia

Schizophrenia is a complex, debilitating mental illness that affects approximately 0.4 - 1% of the population worldwide[1-3]. Schizophrenia has a high comorbidity rate with other psychiatric disorders as well as metabolic disorders, and mortality rate is 1.5 - 3.3 fold higher among those with schizophrenia than in the general population[4]. Both genetic and environmental factors contribute to risk for developing schizophrenia, but no one gene has been consistently linked to the illness[3].

The symptoms of schizophrenia are broken into three clusters: positive symptoms, negative symptoms, and cognitive deficits[1]. Positive symptoms include delusions, hallucinations and paranoia. Negative symptoms include flattened affect, poverty of speech, social withdrawal, and anhedonia[1]. Cognitive deficits include disorganized thought processes and deficits in executive functioning. Although there is no cure for schizophrenia, there are

treatments for the illness, but the treatments are not fully effective, can be expensive, and often have adverse side-effects[4, 5].

1.2 The glutamate hypothesis of schizophrenia

For decades, schizophrenia research has focused on the dopamine hypothesis of schizophrenia, which postulates that dysregulated dopaminergic neurotransmission is a key feature of the pathophysiology of the illness. The dopamine hypothesis is based on the observation that antipsychotic efficacy for reducing positive symptoms is associated with D2 dopamine receptor blockade. Although numerous studies point to dopaminergic abnormalities in schizophrenia, dopamine dysfunction does not completely account for all of the symptoms seen in this illness. Antipsychotics typically are effective only for the positive symptoms of the illness, while negative symptoms and cognitive deficits are relatively refractory to treatment [6, 7].

Consequently, alternative neurotransmitter systems that may also be involved in the pathophysiology of schizophrenia have been sought, and a growing body of evidence now implicates glutamatergic dysfunction in this illness. The strongest evidence is that phencyclidine (PCP) and similar compounds, which are noncompetitive antagonists of the NMDA receptor, can induce positive negative and cognitive symptoms in unaffected healthy subjects [8, 9]. Moreover, these compounds can exacerbate both positive and negative symptoms in schizophrenia [10]. Chronic administration of PCP-like

compounds reduces frontal lobe blood flow and glucose utilization, which is similar to the "hypofrontality" described in schizophrenia [11]. More specifically, the most widely held hypotheses posit diminished NMDA receptor function in limbic brain structures, including the prefrontal cortex. I will discuss evidence supporting this hypothesis, and expand the glutamate hypothesis to encompass other glutamatergic abnormalities beyond NMDA receptor dysfunction.

1.2.1 Glutamate abnormalities in the prefrontal cortex

A large number of postmortem stereological, electrophysiologic, neuroimaging, and gene expression studies implicate cortical dysfunction in schizophrenia[12-15]. Two regions in particular that are implicated in this illness are the dorsolateral prefrontal cortex (DLPFC) and anterior cingulate cortex (ACC). Disturbances in executive functioning and working memory in schizophrenia have been linked to dysfunction of the DLPFC, and structural abnormalities in the DLPFC such as increased cell packing density, decreased somal size, and decreased neuropil are found[12]. Patients with chronic schizophrenia show decreased NAA, glutamine, and glutamate levels in the DLPFC as shown through magnetic resonance spectroscopy[16]. The ACC participates in selective attention, working memory, anticipation, and behavioral monitoring. Structural abnormalities such as neuronal loss in the ACC have been shown in schizophrenia, and deficiencies in insight and judgment are linked to ACC dysfunction[17]. The ACC may be particularly

vulnerable to changes in glutamatergic signaling as administration of subanesthetic doses of ketamine to patients with schizophrenia results in increased regional cerebral blood flow to the ACC as measured by positron emission topography[10].

1.3 Glutamate neurotransmission

Glutamate is the major excitatory neurotransmitter in the central nervous system. Glutamatergic neurons project throughout the brain, and deficits in glutamatergic neurotransmission can result in serious disease or death[18-25]. Below I describe the circuitry of glutamatergic neurons in the prefrontal cortex and the components of the glutamate synapse.

1.3.1 Prefrontal cortical glutamate circuits

Glutamatergic projection neurons, called pyramidal neurons, comprise approximately two thirds of cortical neurons and exist in all layers of neocortex except layer I[26]. Pyramidal neurons in superficial layers of cortex send efferents to projection neurons and interneurons in other areas of cortex[26]. Pyramidal neurons in deep layers of cortex send their projections to subcortical structures[26]. The prefrontal cortex in particular has dense reciprocal innervation with the dorsal thalamus[27-30], another brain area in which glutamatergic abnormalities have been implicated in schizophrenia[26, 31-38].

1.3.2 Components of the glutamate synapse

The release, activity as a ligand, and reclamation of glutamate involves three distinct cell types: the astrocyte, presynaptic neuron and postsynaptic neuron [39]. In the presynaptic neuron, glutamine can be converted to glutamate by the enzyme glutaminase, and packaged into vesicles by a family of vesicular glutamate transporters (VGLUT1-3) for release into the synapse [40, 41]. Glutamate may also be synthesized by the hydrolysis of N-acetyl-alpha-L-aspartyl-L-glutamate (NAAG) into NAA and glutamate by the enzyme carboxypeptidase II (GCP II) [42]. Once released into the synapse, glutamate may occupy and activate ionotropic (NMDA, AMPA, and kainate) or metabotropic (mGluR1-8) glutamate receptors located on both neurons and astrocytes [43]. Rapid removal of glutamate from the synapse is facilitated by a family of plasma membrane excitatory amino acid transporters (EAATs), generally localized to postsynaptic neurons and astrocytes [44]. Recovered glutamate may enter the citric acid cycle via conversion to alpha-ketoglutarate by glutamate dehydrogenase, be converted to glutamine by glutamine synthetase and transported back into the synapse, or be released into the extracellular space by a cystine/glutamate antiporter [39, 45, 46]. Finally, several families of novel glutamate receptor and transporter associated molecules mediate integration of intracellular signaling and glutamate reuptake [47-50].

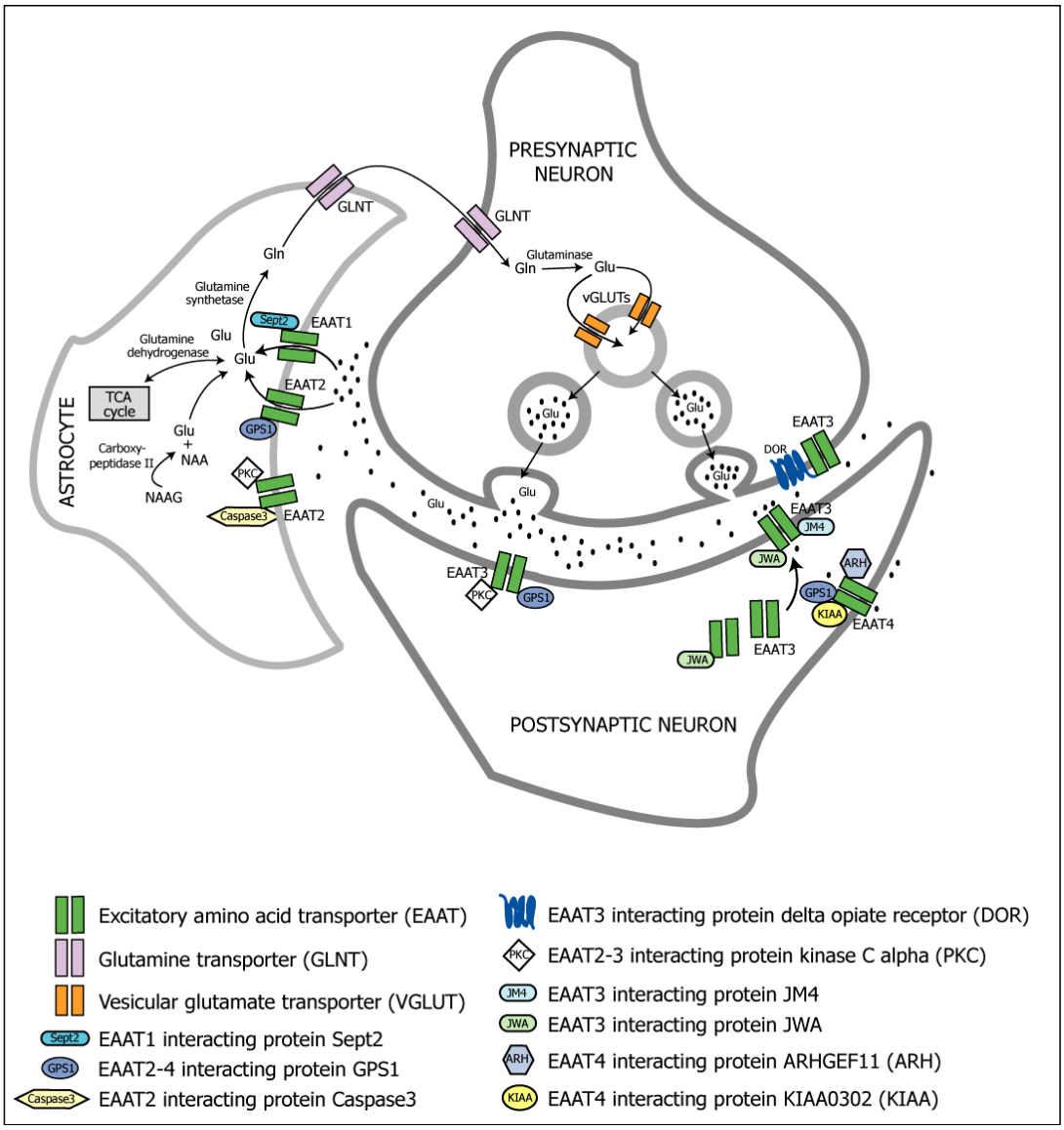

Figure 1.1: The Glutamate Synapse. Glutamate is packaged into presynaptic vesicles by vesicular glutamate transporters (VGLUTs), and is released into the synaptic cleft where it can activate glutamate receptors. Glutamate is rapidly removed from the synapse by excitatory amino acid transporters (EAATs) localized on astrocytes (EAAT1-2) and neurons (EAAT3-4).

1.3.3 Vesicular glutamate transporters

The vesicular glutamate transporters (VGLUTs), are responsible for the packaging of glutamate into synaptic vesicles. Vesicular uptake of glutamate is an ATP–dependent process independent of sodium and potassium. It has a biphasic dependence on chloride concentration, and is driven by an internal positive membrane potential. The VGLUTs are highly selective for L-glutamate, but have a lower affinity for glutamate in comparison to the EAATs [51, 52]. Alterations in synaptic activity can be induced by the modulation of the amount of glutamate released from synaptic vesicles. Therefore, VGLUT mediated transport of glutamate into vesicles for release into the synaptic cleft is a pivotal control point for synaptic activity [53]. Three vesicular transporters (VGLUT1, VGLUT2, and VGLUT3) have been cloned and characterized [54].

1.3.4 Ionotropic and Metabotropic Glutamate Receptors

There are four classes of functionally and pharmacologically distinct glutamate receptors. The ionotropic glutamate receptors, AMPA, kainate, and NMDA are comprised of four or five subunits that form ligand-gated ion channels, while the metabotropic glutamate receptors (mGluRs) are seven transmembrane domain G-protein coupled receptors [43, 55].

The AMPA receptor subunits are derived from a family of four genes termed GluR1-GluR4 that confer heterogeneity in assembled AMPA receptors by alternative splicing and post-translational editing [43, 55]. Assembled AMPA

receptors contain discrete binding sites for glutamate, competitive antagonists such as CNQX, and desensitization modulators such as aniracetam.

Kainate receptors are also ligand gated ion channels composed of subunits derived from genes for the low affinity GluR5-GluR7 and high affinity KA1-KA2 subunits [43, 55]. These subunits also undergo alternative splicing and post-translational editing. Assembled kainate receptors may be composed of five identical subunits (homomers) or composed of low and high affinity subunits.

The NMDA receptor subunits are encoded by seven genes termed NR1, NR2A-NR2D, and NR3A-NR3B. NR1 is expressed as one of eight isoforms, due to the alternative splicing of exons 5, 21, and 22 [43, 55-57]. NMDA receptors exhibit subunit and splice variant specific properties, and pharmacological regulation of this receptor depends on the unique combination of glutamate, glycine/D-serine, polyamine, H^+, $Zn^{2+,}$ and Mg^{2+} binding sites [43, 55]. In addition, there is an intrachannel binding site for uncompetitive antagonists of the NMDA receptor, such as PCP, ketamine, and MK801.

The eight metabotropic glutamate receptors (mGluRs) are divided into three groups (I, II, and III) based on pharmacology, sequence homology, and which signal transduction pathways they activate in heterologous systems [56, 58-63]. The mGluRs belong to a unique subset of G-protein coupled receptors with seven transmembrane domains and large extracellular amino termina. Group I mGluRs have been shown to stimulate phospholipase C,

phosphoinositide hydrolysis, and cAMP formation, while group II and III mGluRs inhibit forskolin-stimulated cAMP formation and adenylyl cyclase, via G proteins [58, 62-68].

1.3.5 Glutamate receptor interacting proteins

AMPA receptors may be mobilized to the synapse by three distinct mechanisms: a constitutive pool, a regulated pool, and a golgi-derived newly synthesized pool [69]. Cycling and integration of AMPA receptors to the postsynaptic density by these mechanisms involve a number of molecules that contain AMPA receptor subunit-specific protein binding domains and are regulated by posttranslational modification such as phosphorylation or palmitoylation [69-72]. GRIP, PICK, NSF, SAP97, stargazin, JNK, Rap2, and ABP-L have all been implicated in AMPA receptor trafficking [69, 70, 73, 74].

Receptor-associated molecules have also been identified for the NMDA receptor, including NF-L, SAP102, yotiao, PSD95, and PSD93 [69, 70]. Some of these molecules specifically bind C-terminal consensus sequences called PDZ domains, named for three proteins with this motif: PSD95, *Drosophila* disc-large tumor suppressor gene (Dlg-A) product, and ZO-1, a tight junction protein [75-77]. PDZ and related binding domains link neurotransmitter receptors with kinases, phospholipases, and other signal transduction and receptor trafficking pathways. Several of the AMPA interacting molecules, including GRIP1 and PICK1[78, 79] as well as calcineurin [80], also interact with the kainate receptors. In addition, a family of molecules

that interacts with the metabotropic receptors has been identified, including RGS4 [81-83] and Homer1-3 [84-87].

The glutamate receptor interacting proteins modulate a variety of processes related to synthesis, trafficking, insertion, activation, recycling, and degradation of glutamate receptors. Thus, the expression and regulation of this family of molecules has a critical role in cortical glutamatergic synapses.

1.3.6 Cortical glutamate receptor abnormalities in schizophrenia

Glutamate receptor expression has been extensively studied in schizophrenia and the results of cortical studies of glutamate receptor expression and binding are summarized in Tables 1.1 - 1.4. Most abnormalities of glutamate receptor expression in schizophrenia have been reported in limbic regions including the DLPFC and ACC. With a few exceptions, findings of altered glutamate receptor expression in schizophrenia are typically region-specific and brain-collection-specific, with many studies yielding conflicting results.

Alterations in NMDA receptor subunit and binding site expression are complex and region specific (Table 1.1). There is evidence for shifts in subunit stoichiometry and increased binding to at least some of the NMDA binding sites, primarily in cortical areas [88-93].

Table 1.1: NMDA receptor binding and expression in schizophrenia

Level of Gene Expression	Technique	Probe(s)*	Finding	Brain Region	Reference
Receptor binding sites	homogenate binding	MK-801	unchanged	FC	[90]
	homogenate binding	L-689,560 L-689,560 CGP 39653 ifenprodil ifenprodil	↑ unchanged unchanged ↑ unchanged	TC MC TC, MC TC MC	[93]
	homogenate binding	L-689,560 L-689,560	↑ unchanged	TC PFC	[94]
	homogenate binding	glycine	↑	cerebral cortex	[95] [96]
	autoradiography	MK-801	↑	ACC	[97]
	autoradiography	MK-801	unchanged	PFC	[98]
	autoradiography	MK-801	↑	PCC	[99]
	autoradiography	MK-801 CGP 39653	unchanged unchanged	DLPFC DLPFC	[100]
Subunit mRNA expression	ISH	NR2D NR1, NR2A-C NR1, NR2A-D	↑ unchanged unchanged	PFC PFC PTC	[92]
	ISH	NR1 w/exon 5	↑	FC, OCC, TC	[101]
	ISH	NR3A	↑ unchanged	PFC ITC	[102]
	Northern blot	NR1	↓	TC	[91]
	qPCR	NR1 NR1	↓[#] unchanged[@]	FC FC	[89]
	qPCR	NR1, NR2A NR2B	↑ unchanged	PFC, OCC PFC, OCC	[88]
	Double ISH	NR2A/GAD67	unchanged	PFC	[103]
Subunit protein expression	Western blot	$NR1^{C2'}$ $NR1^{C2'}$ $NR1^{C2}$, NR2A-D $NR1^{C2}$, NR2A-D	↑ unchanged unchanged unchanged	ACC DLPFC ACC DLPFC	[104]

*All binding studies utilized ^{3}[H].
[#]Neuroleptic free schizophrenics vs. controls.
[@]Neuroleptic treated schizophrenics vs. control.

Abbreviations: *in situ* hybridization (ISH), quantitative polymerase chain reaction (qPCR) anterior cingulated cortex (ACC), dorsolateral prefrontal cortex (DLPFC), prefrontal cortex (PFC), occipital cortex (OCC), visual cortex (VC), motor cortex (MC), frontal cortex (FC), parietal cortex (PC), temporal cortex (TC), inferior temporal cortex (ITC), parietotemporal cortex (PTC), posterior cingulate cortex (PCC), ($NR1^{C2'}$) variably spliced NR1exon cassettes C2 and C2'

Studies of AMPA receptor expression in schizophrenia (Table 1.2) result in one of the more robust and reproducible sets of findings in this field; many past studies have found that AMPA receptor expression is decreased in the hippocampus and related structures in schizophrenia, occurring at the levels of both transcript and subunit protein expression [105-110]. With few exceptions, AMPA receptor expression tends to be unchanged in other cortical areas [89, 105, 106, 108, 111, 112].

Unlike the AMPA receptor, findings for the kainate receptor (Table 1.3) are contradictory, with decreased binding reported in two studies, increased binding reported in three studies, and no changes reported in another study [113-115]. In addition, one study found increased GluR7 mRNA in the prefrontal cortex (PFC) while another found decreased GluR7 in the frontal cortex.

Three studies have examined mGluR expression in the cortex in schizophrenia (Table 1.4). Increased mGluR1a and mGluR2/3 immunoreactivity was found in Brodmann areas 9, 11, 32, and 46 in schizophrenia [116], while another group found decreased expression of mGluR3 in DLPFC [117], and a third group found increased mGluR5 mRNA in the PFC in this illness [118].

Table 1.2: AMPA receptor binding and expression in schizophrenia

Level of Gene Expression	Technique	Probe(s)*	Finding	Brain Region	Reference
Receptor binding sites	autoradiography	CNQX	↑	PFC	[98]
	homogenate binding	AMPA	unchanged	FC	[111]
	autoradiography	AMPA	↑	ACC	[97]
	homogenate binding	AMPA	unchanged	FC, PC, OCC, TC, LC	[119]
	autoradiography	AMPA	unchanged	PCC	[99]
	autoradiography	AMPA	unchanged	DLPFC	[100, 112]
Subunit mRNA expression	qPCR	GluR1 GluR1	↓# unchanged@	FC FC	[89]
	qPCR	GluR1, GluR4 GluR2, GluR3 GluR4 GluR1-3	↑ unchanged ↑ unchanged	DLPFC DLPFC OCC OCC	[120]
	Microarray	GluR2	↓	PFC	[121]
	ISH	GluR1, GluR3 GluR2, GluR4	unchanged ↓	DLPFC	[112]
Subunit protein expression	Western blot	GluR2, GluR3	unchanged	cingulate cortex	[105]

*All binding studies utilized ^{3}[H]AMPA or ^{3}[H]CNQX.
#Neuroleptic free schizophrenics vs. controls.
@Neuroleptic treated schizophrenics vs. control.

Abbreviations: *in situ* hybridization (ISH), quantitative-polymerase chain reaction (qPCR) anterior cingulated cortex (ACC), dorsolateral prefrontal cortex (DLPFC), prefrontal cortex (PFC), frontal cortex (FC), parietal cortex (PC), occipital cortex (OCC), temporal cortex (TC), limbic cortex (LC), posterior cingulate cortex (PCC)

Table 1.3: Summary of Kainate receptor binding and expression data in schizophrenia

Level of Gene Expression	Technique	Probe(s)*	Finding	Location	Reference
Receptor binding sites	autoradiography	KA	↓	PHG	[122]
	homogenate binding	KA	↑	PFC	[113]
	homogenate binding	KA	↑	FC	[114]
		KA	unchanged	TC	
	homogenate binding	KA	↑	PFC	[115]
	autoradiography	KA	↓	PFC	[123]
	autoradiography	KA	unchanged	PCC	[99]
	autoradiography	KA	↓	DLPFC	[100]
	autoradiography	KA	unchanged	ACC	[97]
Subunit mRNA expression	ISH	GluR7	↑	PFC	[123]
		KA2	↓	PFC	
		GluR5,6, KA1	unchanged	PFC	
	RT-PCR	GluR7	↓#	FC	[124]
		GluR7	unchanged@	FC	
		KA1	↓#	FC	
		KA1	unchanged@	FC	

*All binding studies utilized ^{3}[H]kainate.
#Neuroleptic free schizophrenics vs. controls.
@Neuroleptic treated schizophrenics vs. control.

Abbreviations: kainate (KA), *in situ* hybridization (ISH), reversed transcribed-polymerase chain reaction (RT-PCR), immunoreactive dendrites (IR) anterior cingulated cortex (ACC), dorsolateral prefrontal cortex (DLPFC), prefrontal cortex (PFC), frontal cortex (FC), occipital cortex (OCC), temporal cortex (TC), parahippocampal gyrus (PHG), posterior cingulate cortex (PCC)

Table 1.4: Summary of metabotropic glutamate receptor binding and expression data in schizophrenia

Level of Gene Expression	Technique	Probe(s)	Finding	Location	Reference
Subunit mRNA expression	Western	mGluR1a, mGluR2/3	↑	PFC	[116]
		mGluR4a, mGluR5	unchanged	PFC	
	ISH	mGluR3	unchanged	PFC	[118]
		mGluR5	↑	PFC	

Abbreviations: in situ hybridization (ISH), prefrontal cortex (PFC)

1.3.7 Cortical Glutamate Receptor interacting protein abnormalities in schizophrenia.

A number of studies have moved beyond measuring receptors in the plasma membrane to examine the molecules which functionally link glutamate receptors to cellular processes. Published data on receptor interacting proteins revealed that cortical regions are differentially affected in schizophrenia (Table 1.5). Studies of glutamate receptor interacting proteins suggest that glutamatergic dysfunction in schizophrenia is not simply a problem of too many or too few receptors, but is likely a problem of how these receptors are assembled, transported, and functionally linked to receptor signaling complexes.

Table 1.5: Cortical Glutamate Receptor Interacting Protein Abnormalities in Schizophrenia

Receptor-Interacting Protein	Associated Receptor(s)	Level of gene expression	Technique	Finding	Cortical Region	Reference
PSD95	NMDA, AMPA, kainate	mRNA	ISH	↓	DLPFC	[125]
		mRNA	ISH	↑ unchanged	ACC DLPFC	[104]
		protein	Western	unchanged	DLPFC	[79]
			Western	↓ unchanged	ACC DLPFC	[104, 126]
PSD93	NMDA	mRNA	ISH	↑ unchanged	ACC DLPFC	[104]
		protein	Western	↓ unchanged	ACC DLPFC	
			Western	unchanged	DLPFC	[79]
NF-L	NMDA	mRNA	ISH	unchanged ↑	ACC DLPFC	[104]
		protein	Western	unchanged ↓	ACC DLPFC	
SAP-102	NMDA, AMPA	mRNA	ISH	unchanged unchanged	ACC DLPFC	[104]
		protein	Western	unchanged unchanged	ACC DLPFC	
		protein	Western	unchanged	DLPFC	[79]
NSF	AMPA	mRNA	ISH, microarray	↓	DLPFC	[127]
		mRNA	ISH	unchanged	DLPFC	[112]
		mRNA	QPCR	unchanged	DLPFC	[128]
		protein	Western	unchanged	DLPFC, FP, PC	[129]
		protein	2D-DIGE	↑	DLPFC	[130]
		protein	Western	unchanged	DLPFC	[128]
SAP97	AMPA	mRNA	QPCR	unchanged unchanged	DLPFC OCC	[78]
		protein	Western	↓	DLPFC	[79]
GRIP1	AMPA, kainate, mGluR	mRNA	QPCR	↑ ↑	DLPFC OCC	[78]
		protein	Western	unchanged	DLPFC	[79]
PICK1	AMPA, kainate	mRNA	QPCR	unchanged unchanged	DLPFC OCC	[78]
		mRNA	ISH	↓	DLPFC	[112]
ABP	AMPA	mRNA	QPCR	unchanged ↑	DLPFC OCC	[78]
stargazin	AMPA	mRNA	ISH	↓	DLPFC	[112]
syntenin	AMPA	mRNA	ISH	unchanged	DLPFC	[112]

Receptor-Interacting Protein	Associated Receptor(s)	Level of gene expression	Technique	Finding	Cortical Region	Reference
calcineurin	kainate	protein	Western	unchanged	DLPFC	[80]
RGS4	mGluR	mRNA	QPCR	unchanged	DLPFC	[82]
		mRNA	ISH	↓ unchanged	CG, SFG, IC IFS, IFG	[81]
		mRNA	Microarray, ISH	↓	DLPFC, VC, MC	[83]
		protein	Western	↓ unchanged	FC IC	[81]
Homer1b	mGluR1/5	mRNA	ISH	unchanged	ACC, DLPFC	[87]
Homer2	mGluR1/5	mRNA	ISH	↑	ACC, DLPFC	[87]

Abbreviations: *in situ* hybridization (ISH), quantitative real time polymerase chain reaction (QPCR), two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), anterior cingulated cortex (ACC), dorsolateral prefrontal cortex (DLPFC), prefrontal cortex (PFC), occipital cortex (OCC), visual cortex (VC), motor cortex (MC), cingulated gyrus (CG), superior frontal gyrus (SFG), insular cortex (IC), frontal cortex (FC), inferior frontal sulcus (IFS), inferior frontal gyrus (IFG), frontal pole (FP), parietal cortex (PC)

1.4 Plasma Membrane Transporters

Extracellular glutamate levels are maintained at very low levels by functionally distinct low and high affinity systems that facilitate glutamate reuptake. High affinity Na^+-dependent synaptic reuptake of glutamate and aspartate is mediated by a family of excitatory amino acid transporters (EAAT1-EAAT5), which are localized in the plasma membrane and transport glutamate into neurons and glia [131, 132]. EAATs mediate glutamate transport by an electrogenic exchange of 3 Na^+, 1 H^+, and 1 glutamate molecule into the cell and 1 K^+ ion out of the cell, with the net inward movement of one positive charge [133, 134]. EAATs are expressed natively as homomeric trimers comprised of non-covalently linked subunits that have 6-10 transmembrane domains [51, 135, 136].

The transporters have specific patterns of cellular localization: EAAT1 and EAAT2 have primarily been localized to astroglia, whereas EAAT3 and EAAT4 are primarily localized to neurons[137-140]. EAAT5 expression is limited to the retina. EAAT expression may be regulated at multiple levels, including transcription, translation, post-translational modification, trafficking, substrate affinity and transport kinetics [51]. Expression, intracellular localization, regulation and function of EAAT1-4 are discussed below.

1.4.1 *EAAT1*

Called GLAST in the rodent, EAAT1 is expressed on astrocytes throughout the CNS, with the highest levels of expression in the Bergmann

glia in the cerebellum and the retina [137, 141-143]. Immunocytochemical studies suggest that GLAST/EAAT1 expression is limited to the plasma membranes of astrocyte cell bodies and processes, although one study suggests that EAAT1 is expressed in a subset of cortical neurons in subjects with Alzheimer's-type pathology [142, 144]. GLAST/EAAT1 expression is enriched in regions of the plasma membrane proximal to nerve terminals [145]. Immunogold labeling demonstrated relatively little intracellular GLAST/EAAT1 protein expression, suggesting that localization of GLAST/EAAT1 to the plasma membrane is not regulated via mobilization of an intracellular pool of transporter protein [145]. Cell surface expression of GLAST/EAAT1 was increased by insulin-like growth factor-1 via a protein kinase dependent mechanism, and region-specific GLAST/EAAT1 expression is increased by a number of peptide growth factors likely mediated by serine and tyrosine kinases [118, 146-149]. Recent studies have identified consensus tyrosine kinase phosphorylation sites targeted by specific glucocorticoid-sensitive kinase isoforms that facilitate increased EAAT1 activity.

1.4.2 EAAT2

Called GLT-1 in the rodent, EAAT2 is the most comprehensively studied of the glutamate transporters. GLT-1/EAAT2 accounts for approximately 90% of rodent forebrain glutamate reuptake [18, 23]. Expression of EAAT2 protein and mRNA has been observed throughout the human brain, but is highest in the forebrain [141, 150]. Astrocytes express EAAT2 transcripts

and protein, while EAAT2 mRNA is detectable in neurons found in the neocortex and thalamus [138, 139, 142, 151]. EAAT2 protein is expressed in neurons of the retina and embryonic neuronal cultures; in one recent report, protein expression of an EAAT2 splice variant (GLT-1a) was detected in rodent hippocampal neurons, while a PDZ-domain containing variant (called EAAT2b) was expressed in human neurons [51, 152-154]. GLT-1/EAAT2 protein is enriched in the portion of the astrocytic plasma membrane facing neuropil [145]. Less than 5% of GLT-1/EAAT2 immunoreactivity is found in structures other than the plasma membrane, including mitochondria and the endoplasmic reticulum [145]. GLT-1/EAAT2 mediated glutamate uptake is modulated by a number of paracrine factors including arachidonic acid, cytokines, oxygen radicals, and peptide growth factors [147, 155-157].

1.4.3 EAAT3

Called EAAC1 in the rodent, EAAT3 is expressed on neurons in the neocortex, striatum, thalamus, cerebellum and other structures [158, 159]. EAAT3 protein expression has been demonstrated on glutamatergic, GABAergic and aminergic neurons, as well as astrocytes of the cortex and in white matter [158, 159]. In contrast to EAAT1 and EAAT2, subcellular distribution of EAAT3 includes the cytoplasm, where it is available for rapid mobilization to perisynaptic regions of the plasma membrane [158-161]. Dendritic trafficking of EAAT3 is mediated by a novel c-terminal sorting motif, but mutation of this motif did not impair clustering of EAAT3 on dendritic spines [162]. Surface

expression of EAAT3 (and glutamate uptake) is rapidly increased by activation of PKC in C6 glioma cells constitutively expressing this transporter, and PKC activation increased glutamate uptake in cortical neuron cultures [163-166]. Platelet-derived growth factor (PDGF) increased surface expression of EAAT3 and glutamate uptake in C6 glioma cells, an effect mediated by activation of PI3K, and blocked by the PI3K inhibitor wortmanin [164, 167]. Activation of the neurotensin receptor NTS1 also increased EAAC surface expression, an effect that did not require PKC or PI3K activity [168]. The transcription factor RFX1 has also been shown to increase EAAT3 protein expression [169]. Similar to EAAT2, EAAT3 appears to be regulated by converging signaling pathways that utilize independent signaling substrates [51].

1.4.4 EAAT4

In the adult CNS, human and rodent EAAT4 transcripts and protein are only robustly expressed in the Purkinje cells of the cerebellar molecular layer [140, 141, 170, 171]. We have detected EAAT4 transcript expression in the human striatum by *in situ* hybridization, and low levels of EAAT4 mRNA and protein have been detected in rodent cortical neurons and astrocyte cultures [172-174]. Unlike the other transporters, EAAT4 protein has an uneven subcellular distribution and is typically localized in extrasynaptic regions [171, 175].

1.5 Refining the Glutamate Hypothesis of Schizophrenia

Although the glutamate hypothesis of schizophrenia is focused primarily on NMDA receptor dysfunction, recent data implicate glutamate transporters in schizophrenia. Knockout mice for the glutamate transporter EAAT1 exhibit schizophrenia-like endophenotypes including self-neglect, social withdrawal, and impaired learning [20]. This hypothesis is further supported by a report of a subject with schizophrenia who has a partial deletion of the EAAT1 gene [176]. These data suggest that maintenance of synaptic glutamate levels may play an important role in this illness. In fact, one study using an mGluR2/3 agonist, which decreases glutamate release, had antipsychotic properties in schizophrenia [177]. Below I discuss postmortem studies that have investigated EAAT expression in schizophrenia.

1.5.1 Abnormalities in Glutamate Transporters in Schizophrenia

Fewer studies have focused on glutamate transporters (Table 1.6) than glutamate receptors, and studies of glutamate transporter expression in the cortex have yielded conflicting results. Of the transporters, EAAT2 has been the most extensively studied. Three groups have investigated changes in mRNA expression of EAAT2 in the PFC in schizophrenia, all with different results. Ohnuma et al. found decreased EAAT2 mRNA in the PFC using *in situ* hybridization [178], while Matute et al. found increased[179] and Lauriat et al. found no changes in EAAT2 mRNA in the PFC using QPCR[180]. These

discrepant findings might be explained by differences in subjects studied, as the samples varied widely in age, postmortem interval (PMI), and neuroleptic treatment. The differences could also be explained by the use of different methodologies, as QPCR is more sensitive than ISH for detecting mRNA expression.

Another possible source of variation in these studies is the type of probe used to detect EAAT2. EAAT2 is differentially spliced, and the majority of EAAT splice variants are either EAAT2a or EAAT2b, which differ in their carboxyl termini. EAAT2a is more abundant and is generally found in astrocytes [142, 181], whereas EAAT2b is less abundant and generally found in neurons [154, 182]. These variants appear to be abnormally expressed in disease states such as amyotrophic lateral sclerosis [183]. Since Ohnuma et al. detected EAAT2 by ISH using a probe near the 3' end of EAAT2, they likely detected only the EAAT2a isoforms[184], while Matute et al. did not report the location of the EAAT2 probe used in their QPCR study[179]. It is possible that the groups were measuring different mRNA isoforms altogether, accounting for their discrepant results.

In addition to these conflicting EAAT2 data, the only other abnormality in glutamate transporter expression detected in the cortex in schizophrenia thus far is in the presynaptic vesicular glutamate transporter VGLUT1, which is involved in packaging of presynaptic glutamate into vesicles. Decreased VGLUT1 mRNA expression was found in superficial layers of the DLPFC in schizophrenia [185]. This finding suggests a decrease in glutamatergic

neurotransmission in the DLPFC, given that VGLUT1 expression has been shown to directly influence quantal glutamate release [186]. Such a change in presynaptic innervation might be consistent with deficits in cortical function in schizophrenia. Given the small number of studies of EAAT expression in schizophrenia, this represents a rich area for exploration in this dissertation.

Table 1.6: Cortical Glutamate Transporter Abnormalities in Schizophrenia

Transporter	Level of gene expression	Technique	Finding	Cortical Region	Reference
EAAT1	mRNA	QPCR	unchanged	DLPFC, PVC	[180]
EAAT2	mRNA	QPCR	unchanged	DLPFC, PVC	[180]
	mRNA	ISH	↓	PFC	[178]
	mRNA protein glutamate transport	QPCR ICC expression in oocytes	↑ ↑ ↑	PFC PFC PFC	[179]
EAAT3	mRNA	QPCR	unchanged	DLPFC, PVC	[180]
VGLUT1	mRNA	ISH	unchanged ↓	STC DLPFC	[185]

Abbreviations: quantitative real time polymerase chain reaction (QPCR), *in situ* hybridization (ISH), immunocytochemistry (ICC), dorsolateral prefrontal cortex (DLPFC), primary visual cortex (PVC), prefrontal cortex (PFC), superior temporal cortex (STC)

1.6 Modulation of EAAT function

Although a few studies have investigated EAAT expression in schizophrenia, other mechanisms of EAAT regulation have not been investigated in this illness. EAAT function can be modulated through a number of mechanisms. Several interacting partners for the EAATs have

been identified through yeast two hybrid screening and co-immunoprecipitation. Several posttranslational modifications have also been identified to affect EAAT function including phosphorylation, ubiquitination, and glycosylation. Lastly, there exist several alternative splice forms of the EAATs that affect transport function. Below we describe these mechanisms of EAAT regulation.

1.6.1 EAAT interacting molecules

Like the glutamate receptors, the EAATs are also regulated by a variety of interacting molecules that can affect transport function through trafficking, anchoring, phosphorylation, glycosylation, and degradation. Known EAAT1, EAAT2, EAAT3, and EAAT4 interacting proteins are discussed below.

1.6.2 EAAT1 interacting proteins

Disruption of putative c-terminal EAAT1 protein-protein interaction domains with a c-terminal peptide increased the affinity of EAAT1 for glutamate in salamander retinal glial cells, suggesting that protein-protein interactions modulate EAAT1 function [187]. Consistent with this finding, another study has demonstrated that sept2, a member of the septin family of GTPases, negatively modulates GLAST/EAAT1 by direct binding of the carboxy-terminal region [188]. GLAST/EAAT1 also forms a complex with glial fibrillary acidic protein (GFAP), Na^+/H^+ exchanger regulatory factor 1

(NHERF1), and ezrin, which anchor it at the plasma membrane and increase transport function [189, 190]

1.6.3 EAAT2 interacting proteins

A number of studies have found effects of the modulation of signaling mechanisms on GLT-1/EAAT2 expression and function, suggesting that there may be intermediary regulatory molecules that facilitate transporter localization and activation. PKC alpha, however, has been shown to directly interact with GLT-1/EAAT2, mediating transporter internalization [191]. Using the yeast-two hybrid technique, at least two additional EAAT2 interacting proteins have been identified. Ajuba is a cytosolic Lin11, Isl-1, and Mec-3 (LIM) protein that translocates to the plasma membrane and colocalizes with GLT-1/EAAT2 [49]. Ajuba interacts directly with F-actin, and may contribute to cell-cell junctions by bridging cadherin adhesive complexes [192]. Transcript expression of ajuba, however, was undetectable in the cortex by *in situ* hybridization (our unpublished observations). Another GLT-1/EAAT2 interacting protein, G-protein pathway suppressor 1 (GPS-1), is a subunit of the COP9 signalsome [50]. Transfection with GPS-1 of HEK cells stably expressing GLT-1/EAAT2 downregulated glutamate transport activity [50]. GPS-1 also interacts with EAAT3 and EAAT4 [50]. Caspase 3 also interacts with EAAT2. Caspase 3 can bind to and cleave EAAT2 at aspartate 505, located in the cytoplasmic C-terminal domain, strongly and selectively impairing transport activity [193].

1.6.4 EAAT3 interacting proteins

Like EAAT2, EAAT3 interacts with PKC alpha [194], but unlike EAAT2, EAAT3 membrane expression is increased by PKC alpha activation. GTRAP3-18 has been identified as an EAAT3 interacting protein. GTRAP3-18 has 95% nucleic acid homology with the human gene JWA, and specifically interacts with the intracellular c-terminus of EAAT3 [48]. Increases in GTRAP3-18 expression lowered EAAT3 substrate affinity, an effect mediated by modulation of transporter N-linked glycosylation [48, 195]. GTRAP3-18 transcripts and protein are ubiquitously expressed in the rodent, in both neuronal and non-neuronal cells [196]. Recently, Jena-Muenchen 4 (JM4), a JWA homolog sharing 62% homology with JWA and GTRAP3-18, has been shown to heterodimerize with JWA, and may interact with EAAT3 [197]. Interaction with the delta opiate receptor has been shown to decrease EAAT3 function [198]. The SNARE protein Syntaxin 1A has been shown to interact with EAAT3 when coexpressed in oocytes, increasing membrane expression through trafficking, but decreasing transport activity by disrupting the structure of the conductance pathway [199, 200]. However, another group showed that syntaxin 1A is not necessary for EAAT3 membrane trafficking in C6 glioma cells, and that another SNARE protein SNAP-23 was involved in the constitutive recycling of EAAT3 [201]. EAAT3 has also been shown to interact with the mitochondrial protein holocytochrome c synthetase (HCCS), serving as an anti-apoptotic mechanism, but no effect on transport activity has been

shown [202].

1.6.5 EAAT4 interacting proteins

The c-terminal end of rat EAAT4 interacts with GTRAP-41 and GTRAP-48, both of which increase EAAT4 mediated glutamate uptake by increasing and stabilizing cell surface transporter expression [47]. GTRAP-41 links EAAT4 with the cytoskeleton, while GTRAP-48 is associated with Rho-GTPase signaling [47]. Transcripts for the human isoforms of GTRAP-41 (KIAA0302) and GTRAP-48 (ARHGEF11) are robustly expressed throughout the CNS, including the thalamus and neocortex (our unpublished observations). The mismatch in the expression of EAAT4 and these interacting proteins suggests they may facilitate intracellular trafficking of other elements of the glutamate synapse. GPS1 is also known to interact with EAAT4, although its effect on function has not been demonstrated.

1.6.6 Post translational modification of the EAATs

The EAATs are also regulated at the level of post-translational modification. The EAATs have been shown to be phosphorylated [146, 203-206], ubiquitinated [207, 208], and glycosylated [135, 139, 209-213].

Glycosylation is a posttranslational modification that plays a role in molecular trafficking, protein folding, endocytosis, receptor activation, signal transduction, and cell adhesion [214]. Abnormalities of glycosylation can lead to a number of cellular storage disorders including Gaucher's, Niemann-Pick

type C, Sandhoff's, and Tay-Sach's diseases, and congenital disorders of glycosylation [214]. Disruptions in glycosylation have also been implicated in Alzheimer's disease, Huntington's disease, and schizophrenia [215].

N-linked glycosylation is the covalent linkage of oligosaccharides to asparagine residues of nascent proteins. N-glycosyl residues are processed as proteins are trafficked through the endoplasmic reticulum (ER) and Golgi. There exists a small literature showing that the EAATs are n-glycosylated, however, glycosylation of the EAATs in human brain has not yet been demonstrated. GLAST is n-glycosylated at Asn206 and Asn195 [209]. GLAST exists in two isoforms, 70-kDa and 64-kDa, which differ only by the degree of N-glycosylation [212]. Non-glycosylated GLAST does not dimerize [209]. There is conflicting literature describing the functional effects of GLT-1 glycosylation. One group found that glycosylation-deficient GLT-1 resulted in decreased transport rate attributable to decreased expression at the plasma membrane [216]. However, another group found no effect of N-glycosylation on the trafficking or transport activity of GLT-1. In rat C-6 glioma cells, EAAC1 (the rodent isoforms of EAAT3) is n-glycosylated with high mannose sidechains and processed into complex chains, coinciding with insertion into the plasma membrane [213].

1.6.7 Alternative splicing of the EAATs

The EAATs may also be regulated at the level of alternative splicing [154, 182, 183, 217-224]. Alterations in function have been demonstrated for several

of these variants, and it is speculated that others may also have altered function. Abnormalities in EAAT splicing have been demonstrated in mood disorders, anxiety disorders, Alzheimer's disease, epilepsy, hypoxia/ischemia, Huntington's disease, brain injury, glaucoma, HIV-associated dementia, addiction, ALS, and schizophrenia [180, 183, 219, 222, 224-226]. Described below are the known variants of EAAT1, EAAT2, and EAAT3.

EAAT1 contains both an exon 3 skipping [210, 224, 227] and an exon 9 skipping variant [228]. The exon 3 skipping variant, also known as EAAT1a, is thought to have an opposite orientation in the cell membrane to regular EAAT1 and to be unglycosylated [210]. The exon 9 skipping variant, also known as EAAT1b is retained in the ER, is likely unglycosylated, and appears not only to be a non-functioning transporter, but also to exert a dominant negative effect on the transport of the full length transporter [229].

Of the EAATs, the greatest number of splice variants has been described for EAAT2. Exon skipping variants of EAAT2 include an exon 7 skipping variant [219], an exon 9 skipping variant [219, 221, 222] and a variant containing a partial deletion of exons 6 and 7 [219]. Like the exon 9 skipping form of EAAT1, the exon 9 skipping form of EAAT2 is also retained in the ER [230]. There are also several intron retention variants of EAAT2. Five prime untranslated region retention variants of EAAT2 include HBGTII [218, 220], HBGTIIA [220], HBGTIIB [220], HBGTIIC [218, 220], EAAT2/3 [218], and EAAT2/31 [218]. Three prime untranslated region retention variants of EAAT2 include GLT1b, also known as EAAT2-1 or GLT1v which retains intron 9 [182, 183, 220, 221, 231],

GLT1c which retains part of intron 10 [221, 231], Intron 3' to exon 7 which skips exons 8-11 [219], and I7R, an intron 7 retention variant. EAAT2b retains the ability to transport glutamate *in vitro*, but unlike EAAT2 is expressed in both neurons (glutamatergic and GABAergic) and glia, and is expressed cytoplasmically rather than at the plasma membrane [182, 183]. I7R exerts a dominant negative effect on normal EAAT2 functioning marking the transporter for decay [222].

Like EAAT1 and EAAT2, EAAT3 can also exist as an exon 9 skipping variant [224]. It remains unclear, however, whether this variant is also retained in the ER [224].

1.7 Goals of dissertation research

Only a limited number of studies have investigated prefrontal cortical expression of EAATs in schizophrenia. One study found no changes in mRNA expression of EAAT1 or EAAT3 in prefrontal cortex while studies of EAAT2 expression in prefrontal cortex yield conflicting results. The EAATs have yet to be studied in the prefrontal cortex in schizophrenia at the level of protein expression, post-translational modification, splicing, and protein-protein interaction. The goals of this dissertation research are to test the following hypotheses:

1.) Expression of the EAATs and EAAT interacting proteins are altered in schizophrenia.

2.) The EAATs are abnormally glycosylated in schizophrenia.

3.) The EAATs are abnormally spliced in schizophrenia.

Chapter 2

Abnormal Expression of Glutamate Transporter and Transporter Interacting Molecules in Prefrontal Cortex in Elderly Patients with Schizophrenia

2.1 Abstract

Glutamate cycling is critically important for neurotransmission, and may be altered in schizophrenia. The excitatory amino acid transporters (EAATs) facilitate the reuptake of glutamate from the synaptic cleft and have a key role in glutamate cycling. We hypothesized that expression of the EAATs and the EAAT regulating proteins ARHGEF11, JWA, G protein suppressor pathway 1 (GPS1), and KIAA0302 are altered in the brain in schizophrenia. To test this, we measured expression of EAAT1, EAAT2, EAAT3, and EAAT interacting proteins in postmortem tissue from the DLPFC and ACC of patients with schizophrenia and a comparison group using *in situ* hybridization and Western blot analysis. We found increased EAAT1 transcripts and decreased protein expression, increased EAAT3 transcripts

and protein, and elevated protein expression of both GPS1 and KIAA0302 protein. We did not find any changes in expression of EAAT2. These data indicate that proteins involved in glutamate reuptake and cycling are altered in the cortex in schizophrenia, and may provide potential targets for future treatment strategies.

2.2 Introduction

Glutamate is rapidly removed from the synapse by plasma membrane EAATs, primarily localized to postsynaptic neurons and astrocytes [44]. EAATs are critical for glutamate transmission, because it is reuptake and not enzymatic breakdown that is responsible for clearance of glutamate from the synapse [51]. Of these transporters, EAAT1-3 are expressed abundantly throughout the CNS, while EAAT4 is expressed primarily in the cerebellum, and EAAT5 is found in the retina [170, 232]. EAAT1 and EAAT2 are generally localized to the plasma membranes of glial cells, and are responsible for the majority of glutamate reuptake in the forebrain [18, 23, 142, 233]. EAAT3 is localized to pre- and postsynaptic neurons in the plasma membrane and cytoplasm, and has been implicated in the regulation of synaptic plasticity [234, 235].

The EAATs are regulated by a variety of mechanisms, including phosphorylation, glycosylation, enzymatic degradation, and protein-protein interactions. A number of EAAT interacting proteins have been identified, including G-protein suppressor pathway 1 (GPS1), JWA, ARHGEF11, and

KIAA0302 (also called beta III spectrin). GPS1 interacts with GLT-1 (the rodent isoform of EAAT2), EAAC1 (EAAT3), and rodent EAAT4. GPS1 decreases GLT-1 mediated glutamate reuptake through a direct protein-protein interaction [50]. JWA is the human homolog of GTRAP3-18, which interacts with and downregulates EAAC1 mediated glutamate reuptake [48]. GTRAP41 (the rodent isoform of ARHGEF11) and GTRAP48 (the rodent isoform of KIAA0302) interact with rodent EAAT4, increasing glutamate transport [47].

Disruptions in cortical functioning and glutamate transmission have both been implicated in schizophrenia [8-10, 17, 36, 42, 54, 78, 88, 123, 132, 142, 178, 236-261]. A genetic variant of EAAT1 has been associated with schizophrenia, and expression of a high-risk for schizophrenia allele of the GRM3 metabotropic glutamate receptor is associated with decreased EAAT2 mRNA expression in human prefrontal cortex [176, 262]. Because the glutamate transporters maintain extracellular glutamate, we hypothesized that EAAT1-3 expression is altered in the DLPFC and ACC in schizophrenia. We also predicted that expression of molecules regulating EAAT function, including ARHGEF11, JWA, GPS1, and KIAA0302, are altered in schizophrenia. To test these hypotheses, we investigated both the transcript and protein expression of these EAATs and EAAT interacting proteins in schizophrenia using *in situ* hybridization and Western blot analysis.

2.3 Materials and Methods

2.3.1 Subjects

Two groups of subjects provided separately from the Mount Sinai Medical Center Brain Bank were studied (Tables 2.1 and 2.2) for mRNA and protein expression, respectively. 11 subjects (3 control subjects and 8 subjects with schizophrenia) were common to both groups. Neuropathological examination revealed no neurodegenerative disorders including Alzheimer's disease in any subject. Brain samples were prepared as previously described [263]. ACC was dissected at the level of the genu of the corpus callosum. Tissue blocks were dissected from the dorsal surface of the corpus callosum extending 12–15 mm dorsally and extending 12–15 mm laterally from the midline. DLPFC was dissected as described by Rajkowska and Goldman-Rakic [264].

Table 2.1: Subject Characteristics for *In Situ* Hybridizations

Age (Years)	Sex	PMI (hours)	pH	Cause of Death			
Comparison Group							
79*	F	3.0	6.3	CPF			
96*	F	3.3	6.7	CPF			
90*	F	2.3	6.0	CPF			
69	M	4.3	6.3	Unknown			
64	F	19.1	6.1	Pulmonary edema			
93	M	19.0	6.4	Congestive heart failure			
102	F	7.1	6.5	Acute Myocardial Infarction			
73	F	3.4	6.3	Acute Myocardial Infarction			
79	F	7.7	6.5	Acute Myocardial Infarction			
84	F	18.5	6.2	Unknown			
101	M	4.7	6.8	Coronary artery disease			
mean ± SD: 85 ± 13	3M/8F	8.6 ± 6.8	6.4 ±0.2				
Schizophrenia					**Subtype**	**Rx**	**Weeks Rx free**
84	F	15.6	6.2	Unknown	par	thi/chl	115
61*	M	3.5	6.5	CPF	undif	per/hal/ser	1
69*	M	4.5	6.4	Cardiac infarction, renal failure	par	hal/flu/tri/chl	6
63*	M	6.2	5.9	CPF	undif	hal	0
69*	F	13.9	6.2	CPF	undif	thi/tri	4
87*	M	11.2	6.5	CPF	undif	thi/tri	11
68*	M	5.6	6.8	CPF	par	pro	2
85	M	5.3	6.3	CPF	undiff	flu/chl	0
73	M	7.9	6.5	CPF	disorg	hal	0
66	M	12.1	6.5	Acute cardiac failure	undif	unknown	unknown
76	F	21.2	6.1	Cardiogenic shock	par	flu/tri/chl	0
97	M	9.3	6.5	CPF	undif	hal	0
66	M	8.4	6.7	CPF	sa	hal	0
82	F	18.8	6.6	CPF	res	ris	124
79	F	9.9	6.8	Cardiac Arrest	undif	thx	364
68	M	17.3	6.6	CPF	cat	flu	0
86	F	6.9	5.8	Respiratory insufficiency, renal failure	cat	thx/chl	36
65*	F	5.8	5.9	CPF	undif	unknown	unknown
79*	F	20.4	7.1	CPF, cancer of pancreas	undif	thi/thx	9
84*	M	6.2	6.5	CPF	undif	hal/per/thi/chl	106
mean ± SD: 75 ± 10	12M/8F	10.5 ± 5.6	6.5 ±0.3				

Abbreviations: post-mortem interval (PMI), standard deviation (SD), female (F), male (M), cardiopulmonary failure (CPF), paranoid (par), undifferentiated (undif), disorganized (disorg), schizoaffective (sa), residual (res), catatonic (cat), antipsychotic medication (Rx), Thioridazine (thi), Chlorpromazine (chl), Perphenazine (per), Haloperidol (hal), Trifluoperazine (tri), Prochlorperazine (pro), Fluphenazine (flu), Thiothixene (thx), Risperidone (ris). Asterisks indicate subject is present in both *in situ* hybridization and Western blot analysis cohorts.

Table 2.2: Subject Characteristics for Western Blot Analyses

Age (Years)	Sex	PMI (hours)	pH	Cause of Death			
Comparison Group							
88	M	4.8	5.9	Cardiac			
86	F	4.7	6.5	Unknown			
55	M	10.0	5.7	Cancer			
79*	F	3.0	6.3	CPF			
96*	F	3.3	6.7	CPF			
90*	F	4.2	6.0	CPF			
74	F	2.3	6.0	CPF			
70	M	8.0	6.0				
mean ± SD: 74 ± 13	3M / 5F	5.1 ± 2.6	6.1 ± 0.3				
Schizophrenia					**Subtype**	**Rx**	**Weeks Rx free**
54	M	8.2	6.0	Acute myelocytic leukemia	disorg	unknown	unknown
61*	M	3.5	6.5	CPF	undif	per/hal/ser	1
84*	M	6.2	6.5	CPF	undif	hal/per/thi/chl	106
69*	M	4.5	6.4	Cardiac infarction, renal failure	par	hal/flu/tri/chl	6
76	F	8.5	6.1	CPF, breast cancer	par	thi	0
87*	M	11.2	6.5	CPF	undif	thi/tri	11
68*	M	5.6	6.8	CPF	par	pro	2
86	F	5.5	6.2	Cardiac, pneumonia	undif	unknown	unknown
72	M	20.6	6.6	CPF	undif	neuroleptic	12
65*	F	5.8	5.9	CPF	undif	unknown	unknown
79*	F	20.4	7.1	CPF, pancreatic cancer	undif	thi/thx	9
63*	M	6.2	5.9	CPF	undif	hal	0
69*	F	13.7	6.2	CPF	undif	thi/tri	4
mean ± SD: 72 ± 13	8M / 5F	9.2 ± 5.7	6.4 ± 0.4				

Abbreviations: post-mortem interval (PMI), standard deviation (SD), female (F), male (M), cardiopulmonary failure (CPF), paranoid (par), undifferentiated (undif), disorganized (disorg), antipsychotic medication (Rx), Thioridazine (thi), Chlorpromazine (chl), Perphenazine (per), Haloperidol (hal), Fluphenazine (flu), Trifluoperazine (tri), Prochlorperazine (pro), Thiothixene (thx). Asterisks indicate subject is present in both *in situ* hybridization and Western blot analysis cohorts

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2.3.2 In Situ Hybridization

mRNA expression was measured using *in situ* hybridization for the following subclones: GPS-1 (Genebank accession no. **BC064503**; nucleotide coding region 1396-1602), ARHGEF11 (**NM_014784**; 1935-2309), JWA (**NM_006407**; 181-520), KIAA0302 (**AB008567**; 5958-6345), EAAT1 (**U03504**; 526-825), EAAT2 (**NM004171**; 601-1026), and EAAT3 (NM004170; 156-979), GLAST (rat EAAT1, **X63744 S49018**; 569–1001) as previously described [34, 173]. We have previously shown specificity of the probes for EAAT1, EAAT2, and EAAT3 using sense and antisense probes [173]. Specificity of the probes for GPS1 and KIAA0302 is shown in Figure 2.1.

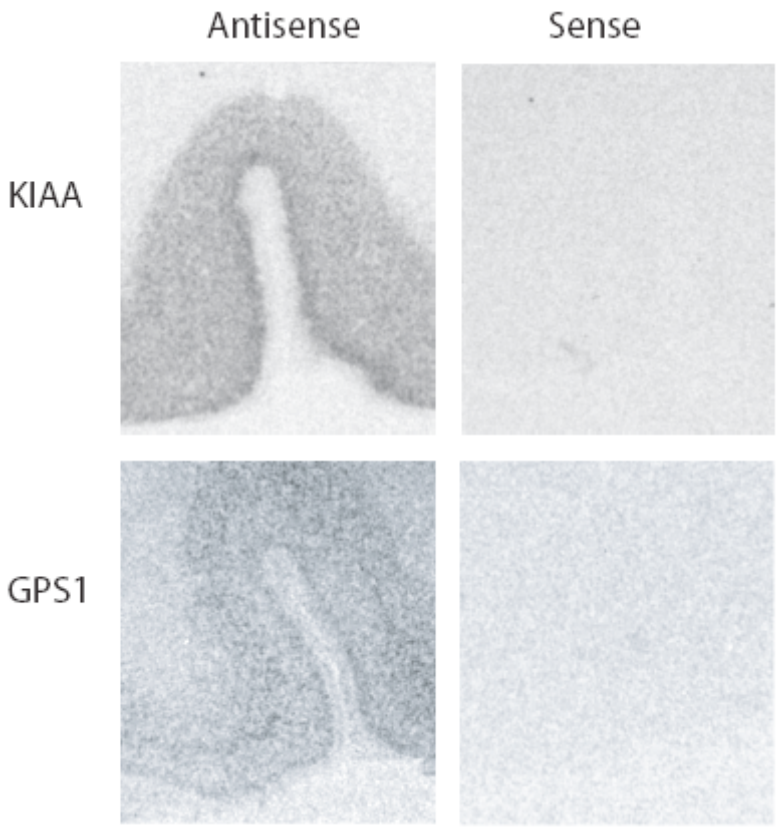

Figure 2.1: Probe specificity of KIAA0302 and GPS1. *In situ* hybridization analysis of KIAA0302 and GPS1 mRNA expression using sense and antisense probes in anterior cingulate cortex. Abbreviations: KIAA0302 (KIAA).

2.3.3 Western Blot Analysis

Western Blot analysis was performed as described previously [263]. For each assay, protein was transferred to membranes from multiple gels together in the same apparatus, and subsequent treatments were performed in parallel under identical conditions to minimize interblot variability. Membranes were incubated in blocking solution (5% milk powder in Tris buffered saline with 0.1% Tween (milk/TBST) for GPS1 and KIAA0302, 5% milk powder in phosphate buffered saline with 0.1% Tween (milk/PBST) for EAAT1 and EAAT2, and 3% milk/TBST for EAAT3) for 1 hour at room temperature. Membranes were incubated in primary antibody diluted 1:1000 for GPS1 (Abcam, ab4535), EAAT1 (Santa Cruz, sc-7758), and EAAT2 (Chemicon, AB1783), 1:200 for KIAA0302 (Santa Cruz, sc-28273) 1:250 for EAAT3 (Alpha Diagnostics, EAAC11-A), and 1:10,000 for beta-tubulin (Upstate, 05-661) in blocking solution overnight at 4^{o}C. The GLT-1 antibody was raised to a peptide sharing 100% sequence homology with human EAAT2, and cross-reacts with human EAAT2 [265]. The EAAC1 and spectrin β III antibodies were raised to peptides sharing 100% sequence homology with human EAAT3 and KIAA0302. Membranes were washed in TBST or PBST, then incubated for 1 hour with horseradish peroxidase (HRP) coupled secondary antibody diluted 1:5000 in blocking solution for EAAT1, GPS1, KIAA0302, and beta-tubulin, 1:4000 for EAAT2, and 1:400 for EAAT3. Membranes were washed in TBST or PBST followed by high purity water. Prior to examining protein expression, we tested our EAAT1, EAAT2, EAAT3,

GPS1, KIAA0302, and beta-tubulin Western blot assays using varying concentrations of total protein of human cortical tissue homogenate. These control studies demonstrated that our assay was linear for the protein concentrations used in our studies.

2.3.4 Statistical Analysis

Values from two sections (for *in situ* hybridizations) or bands (for Western blots) per subject for each region were averaged and converted to optical density. For *in situ* hybridizations, background from white matter (EAAT1, EAAT2, EAAT3, ARHGEF11, KIAA0302, and JWA) or slide background (GPS1) was subtracted from cortical expression grayscale values before conversion to optical density. For Western blots, optical density was divided by optical density of beta-tubulin from the same lane as a loading control and analyzed as a ratio. Outliers, defined as values more than 2 standard deviations from the mean, were excluded. Because tissue pH is a predictor of RNA integrity, correlation analysis was performed to investigate possible associations between transcript expression and tissue pH [266]. Protein integrity is generally not affected by pH [266]. Diagnostic groups were matched for age, pH, and PMI, and t-tests revealed no statistically significant differences between groups for any of these variables. When significant associations between transcript expression and pH were found, analysis of covariance (ANCOVA) was utilized; otherwise analysis of variance (ANOVA)

was utilized with diagnosis as the independent variable and optical density or optical density ratio as the dependent variable. For all tests $\alpha = 0.05$.

2.4 Results

2.4.1 Transcript Studies

Using *in situ* hybridization, transcript expression of EAAT1-3, ARHGEF11, GPS1, JWA, and KIAA0302 were measured. There were no associations between EAAT1, EAAT2, ARHGEF11, or KIAA0302 transcript expression and pH in either the ACC or the DLPFC. Correlation analysis revealed an association between EAAT3 transcript expression and pH in ACC ($R= 0.49$, $p < 0.05$) but not DLPFC. Correlation analysis revealed an association between JWA transcript expression and pH in ACC ($R = 0.45$, $p < 0.05$) but not DLPFC.

EAAT1 transcript expression was increased in schizophrenia in ACC ($F(1,21) = 4.54$, $p < 0.05$), but not DLPFC (Figure 2.2). EAAT3 transcript expression was increased in schizophrenia in ACC ($F(1, 19) = 18.07$, $p < 0.05$), but not DLPFC (Figure 2.2). JWA transcript expression was increased in schizophrenia in ACC ($F(1,24) = 5.73$, $p < 0.05$). KIAA0302 transcript expression was increased in schizophrenia in ACC ($F(1,24) = 8.59$, $p< 0.05$), but not DLPFC. There were no significant differences between groups for EAAT2, ARHGEF11, or GPS1 (Figures 2.2 and 2.3). Sense controls confirmed the specificity of our antisense probes for KIAA0302 and GPS1 (Figure 2.1). Emulsion dipping in the vicinity of layer V of the ACC confirmed

the specificity of EAAT2 expression to small cells, consistent with astrocytic expression (Figure 2.4).

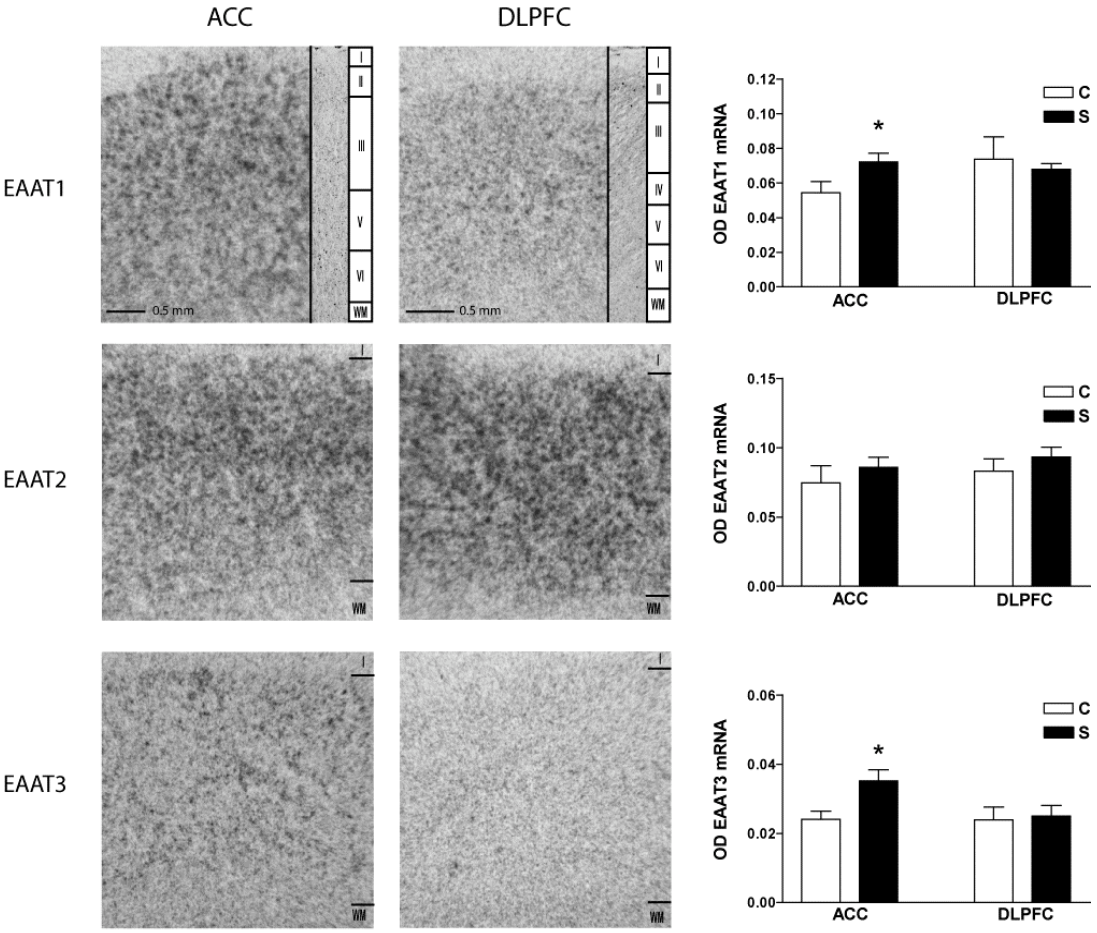

Figure 2.2: EAAT transcript expression. *In situ* hybridization analysis of EAAT1-3 mRNA expression in the anterior cingulate cortex and dorsolateral prefrontal cortex from control and schizophrenia subjects. Nissl stained sections are shown for comparison. Roman numerals indicate cortical layers. Data expressed as means +/- standard error of the mean. Asterisks indicate a significant difference between control and schizophrenia ($p < 0.05$). Abbreviations: Optical density (OD), control (C), schizophrenia (S), anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), excitatory amino acid transporter (EAAT), layer I (I), white matter (WM).

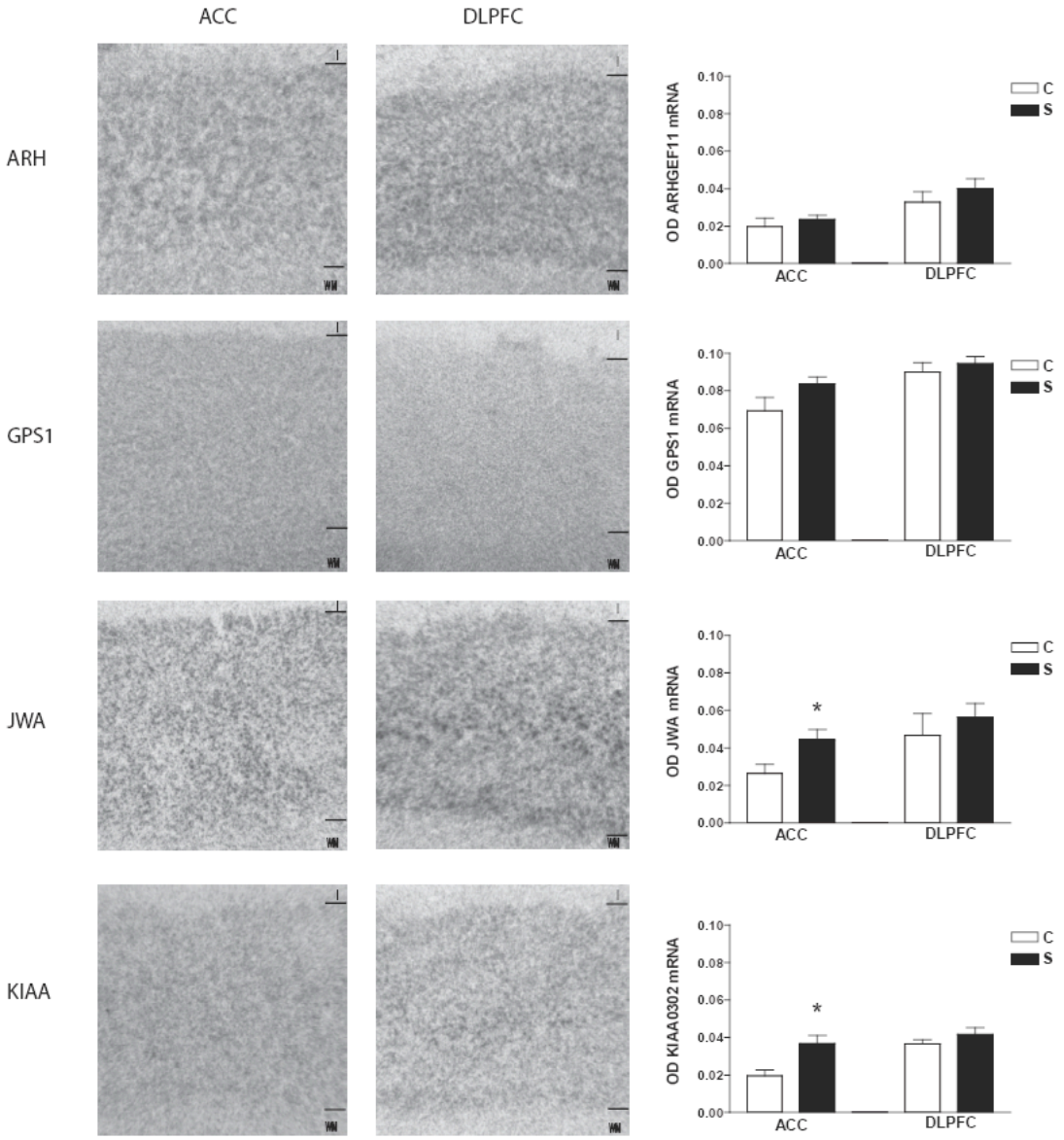

Figure 2.3: EAAT interacting partner transcript expression. *In situ* hybridization analysis of ARHGEF11, GPS1, JWA, and KIAA0302 mRNA expression in the anterior cingulate cortex and dorsolateral prefrontal cortex from control and schizophrenia subjects. Data expressed as means +/- standard error of the mean. Abbreviations: Optical density (OD), control (C), schizophrenia (S), anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), ARHGEF11 (ARH), G-protein suppressor pathway 1 (GPS1), KIAA0302 (KIAA), layer I (I), white matter (WM).

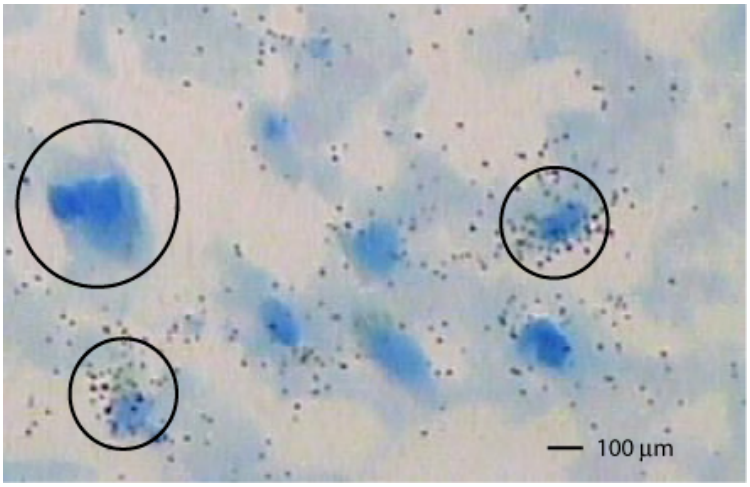

Figure 2.4: Cellular expression pattern of EAAT2. Emulsion dipped *in situ* hybridization of EAAT2 mRNA in nissl stained tissue from the vicinity of layer V of anterior cingulate cortex. Large circle indicates large cell, small circles indicate small cells.

2.4.2 Protein Studies

Using Western blot analysis, protein expression of EAAT1-3, GPS1, and KIAA0302 were measured. For EAAT1-3, protein bands were present for both monomeric and multimeric forms (Figure 2.5). Each of these bands was analyzed separately and in sum. Decreased total EAAT1 expression was found in the DLPFC ($F(1,14) = 6.25$, $p < 0.05$), associated with decreased expression of the monomer ($F(1, 15) = 7.31$, $p < 0.05$), but not the multimer (Figure 2.6). There were no changes in EAAT1 protein expression in the ACC. There were no changes in EAAT2 protein expression in the DLPFC or ACC (Figure 6). Increased expression of the EAAT3 multimer was found in the ACC ($F(1, 16) = 4.92$, $p < 0.05$) but not the DLPFC in schizophrenia

(Figure 2.6). There were no changes in expression of the monomer or the total amount of EAAT3 protein.

GPS1 protein expression was increased in the ACC in schizophrenia ($F(1, 17) = 6.41$, $p < 0.05$) (Figure 3), but unaltered in the DLPFC. KIAA0302 protein expression was increased in the DLPFC ($F(1, 16) = 10.56$; $p < 0.05$), but not the ACC, in schizophrenia (Figure 2.6).

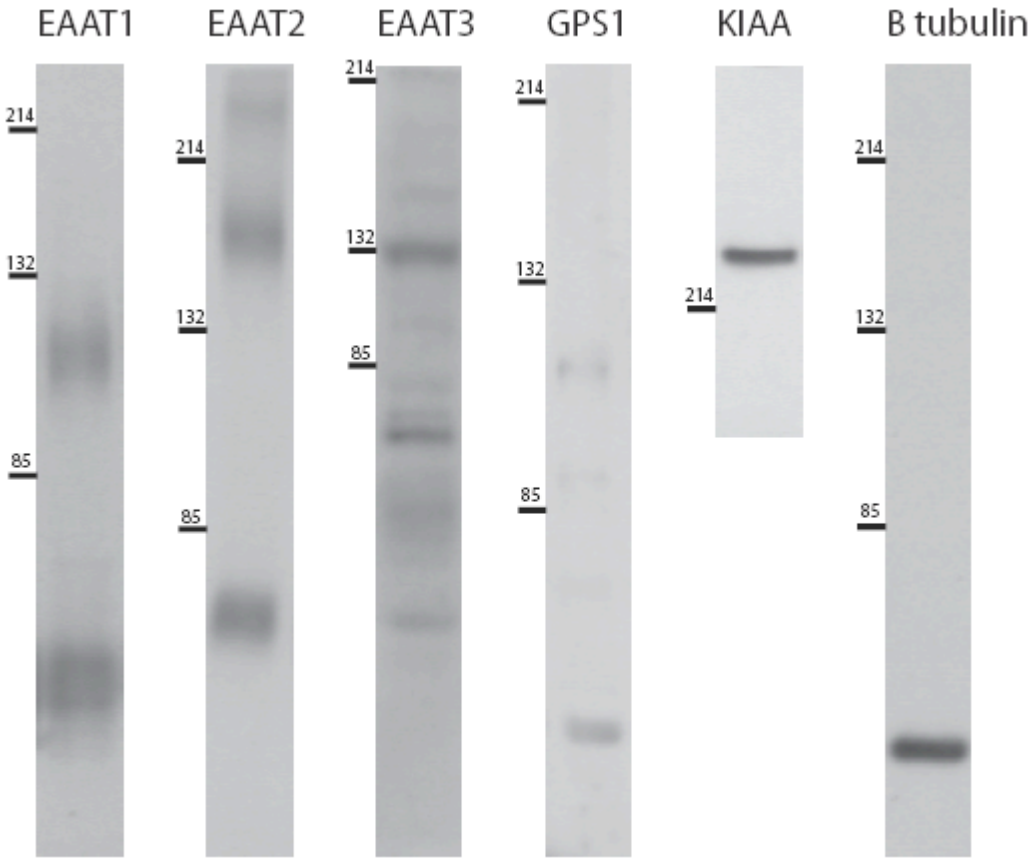

Figure 2.5: Protein expression profiles. Western blot analysis of all proteins studied. Numbers on left indicate molecular weight in kDa. Abbreviations: KIAA0302 (KIAA).

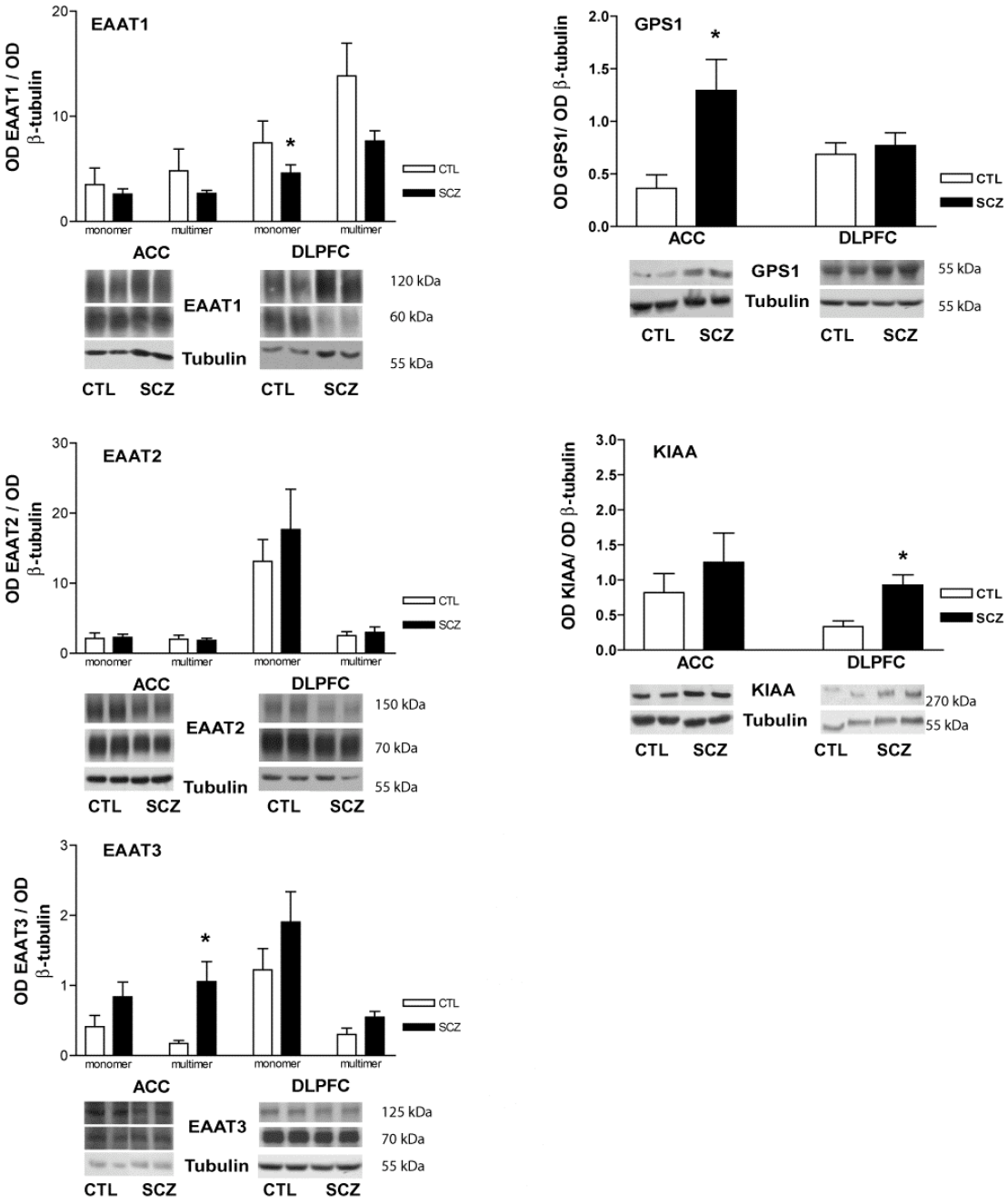

Figure 2.6: EAAT and EAAT interacting partner protein expression. Western blot analysis in the anterior cingulate cortex and dorsolateral prefrontal cortex from control and schizophrenia subjects for EAAT1-3, GPS1, and KIAA0302. Values are normalized to tubulin. Asterisks indicate a significant difference between control and schizophrenia ($p < 0.05$). Data expressed as means +/- standard error of the mean. Abbreviations: Optical density (OD), control (CTL), schizophrenia (SCZ), anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), excitatory amino acid transporter (EAAT), G-protein pathway suppressor 1 (GPS1), KIAA0302 (KIAA).

2.4.3 Sex Effects

There was a sex imbalance between diagnostic groups, so the dependent variables that differed by diagnosis were tested for sex effects. There were generally no sex effects for these variables with the exception of EAAT3 transcripts. EAAT3 transcript expression was increased in male subjects compared to female subjects in the ACC ($F(1,20) = 11.07$, $p < 0.05$). When split by sex, EAAT3 transcript expression was increased in males ($F(1,8) = 15.04$, $p < 0.05$) and females ($F(1,10) = 5.04$, $p < 0.05$) with schizophrenia compared to same sex controls.

2.4.4 Antipsychotic Effects

A number of patients with schizophrenia were medication-free for at least 6 weeks prior to death. No differences in transcript expression were detected between medicated and unmedicated patients for most of the transcripts found altered with the exception of JWA. An increase in JWA expression in the ACC was found in medicated versus unmedicated patients ($F(1,4) = 13.13$, $p < 0.05$). No changes in transcript expression detected between control subjects and unmedicated patients with schizophrenia for any of the transcripts found altered in schizophrenia. No differences in protein expression were detected between medicated and unmedicated patients with schizophrenia for any of the proteins found altered in schizophrenia. No changes in protein expression were detected between control subjects and unmedicated patients with schizophrenia for EAAT1,

GPS1, or KIAA0302. EAAT3 protein expression was increased in the ACC in unmedicated patients with schizophrenia compared to controls ($F(1, 9) = 7.07$, $p < 0.05$).

2.5 Discussion

We found alterations in expression of molecules involved in glutamate transport in schizophrenia, suggesting abnormal glutamate reuptake is involved in the pathophysiology of this illness. Of particular interest are the changes in protein expression of the glial transporter EAAT1, the neuronal transporter EAAT3, and the negative modulator of EAAT2 mediated glutamate reuptake, GPS1. These results suggest that neuronal glutamate reuptake may be increased in schizophrenia, while glial glutamate reuptake may be diminished.

We detected a decrease in total EAAT1 protein expression in the DLPFC, most likely attributable to decreased expression of the monomer. While the EAATs function natively as homomultimers [136], this result suggests that there may be a decrease in monomeric pools of EAAT1. Decreased EAAT1 protein expression may reflect decreased glutamate reuptake into glial cells or a decrease in the total number of EAAT1 expressing glia. In addition to decreased protein expression in the DLPFC, we detected increased EAAT1 transcript, but not protein, expression in the ACC. Unchanged protein expression with increased mRNA expression may be due

to a number of factors including abnormal translation and folding, abnormal post-translational modifications, or increased protein turnover.

We did not detect changes in transcript or protein expression of EAAT2, the transporter responsible for approximately 90% of glutamate reuptake in the forebrain [267]. Previous studies of EAAT2 expression have yielded discrepant results. Two studies found increased EAAT2 transcript expression in the PFC, while another found no changes [178-180]. While we did not find changes in EAAT2, it is possible that EAAT2 mediated glutamate reuptake is regulated by processes other than simply increasing or decreasing gene expression. Phosphorylation of specific serine residues and degradation pathways are known to influence EAAT2 function [193, 203].

EAAT2 mediated glutamate reuptake may also be regulated by GPS1 which interacts with the C-terminus of GLT-1 (the rat homolog of EAAT2), regulating surface trafficking through a leucine zipper-like motif [50]. Coexpression of GPS-1 with GLT-1 downregulates glutamate reuptake in HEK cells [268]. We found increased GPS1 protein, but not mRNA, in the ACC, suggesting decreased EAAT2 mediated glutamate reuptake. GPS1 protein levels have not previously been examined in schizophrenia, but in a different study [34], we found no changes in GPS1 mRNA expression in the thalamus, suggesting that changes in GPS1 may be region specific.

GPS1 may have other functions besides regulating GLT-1/EAAT2. GPS1 also associated with EAAC1 and rodent EAAT4 in an *in vitro* binding assay, and it might regulate these transporters in a manner similar to GLT-1

[50]. It has also been suggested that GPS1 functions in humans as a suppressor of G-protein pathway signaling [269]. Thus, it is possible that GPS1 could also have effects on G-protein linked receptors, including the mGluRs. mGluRs play a role in glutamate cycling because activation of group I mGluRs or group II-III mGluRs enhances and inhibits glutamate release, respectively [270].

While it appears that EAAT2 is not regulated simply by changes in gene expression, EAAT3 expression was increased at both transcript and protein levels. Animal and cell culture experiments have shown that the EAATs exist natively as homomultimers [136]. EAAT trimers are formed immediately after biosynthesis, and are the only functioning forms of EAAT3 in native systems [135]. It is unclear whether the multimer bands we measured are dimers or trimers, given that undissociated proteins do not migrate with a uniform surface area to charge ratio [271]. Our data suggest increased EAAT3 mediated glutamate reuptake in the ACC in schizophrenia, since we found an increase in multimer protein expression.

We propose that the changes in EAAT3 expression are secondary to changes in EAAT1 and GPS1 expression. Decreased EAAT1 and increased GPS1 expression may cause decreased glutamate reuptake in glial cells, leading to increased basal synaptic glutamate levels and synaptic spillover. EAAT3 is largely localized in perisynaptic regions and contributes to clearance of glutamate spillover [272, 273]. Thus, EAAT3 expression may be increased in neurons to compensate for these changes. Consistent with this

notion, EAAT3 protein is localized to regulatable cytosolic pools that may be rapidly mobilized to the plasma membrane, suggesting a biological process that is highly responsive to changes in glutamate levels [164]. It is also possible that increased EAAT3 expression is an effect of direct interaction with GPS1, or another EAAT regulating molecule. However, it is not known what effect, if any, GPS1 has on EAAT3 mediated glutamate reuptake.

In addition to the changes in GPS1, EAAT1, and EAAT3, we detected increased KIAA0302 protein expression in the DLPFC. This increase is difficult to interpret as it relates to glutamate transport because KIAA0302 is reported to interact only with EAAT4 [47]. Although both transcript and protein levels of KIAA0302 are highly expressed in the cortex, EAAT4 is expressed at very low levels, and is predominantly expressed in the cerebellum [171, 274]. This anatomical mismatch suggests that KIAA0302 has other functions. KIAA0302 is a structural molecule involved in Golgi and vesicular membrane skeletons, binds to dynein and dynactin, and it has been implicated in vesicular trafficking along microtubules [47, 275, 276]. Given the scarcity of EAAT4, it is likely that KIAA0302 serves structural, trafficking, or other roles in the prefrontal cortex.

We did not detect changes in transcript expression for any of the EAAT interacting proteins that we studied. This suggests that these molecules are not altered in schizophrenia at the mRNA level. We were unable to measure protein expression of JWA or ARHGEF11 with commercially available

antibodies. It is therefore possible that, similar to GPS1 and KIAA0302, they are altered in schizophrenia at the level of protein, but not mRNA, expression. Another point of interest from these data is that all significant changes were detected in the ACC but not the DLPFC. Although the DLPFC has been a major focus of studies involving altered gene expression in schizophrenia, many other regions have also been implicated in this disease, and may have more robust changes. In a microarray study of multiple brain regions in schizophrenia, the ACC had ~20 fold more altered genes than the dorsolateral prefrontal cortex [277]. Thus, our findings are consistent with the hypothesis that the ACC may be a particularly vulnerable site in the pathophysiology of schizophrenia.

There are several potential limitations of this study. One concern is the advanced age of the subjects. Disorder-specific alterations in gene expression could be masked by age effects on those genes. However, we have previously detected robust changes in glutamate receptor transcripts, proteins, and binding sites in a similar sample from the same brain bank, demonstrating significant changes in gene expression in older subjects [32-34, 36, 38]. Sex effects may also be a confounding factor. In our entire sample, we found an increase in EAAT3 transcript expression in males compared to females in the ACC. However, EAAT3 expression was increased in schizophrenia compared to controls in both males and females when analyzed separately, suggesting that our diagnosis-related results are likely not attributable to sex differences.

Another limitation of this study is the potential effects of treatment with antipsychotic medications [278, 279]. We generally did not detect any differences in expression between medicated and unmedicated patients for any transcripts or proteins we found increased in schizophrenia with one exception. We detected increased JWA transcript expression in the ACC in medicated versus unmedicated subjects. These analyses, however, are underpowered. The increased JWA transcript expression in schizophrenia may therefore be due to either disease specific or medication specific effects. However, we did find increased EAAT3 protein expression in the ACC in unmedicated patients with schizophrenia, compared to control subjects, suggesting that our EAAT3 findings are not due to a medication effect.

Our results shed light on several aspects of glutamate cycling in schizophrenia. Increased GPS1 protein and decreased EAAT1 protein suggest decreased glutamate reuptake capacity in glia, leading to increased synaptic glutamate levels and synaptic spillover. The increase in EAAT3 expression might be compensatory to decreased glutamate reuptake by glia. This scenario suggests a state of increased basal synaptic glutamate levels in the prefrontal cortex in schizophrenia. It remains unclear how KIAA0302 fits into this model, as the transporter subtype it regulates is expressed at very low levels in the cortex. These findings have important implications for neuronal plasticity in schizophrenia, and provide new targets for the development of novel treatments for this illness.

Chapter 3

Abnormal Glycosylation of EAAT1 and EAAT2 in Prefrontal Cortex of Elderly Patients with Schizophrenia

3.1 Abstract

EAATs may be regulated by N-glycosylation, a posttranslational modification that is critical for many cellular functions including localization in the plasma membrane. We hypothesized that glycosylation of the EAATs is abnormal in schizophrenia. To test this hypothesis, we treated postmortem tissue from the DLPFC and ACC of patients with schizophrenia and comparison subjects with deglycosylating enzymes. We then measured the resulting shifts in molecular weight of the EAATs using Western blot analysis to determine the mass of glycans cleaved from the transporter. We found evidence for less glycosylation of both EAAT1 and EAAT2 in schizophrenia. We did not detect N-linked glycosylation of EAAT3 in either schizophrenia or the comparison subjects in these regions. Our data suggest an abnormality of posttranslational modification of glutamate transporters in schizophrenia that suggests a decreased capacity for glutamate reuptake.

3.2 Introduction

Glycosylation of proteins is a posttranslational modification that plays a role in molecular trafficking, protein folding, endocytosis, receptor activation, signal transduction, and cell adhesion [214]. Abnormalities of glycosylation can lead to a number of cellular storage disorders including Gaucher's, Niemann-Pick type C, Sandhoff's, and Tay-Sach's diseases, as well as other congenital disorders of glycosylation [214]. Disruptions in glycosylation have also been implicated in Alzheimer's disease [280], Huntington's disease [281], and schizophrenia [215].

Two common forms of protein glycosylation include N-linked glycosylation and O-linked glycosylation. N-linked glycosylation is the covalent linkage of oligosaccharides to asparagine residues of proteins. N-glycosyl residues are processed as proteins are trafficked through the ER and Golgi. The EAATs are N-glycosylated proteins that transport extracellular glutamate out of the synapse and thus are critical for glutamatergic signaling. However, glycosylation of the EAATs in human brain has not been evaluated.

EAAT1 is variably expressed throughout the cortex in astroglia [138, 142, 143, 181, 233, 282, 283]. GLAST, the rodent form of EAAT1, exists as two isoforms, 70-kDa and 64-kDa, which differ only by the degree of N-glycosylation at Asn206 and Asn195 [209, 212]. Glycosylation of this transporter may serve an important functional role because nonglycosylated GLAST does not form homomultimers, which are the native conformation of GLAST *in vivo* [209]. In

addition, glycosylation of GLAST has been correlated with trafficking of GLAST to plasma membrane and increased glutamate uptake [284].

EAAT2 is an astrocytic transporter responsible for the majority of glutamate uptake in the cortex. Deglycosylation of the rodent isoforms of EAAT2 (GLT-1) resulted in a ~10-15 kDa shift in molecular weight of the monomer band [230]. There is a conflicting literature describing the functional effects of EAAT2 glycosylation. One group found that glycosylation-deficient GLT-1 had a decreased rate of glutamate transport due to decreased expression in the plasma membrane [216]. This may be attributed to retention of GLT-1 in the ER, because mutant GLT-1 expressing an altered extracellular leucine-based motif is immaturely glycosylated and retained in the ER [230]. However, another group found no effect of N-glycosylation on the trafficking or transport activity of GLT-1 in transfected BHK cells, but increased stability at the plasma membrane, which may be critical for transporter localization *in vivo* [211].

EAAT3 is a neuronal glutamate transporter expressed in the cortex. In rat C-6 glioma cells, EAAC1 (the rodent form of EAAT3) is N-glycosylated with high mannose-containing sidechains and processed into complex chains, coinciding with insertion into the plasma membrane [213]. A shift of approximately 5 kDa was detected when EAAT3 immunoprecipitated from human brain synaptosomes were treated with Endoglycosidase F [285].

We previously reported alterations in EAAT1 and EAAT3 protein in prefrontal cortex in schizophrenia, suggesting diminished EAAT-mediated

glutamate reuptake as a part of the pathophysiology of this illness [286]. However, localization of the transporters may be as important as overall protein levels. Altered EAAT localization may lead to glutamate spillover into the extrasynaptic space and adjacent synapses, causing loss of input specificity [287-289]. Since glycosylation is important for targeting of the EAATs to the plasma membrane, abnormal glycosylation of these proteins may play a role in schizophrenia.

Glycobiology is a growing field with an increasing number of tools. The enzyme peptide-N^4-(N-acetyl-beta-glucosaminyl) asparagine amidase F (PNGase F) cleaves N-linked sugars off of proteins attached at asparagine residues. Endoglycosidase H (Endo H) cleaves hybrid and high mannose containing residues from glycoproteins, and is therefore specific to immaturely glycosylated proteins that have not been processed beyond the ER. The removal of glycans is often substantial enough to detect a change in molecular weight of proteins when measured by Western blot analysis. In this study, we assessed glycosylation of EAAT1, EAAT2, and EAAT3 through enzymatic deglycosylation in schizophrenia and a comparison group.

3.3 Materials and Methods

3.3.1 Subjects

Subjects from the Mount Sinai Medical Center Schizophrenia Brain Bank were studied (Table 3.1), including 35 individuals diagnosed with schizophrenia and 33 comparison subjects. Subjects were diagnosed with schizophrenia if the presence of schizophrenic symptoms was documented before age 40, the medical records contained evidence of psychotic symptoms and at least 10 years of psychiatric hospitalization with diagnosis of schizophrenia, and a DSM-III-R diagnosis of schizophrenia was agreed upon by two experienced clinicians. Diagnostic groups did not significantly differ for age, sex, postmortem interval, and tissue pH. Upon neuropathological examination, no evidence of Alzheimer's or other neurodegenerative disease was found. The brain banking procedures were approved by the Mount Sinai School of Medicine Institutional Review Board.

Table 3.1: Subject Characteristics for Deglycosylation Studies

	Comparison Group		Schizophrenia	
Region	ACC	DLPFC	ACC	DLPFC
N	34	32	34	33
Sex	14 m / 20 f	12 m / 20 f	24 m / 10 f	23 m / 10 f
Tissue pH	6.4 ± 0.2	6.5 ± 0.2	6.4 ± 0.3	6.4 ± 0.3
PMI (hours)	8.3 ± 6.7	8.2 ± 6.8	13.4 ± 8.1	12.5 ± 6.7
Age (years)	78 ± 14	78 ± 14	74 ± 12	74 ± 12
On / Off Rx	0 / 34	0 / 32	23 / 11	22 / 11

Values presented as mean ± standard deviation
Abbreviations: anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), male (m), female (f), antipsychotic medication (Rx), postmortem interval (PMI).

3.3.2 Tissue preparation

Brains were obtained after autopsy and one hemisphere was cut coronally into ~0.8 - 1 cm^3 slabs and flash frozen. Gray matter was dissected from ACC (n = 68) and DLPFC (n = 66). ACC was dissected at the level of the genu of the corpus callosum. Tissue blocks were dissected from the dorsal surface of the corpus callosum extending 12–15 mm dorsally and extending 12–15 mm laterally from the midline. DLPFC was dissected corresponding to Brodmann area 46 and measuring ≈ 1.5 cm along the cortical surface as described by Rajkowska and Goldman-Rakic [264]. Approximately 1 cm^3 of frozen tissue was pulverized in liquid nitrogen, then homogenized (10% wt/vol) in 5 mM Tris-HCl (pH 7.4) with 320 mM sucrose and 1 protease inhibitor tablet (Complete mini, Roche Diagnostics, Manheim, Germany) per 10 mL for 30 sec with a polytron homogenizer (Fisher Scientific, Pittsburgh, Pennsylvania) and stored at -80^oC in 0.5 mL aliquots. To determine protein concentrations, assay by the Bradford method [290] was performed on these homogenates.

3.3.3 Deglycosylation

16 µg of protein for each sample was added to 6.7 µl 5X reaction buffer (QA Bio), 1.7 µl denaturation solution (2% SDS/ 1M β mercaptoethanol) (QA Bio), and adjusted to volume with deionized water. Samples were then incubated at 70^oC for 10 min. Samples were cooled to room temperature and incubated with 1.3 µl Endoglycosidase H or 1.3 µl

PNGase F and 1.7 µl 15% triton X-100 (QA Bio) at 37^{o}C for 12 hours. Non-enzyme-treated samples were prepared identically to the enzyme-treated samples with the same buffers except that they were incubated with water instead of the deglycosylating enzymes.

3.3.4 Electrophoresis

NuPAGE sample reducing agent (Invitrogen), and NuPAGE LDS sample buffer (Invitrogen) were added to the samples, which were then incubated at 70^{o}C for 10 minutes. The Novex Mini Cell NuPAGE system (Invitrogen) with 4-12% Bis-Tris gradient polyacrylamide gels (Invitrogen) was used and 8µg of protein was added per lane. A molecular mass standard was run on each gel (Kaleidescope prestained standards, BioRad). Gels were suspended in a bath of NuPAGE MES SDS running buffer (Invitrogen) with 500µl NuPAGE antioxidant (Invitrogen) during electrophoresis.

3.3.5 Western blot analysis

Following electrophoresis, proteins were transferred onto Immobilon-FL PVDF membranes (Millipore) using a semi-dry transfer apparatus (Bio-Rad). After electroblot transfer, membranes were washed twice and incubated with Li-Cor Blocking Buffer (Li-Cor Biosciences) for 1 hour at room temperature with rocking to block nonspecific antibody binding. Membranes were incubated with either a rabbit polyclonal antibody to EAAT1 (Santa Cruz sc-15316) diluted 1:1,000, rabbit polyclonal antibody to EAAT2 (Santa Cruz

sc-15317) diluted 1:1000, mouse moloclonal antibody to EAAT3 (Chemicon MAB1578) diluted 1:1000, rabbit polyclonal antibody to EAAT3 (Santa Cruz sc-25658) diluted 1:500, or rabbit polyclonal antibody to EAAT3 (Alpha Diagnostics #EAAC11-A) diluted 1:500 in blocking buffer with 0.1% Tween overnight at 4^{o}C with rocking. Next, the membranes were washed three times for ten minutes in tris-buffered saline with 0.1% Tween (TBST), then rocked for 15 minutes at room temperature with anti-rabbit or anti-mouse IR-Dye 800CW secondary antibody (Li-Cor Biosciences) diluted 1:10,000 in blocking buffer with 0.1% Tween. Membranes were washed three times for 10 minutes in TBST then washed 5 times in deionized water and allowed to dry for 3-5 minutes before scanning (infrared imaging system; Li-Cor Biosciences).

3.3.6 Data Analysis

Membranes probed with infrared-labeled secondary antibodies were scanned using a Li-Cor Odyssey scanner, and the migration distance for each protein band was measured in pixels using the Odyssey 2.1 software package. Migration distance was converted to molecular mass by plotting the relative migration of the molecular mass standards against the log of their molecular masses, and fitting the relative migration of the bands of interest to that standard curve [291] (Figure 3.1). Band shift was measured as molecular mass of the control band minus the molecular mass of the enzyme treated band in the adjacent lane, as described previously [292-294] (Figure 3.1). EAAT1

and EAAT2 migrate as both monomers and multimers [286], and the molecular mass shifts of monomers and multimers were analyzed separately.

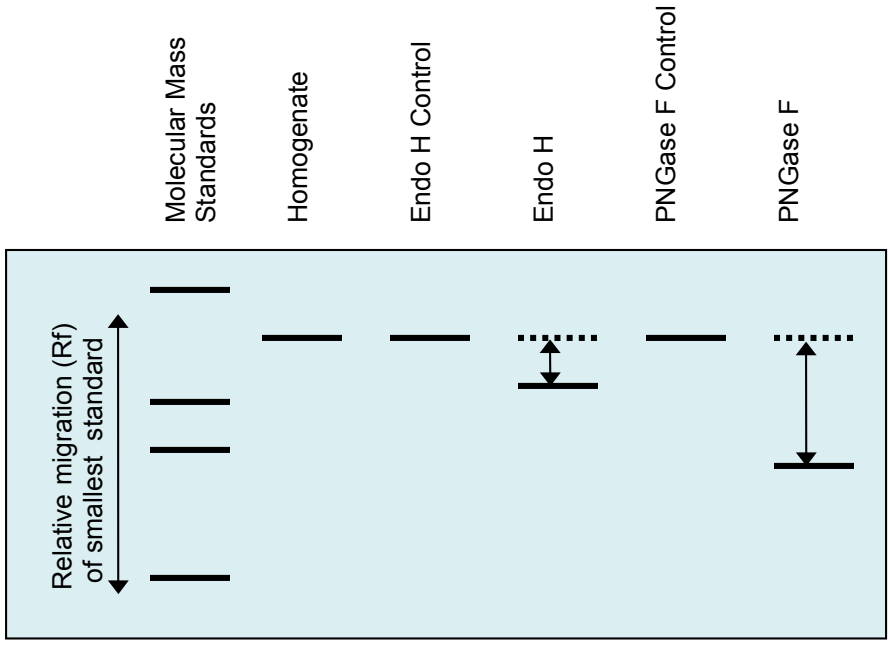

Figure 3.1: Schematic of a gel with control and deglycosylation treatments. Molecular mass was calculated by plotting the relative migration of the molecular mass standards against the log of their molecular masses, and fitting the relative migration of the bands of interest to that standard curve. Molecular mass shift was calculated by subtracting the molecular mass of the adjacent control band from the band of interest. Abbreviations: relative migration (Rf), endoglycosidase H (endo H), peptide-N^4-(N-acetyl-beta-glucosaminyl) asparagine amidase F (PNGase F).

3.3.7 Statistical Analysis

All statistical analyses were performed using Statistica (StatSoft, Tulsa, Oklahoma). Outliers more than 6 standard deviations from the mean were excluded. Correlation analysis was performed to determine associations

between the dependent variable, molecular mass shift and age, PMI, and pH. We analyzed deglycosylation induced changes in molecular mass using analysis of variance (ANOVA), or analysis of covariance (ANCOVA) when significant correlations were detected. To test for possible medication effects, patients with schizophrenia off antipsychotic medication for at least 6 weeks prior to death were compared to patients on antipsychotic medication within 6 weeks of death.

3.4 Results

When samples were treated with EndoH, none of the transporters exhibited shifts in molecular mass of either monomeric or multimeric forms (Figure 3.2). When samples were treated with PNGase F, EAAT1 and EAAT2 exhibited detectable shifts in molecular mass for both monomeric and multimeric forms (Figure 3.2). However, EAAT3 did not shift when treated with PNGase F (Figure 3.2). Because we were concerned that the lack of shift could be due to a loss of an epitope following enzymatic digestion, we performed Western blots with two additional EAAT3 antibodies raised against different epitopes, and did not detect shifts in EAAT3 (data not shown).

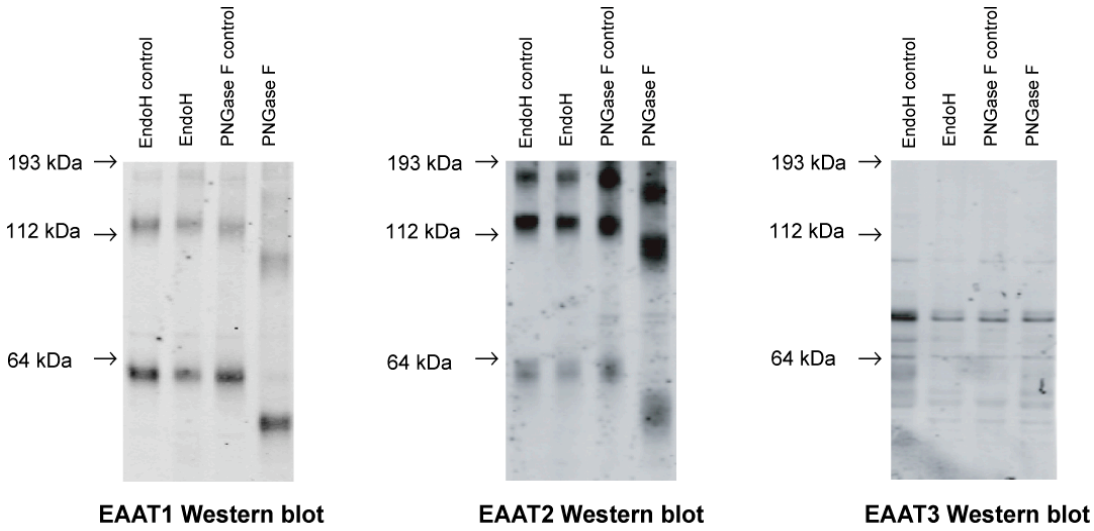

Figure 3.2: Western blots of deglycosylated EAATs. Western blot analysis of EAAT1, EAAT2, and EAAT3 deglycosylated with the enzymes Endoglycosidase H and PNGase F. EndoH and PNGase F lanes indicate enzyme treated samples. EndoH control and PNGase F control lanes were treated identically to the corresponding enzyme treated samples except the enzymes were omitted. Molecular masses of EAAT1 and EAAT2 monomers and multimers were shifted in the PNGase F treated lanes. No shift was detected for EAAT3. Abbreviations: kDa (kilodaltons), excitatory amino acid transporter (EAAT), endoglycosidase H (EndoH), peptide-N^4-(N-acetyl-beta-glucosaminyl) asparagine amidase F (PNGase F).

We examined the effects of PNGase treatment on monomer and multimer forms of EAAT1 and EAAT2 in schizophrenia and comparison subjects. There were generally no correlations detected between age, PMI (which differs between diagnosis groups ($F(1, 66)=7.3766$, $p=.00843$)), or pH and our dependent measures with the exception of shift of EAAT1 monomer in the DLPFC: ($R= 0.28$, $p < 0.05$). We found less of a molecular mass shift for the EAAT1 monomer in schizophrenia in the ACC ($F(1, 61) = 6.40$; $p < 0.05$) (Figure 3.3). We found less of a mass shift for the EAAT2 multimer in schizophrenia in the DLPFC ($F(1,52) = 9.41$; $p < 0.05$) (Figure 3.2). There was no effect of medication status in the subjects with schizophrenia on either of these dependent measures (EAAT1 monomer in ACC ($F(1, 29) = 0.06$, $p = 0.80$), EAAT2 multimer in DLPFC ($F(1, 27) = 1.04$, $p = 0.32$)) (Figure 3.4).

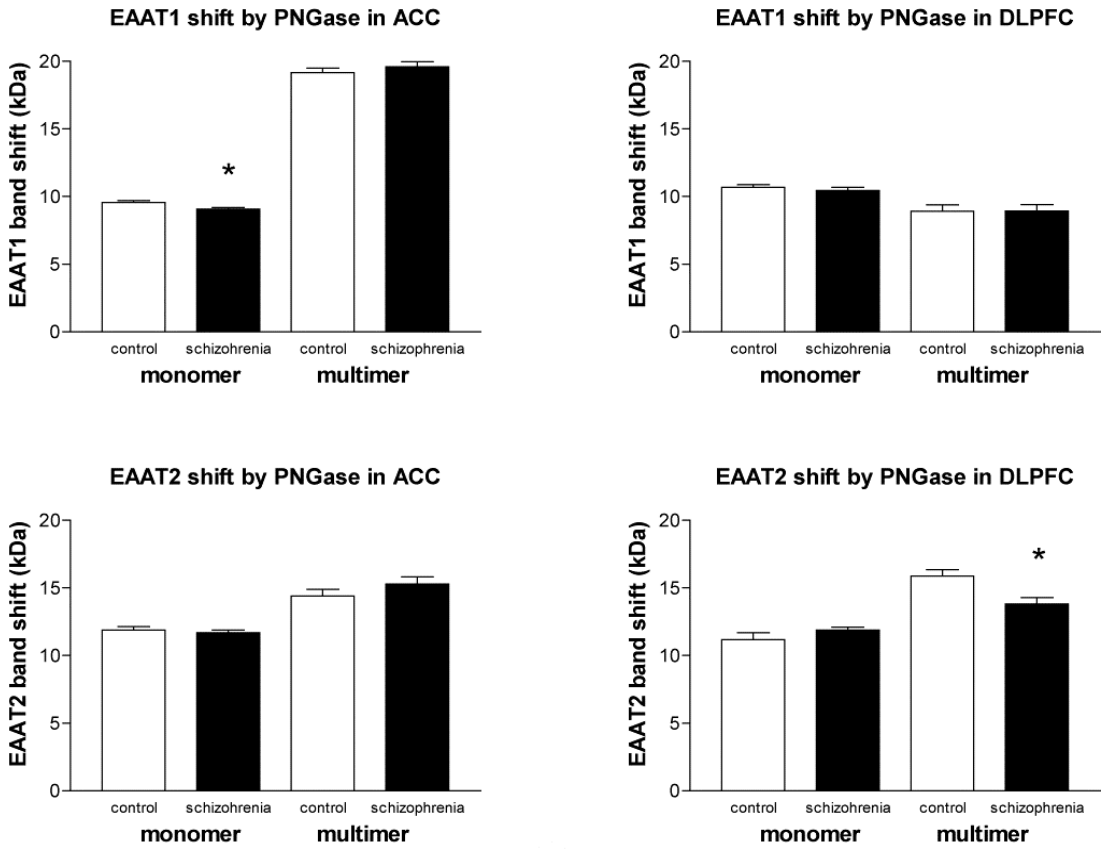

Figure 3.3: Molecular mass shifts of EAAT1 and EAAT2 in schizophrenia and a comparison group following enzymatic deglycosylation with PNGase F. Data expressed as means +/- standard error of the mean. Asterisks indicate a significant difference between schizophrenia and comparison subjects ($p < 0.05$). Abbreviations: kDa (kilodaltons), anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), excitatory amino acid transporter (EAAT), endoglycosidase H (EndoH), peptide-N^4-(N-acetyl-beta-glucosaminyl) asparagine amidase F (PNGase F).

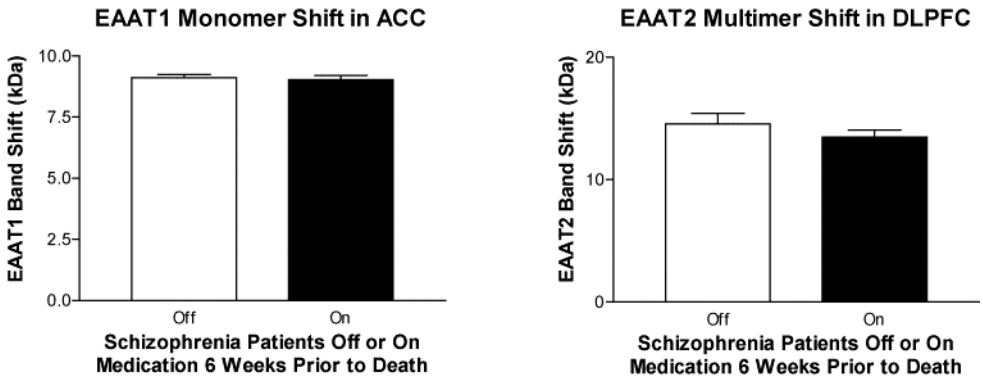

Figure 3.4: Molecular mass shifts of EAAT1 and EAAT2 following enzymatic deglycosylation with PNGase F in patients with schizophrenia off or on medication 6 weeks prior to death. Data expressed as means +/- standard error of the mean. Abbreviations: kDa (kilodaltons), anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), excitatory amino acid transporter (EAAT).

3.5 Discussion

Previous work has demonstrated altered glycosylation of several proteins in schizophrenia. Increases in the plasma activity of the glycosylating enzyme alpha 2,6 sialyltransferase and in serum levels of alpha 2 and beta globulins have been found in schizophrenia [295, 296]. In addition, a decrease in the number of cells expressing polysialated neural cell adhesion molecule (NCAM) was detected in the hilus of the hippocampus without an overall change in NCAM expression [297]. We detected two additional proteins that have altered glycosylation in schizophrenia, suggesting that deficits in glycosylation may have a role in the pathophysiology of this illness.

We found that EAAT1 and EAAT2, but not EAAT3, are N-glycosylated in the human brain. Given that we found changes in the glial (EAAT1 and EAAT2) but not neuronal (EAAT3) transporters, it is possible that the mechanisms for glycosylation deficits in schizophrenia are glia-specific. Our EAAT3 finding is surprising, given that in the rodent EAAT3 (EAAC1) is glycosylated [213], and that another study demonstrated deglycosylation of EAAT3 in an immunoprecipitated fraction from human brain synaptosomes [285]. These divergent findings might be due to the type of deglycosylating enzyme used, or that immunoprecipitating EAAT3 from synaptosomes significantly enriched EAAT3, allowing detection of subtle changes that might not be apparent in tissue homogenate. It may be that a small subset of EAAT3 is glycosylated in human brain, while the majority of EAAT3 is unglycosylated, and thus not detectable with our approach. Alternatively, the absence of a molecular mass shift in EAAT3 could be due to a loss of epitopes associated with glycosylation for the EAAT3 antibodies following enzymatic digestion [234, 298]. For example, an antibody might bind to bind to a glycosylated residue of EAAT3 and lose antigenicity if that glycan is cleaved. However, we feel this is unlikely because we did not detect a shift using any of the three EAAT3 antibodies raised to different epitopes and one of these epitopes does not contain any putative N-glycosylation sites.

We found that EAAT1 has fewer sugar residues added by N-glycosylation in schizophrenia. The change in molecular mass shift of EAAT1 was relatively small (~5%) between schizophrenia and the comparison group.

It is difficult to determine if such a small change in glycosylation is of physiological significance. It is possible that because the change is small, it may be a type I statistical error due to a high coefficient of variation. However, if this change is real, preclinical data suggests that a small change in glycosylation can have a strong effect on glutamate uptake. For example, activation of astrocytes with ciliary neurotrophic factor (CNTF) results in a small increase in glycosylation of GLAST (~8% shift), with increased localization of GLAST to lipid rafts at the cell surface and increased glutamate reuptake, resulting in a 67% decrease in extracellular glutamate levels upon quinolinate evoked glutamate release [284]. This suggests that the decrease in glycosylation of EAAT1 that we detected may significantly impact glutamate reuptake.

We also found evidence for less glycosylation of the other astrocytic transporter, EAAT2, in schizophrenia. The difference in molecular mass shift between groups was larger for EAAT2 (~13%) than for EAAT1, although the functional effect of this larger shift is not known. Less glycosylation of EAAT2 might reflect decreased glutamate reuptake, since altered glycosylation of EAAT2 is associated with ER retention and decreased plasma membrane expression, and trafficking of EAAT2 to the plasma membrane is necessary for EAAT2 mediated glutamate reuptake [216, 230].

One potential mechanism for the decreases in glycosylation could be altered splice variant expression. EAAT1 and EAAT2 can both be alternatively spliced to skip exon 9, which contains an ER exit motif. These

splice variants are retained in the ER, and cause ER retention of any full length variants with which they dimerize [230]. The transporters that are retained in the ER are less glycosylated than transporters that are not retained [230]. Thus, it is possible that the decreases in glycosylation that we found are due to increased expression of these exon skipping variants. In fact, we found increases in the exon 9 skipping variant of EAAT2, which could explain the decrease we found in EAAT2 glycosylation (Chapter 4).

It is also possible that the changes in glycosylation we found are due to changes in the levels or activity of the glycosyl transferases that attach glycans to the proteins. Few studies have investigated glycosyl transferases in schizophrenia. An increase has been detected in the plasma activity of the glycosylating enzyme alpha 2,6 sialyltransferase [295]. An increase in activity of a glycosyl transferase is unlikely to explain a decrease in glycosylation, but it is possible that other glycosyl transferases are decreased in schizophrenia. Since most of the patients with schizophrenia were treated with antipsychotic medications, the reductions we found in glycosylation could be due to a medication effect. However, we did not find any effects of medication on molecular mass shift when comparing patients on medication 6 weeks prior to death to patients off medication at least 6 weeks prior to death.

The reductions in EAAT1 and EAAT2 glycosylation suggest decreased plasma membrane expression of these transporters. Altered localization of EAAT1 and EAAT2, combined with the decreased EAAT1 protein expression we previously described [286], suggest that there is decreased perisynaptic

glutamate reuptake into astrocytes in schizophrenia. The glutamate transporters are important for maintaining low synaptic glutamate levels by buffering and transporting synaptic glutamate [299, 300]. Diminished perisynaptic reuptake and buffering may lead to glutamate spillover and loss of input specificity [287, 288]. Our data suggesting decreased glutamate reuptake support a hypothesis of increased synaptic glutamate levels and/or glutamate spillover in schizophrenia. Consistent with this hypothesis, EAAT1 deficient mice exhibit endophenotypes including self-neglect, social withdrawal, and impaired learning, suggesting that schizophrenia-associated rodent endophenotypes can be modeled by disruption of EAAT1-mediated glutamate reuptake [20]. This hypothesis is further supported by a report of a subject with schizophrenia who has a partial deletion of the EAAT1 gene [176]. Finally, our data suggest that reducing synaptic glutamate could be a useful strategy in the treatment of schizophrenia. One study using an mGluR2/3 agonist, which decreases glutamate release, had antipsychotic effects in schizophrenia [177]. Taken together, these data support a role for diminished glutamate reuptake in the pathophysiology of schizophrenia.

Chapter 4

Abnormal Expression of the Exon 9 Skipping EAAT2 Splice Variant in Schizophrenia

4.1 Abstract

Alternative splicing of the EAATs provides one mechanism by which EAAT function can be regulated. Given that abnormal EAAT expression and evidence for dysregulation of glutamatergic neurotransmission have been found in schizophrenia, we hypothesized that expression of EAAT splice variants may be abnormal in schizophrenia. To test this hypothesis, we measured transcript expression of five EAAT isoforms: EAAT1, EAAT1Δ9, EAAT2, EAAT2B and EAAT2Δ9 by QPCR in postmortem tissue from the prefrontal cortex of patients with schizophrenia and comparison subjects. We found increased expression of EAAT2Δ9 in schizophrenia. Our data suggest abnormal glutamate transporter splicing in schizophrenia, consistent with decreased capacity for glutamate reuptake.

4.2 Introduction

The EAATs are known to be alternatively spliced, which is associated with their functional regulation [183, 218-224]. EAAT1 can be alternatively spliced as an exon 3 skipping [210, 224, 227] or an exon 9 skipping variant [228]. The exon 3 skipping variant, also known as EAAT1a, is thought to have an opposite orientation in the cell membrane to regular EAAT1 and to be unglycosylated [210]. The exon 9 skipping variant, also known as EAAT1Δ9 or EAAT1b is retained in the ER, is likely unglycosylated, and appears to be a non-functioning transporter exerting a dominant negative effect on the native transporter [229].

Several splice variants have also been described for EAAT2. Exon skipping variants of EAAT2 include an exon 7 skipping variant [219], an exon 9 skipping variant [219, 221, 222] and a variant containing a partial deletion of exons 6 and 7 [219]. Like the exon 9 skipping form of EAAT1, the exon 9 skipping form of EAAT2 (EAAT2Δ9) is also retained in the ER [230]. There are also several intron retention variants of EAAT2. For example, EAAT2b is an intron 10-11 retention variant that retains the ability to transport glutamate *in vitro*, but unlike EAAT2, is expressed in neurons and glia, and is expressed in the cytoplasm rather than the plasma membrane [182, 183].

EAAT function must be tightly regulated in order to ensure proper glutamatergic neurotransmission. We previously demonstrated abnormalities in EAAT expression in schizophrenia[286]. Alterations in splicing could also

cause disturbances in EAAT function. Abnormalities in EAAT splicing have been demonstrated in mood disorders, anxiety disorders, Alzheimer's disease, epilepsy, hypoxia/ischemia, Huntington's disease, brain injury, glaucoma, HIV-associated dementia, addiction, ALS, and schizophrenia[225]. Additionally, changes in splicing of several other glutamatergic molecules including AMPA and NMDA receptor subunits have previously been shown in schizophrenia[33, 104, 107]. In this study, we measured expression of EAAT1 and EAAT2 splice variants in postmortem brain from patients with schizophrenia and a comparison group. We hypothesized that there is abnormal splice variant expression of EAAT1 and EAAT2 in schizophrenia.

4.3 Methods

4.3.1 Subjects

Subjects from the Mount Sinai Medical Center Schizophrenia Brain Bank were studied (Table 4.1), including 25 individuals diagnosed with schizophrenia and 25 comparison subjects. Subjects were diagnosed with schizophrenia if the presence of symptoms was documented before age 40, the medical records contained evidence of psychotic symptoms and at least 10 years of psychiatric hospitalization with diagnosis of schizophrenia, and a DSM-III-R diagnosis of schizophrenia was agreed upon by two experienced clinicians. Diagnostic groups did not significantly differ for age, sex, postmortem interval, and tissue pH. Upon neuropathological examination, no evidence of Alzheimer's or other neurodegenerative disease was found. The

brain banking procedures were approved by the Mount Sinai School of Medicine Institutional Review Board.

Table 4.1: Subject Characteristics for Splice Variant Studies

	Comparison Group	Schizophrenia
N	25	25
Sex	12 m / 13 f	16 m / 9 f
Tissue pH	6.6 ± 0.3	6.5 ± 0.2
PMI (hours)	8.6 ± 7.0	17.1 ± 10.0
Age (years)	76 ± 11	75 ± 13
On / Off Rx	0 / 25	18 / 7
Values presented as mean ± standard deviation Abbreviations: male (m), female (f), antipsychotic medication (Rx), postmortem interval (PMI).		

4.3.2 RNA preparation

Brains were obtained after autopsy and one hemisphere was cut coronally into ~0.8 - 1 cm^3 slabs and flash frozen. Gray matter was dissected from ACC (n = 68) and DLPFC (n = 66). ACC was dissected at the level of the genu of the corpus callosum, from the dorsal surface of the corpus callosum extending 12–15 mm dorsally and extending 12–15 mm laterally from the midline. DLPFC was dissected corresponding to Brodmann area 46 measuring approximately 1.5 cm along the cortical surface. Approximately 1 cm^3 of frozen tissue was pulverized in liquid nitrogen. The tissue was then homogenized in Buffer RLT Plus (AllPrep DNA/RNA Mini Kit (Qiagen)) by passing it 10 times through a 20-guage needle and RNA was extracted with

the AllPrep DNA/RNA Mini Kit (Qiagen). RNA concentration was determined using UV spectrophotometry at 260nm.

4.3.2 Reverse Transcription

Equivalent amounts of RNA (1 μg) were treated with DNase I at 37°C for 30 minutes (1 unit DNase per μg RNA; Promega, Madison, WI), DNase was inactivated at 65°C for 15 minutes, and RNA was reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

4.3.3 Primers

The following primers were used for QPCR:

β-actin forward: AGTACTCCGTGTGGATCGGC,

β-actin reverse: GCTGATCCACATCTGCTGGA,

18S forward: CGCCGCTAGAGGTGAAATTC,

18S reverse: TTGGCAAATGCTTTCGCTC,

cyclophilin forward: CTCCTTTGAGCTGTTTGCAG,

cyclophilin reverse: CACCACATGCTTGCCATCC,

EAAT2 forward: GGGCACCGCTTCCAGTG,

EAAT2 reverse: ATACTGGCCGCGCCG,

EAAT2Δ9 forward: GGGCACCGCTTCCAGTG,

EAAT2Δ9 reverse: GACTGAAGTTCTCATCCTGTCCCTT,

EAAT2b forward: GGACAGGATGAGAACTTCAGTCAA,

EAAT2b reverse: ACAAGTCTCGATATCCATGAATGG,

EAAT1 forward: CGAAGCCATCATGAGACTGGTA,

EAAT1 reverse: TCCCAGCAATCAGGAAGAGAA,

EAAT1Δ9 forward: TGCCCTCTATGAGGCTTTGG,

EAAT1Δ9 reverse: GTCCGGAGGCGATCCCT.

4.3.4 QPCR

All amplification reactions were performed in duplicate using 96-well optical reaction plates (Stratagene) on a Stratagene detection system. For each reaction, 3 ul of cDNA (1:3 diluted) was placed in a 20 ul reaction containing 10 ul of SYBR Green PCR Master Mix (Applied Biosystems) and 10 pmol of each primer (Invitrogen). Reactions were performed with an initial ramp time of 3 minutes at 95°C, and 50-60 subsequent cycles of 15 seconds at 95°C and 1minute at the annealing temperature. The annealing temperature was 63^{o}C for EAAT2Δ9 and was 59^{o}C for all other primer sets. 60 cycles were used for EAAT2B and EAAT2Δ9, and 50 cycles were used for all other primer sets. For negative controls for the QPCR reactions, cDNA was omitted. Relative concentrations of the transcripts of interest were calculated with comparison to a standard curve made with dilutions of cDNA from a pooled sampling of all the subjects. Values for the transcripts of interest were normalized to the geometric mean of 18s, actin, and cyclophilin values for the same samples.

4.3.5 Statistical Analysis

All statistical analyses were performed using Statistica (StatSoft, Tulsa, Oklahoma). Outliers more than 6 standard deviations from the mean were excluded. Correlation analysis was performed to determine associations between transcript expression and age, PMI, and pH. We analyzed transcript expression using analysis of variance (ANOVA) or analysis of covariance (ANCOVA) as appropriate. To test for possible medication effects, patients with schizophrenia off antipsychotic medication for at least 6 weeks prior to death were compared to patients on antipsychotic medication within 6 weeks of death in a post hoc analysis.

4.4 Results

Using QPCR, transcript expression of EAAT1, EAAT2, EAAT1Δ9, EAAT2Δ9 and EAAT2B was measured. There were no associations between EAAT1, EAAT2, EAAT1Δ9, or EAAT2B transcript expression and age, pH, or PMI in the ACC or DLPFC. Correlation analysis revealed an association between EAAT2Δ9 transcript expression and age in ACC ($R= 0.31$, $p < 0.05$) but not DLPFC.

EAAT2Δ9 transcript expression was increased in schizophrenia in ACC ($F(1,42) = 6.03$, $p < 0.05$), and DLPFC ($F(1, 43) =4.65$, $p < 0.05$) (Figure 4.1). There was no effect of medication status in the subjects with schizophrenia on EAAT2Δ9 expression. There were no significant

differences between groups for EAAT1, EAAT2, EAAT1Δ9, or EAAT2B (Figure 4.1).

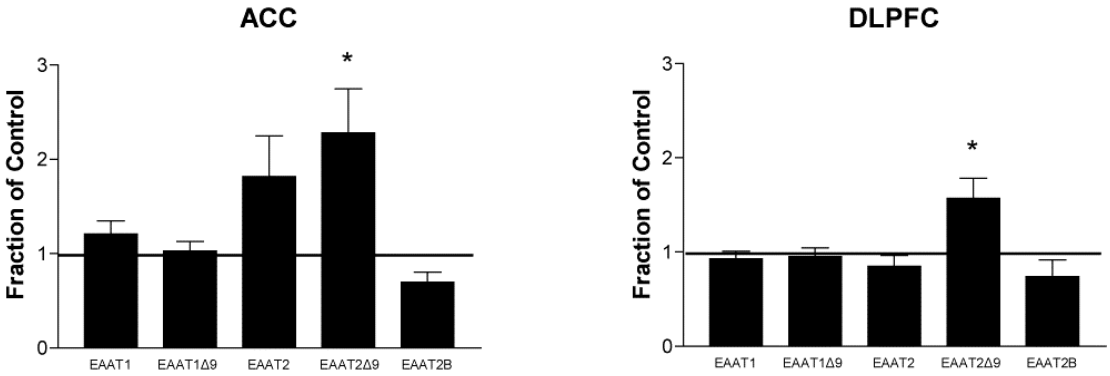

Figure 4.1: EAAT splice variant transcript expression. QPCR analysis of EAAT1, EAAT1Δ9, EAAT2, EAAT2Δ9, and EAAT2B splice variant mRNA expression in the anterior cingulate cortex and dorsolateral prefrontal cortex from subjects with schizophrenia (n = 25) and a comparison group (n = 25). Expression is normalized to the geometric mean of 18s, actin, and cyclophilin. Data expressed as fold change of control +/- standard error of the mean. Asterisks indicate a significant difference between control and schizophrenia ($p < 0.05$). Abbreviations: anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), excitatory amino acid transporter (EAAT), EAAT1 exon 9 skipping (EAAT1Δ9), EAAT2 exon 9 skipping (EAAT2Δ9).

4.5 Discussion

In this study, we found increases in EAAT2Δ9 mRNA expression, but no changes in expression of EAAT1, EAAT1Δ9, EAAT2, or EAAT2B in schizophrenia brain. We previously studied expression of transcripts for EAATs in the ACC and DLPFC using *in situ* hybridization in a different set of subjects from the same brain bank[286]. We found no changes in EAAT2 transcript expression in this smaller set[286]. Another study using subjects from the same brain bank also found no changes in EAAT2 transcript expression using QPCR[180]. Other studies of EAAT2 expression in prefrontal cortex in schizophrenia have found a decreased ratio of EAAT2 to mGluR using *in situ* hybridization[178], while another group found increased EAAT2 expression using QPCR[179]. The balance of these data suggests that native EAAT2 transcripts are unchanged in schizophrenia.

In our previous study, we also measured transcript expression of EAAT1 by *in situ* hybridization[286]. While we found no changes in EAAT1 in the present study using QPCR, with situ hybridization we found an increase in EAAT1 transcript expression in ACC [286]. The difference in EAAT1 findings may be due to differences in the set of subjects used, or because the ACC samples used in the current work were dissected from a portion of the cingulate that is located more caudally than the samples from our previous study[286]. EAAT1 transcript expression in the DLPFC of subjects from the same brain bank was also measured by QPCR, and no difference between diagnostic groups was found[180].

Previously, only one other study has examined EAAT2 splice variants in schizophrenia[180]. EAAT2B, an intron retaining form of EAAT2, was found to be unchanged[180]. We did not find changes in EAAT2B, suggesting that the expression of this splice variant is not affected in schizophrenia.

EAAT2 is responsible for the majority of glutamate reuptake in the forebrain, and is tightly regulated to prevent perturbations in glutamate transmission[51]. EAAT2Δ9 exerts a dominant negative effect on EAAT2 function by heterodimerizing with full length EAAT2 and preventing it from exiting the ER. Thus, increased EAAT2Δ9 expression may lead to decreased glutamate buffering and reuptake, resulting in increased synaptic glutamate levels. Decreases in perisynaptic EAAT expression could also lead to glutamate spillover into adjacent synapses and loss of input specificity[287, 288]. The increase we detected in EAAT2Δ9 is consistent with decreases in EAAT2 glycosylation we previously detected because disruption of exon 9 results in immaturely glycosylated EAAT2[230].

Abnormal expression of splice variants affecting ER trafficking have previously been demonstrated in schizophrenia. One group found an increase in the ratio of the flip : flop isoforms of the GluR2 AMPA subunit in hippocampus in schizophrenia[107]. Because the flop variant contains an ER retention sequence and the flip variant does not, these data suggest AMPA receptors may be forward trafficked too quickly in schizophrenia. Another study found increased expression of the alternatively spliced NR1 NMDA receptor subunit NR1C2' in ACC in schizophrenia[104]. This variant confers

accelerated trafficking of the NMDA receptor through the ER. Decreased expression of exon 22 containing splice variants of the NR1 NMDA receptor subunit was found in the thalamus in schizophrenia[33]. This finding also points to forward trafficking of the NMDA receptor because the long C-terminal tails of these variants block release from the ER. Taken together with the current study, these data suggest alterations in splice variants involved in ER retention and trafficking may play an important role in the pathophysiology of schizophrenia.

Since most of the patients with schizophrenia were treated with antipsychotic medications, the increases we found in EAAT2Δ9 expression could be due to a medication effect. However, we did not find any effects of medication on EAAT2Δ9 expression when comparing patients on medication 6 weeks prior to death to patients off medication at least 6 weeks prior to death.

Expression of the exon 9 skipping variant of EAAT2, EAAT2Δ9, is increased in the ACC and DLPFC of elderly patients with schizophrenia. We suggest that this increase in a dominant negative splice variant could lead to decreased glutamate buffering and reuptake and increased synaptic glutamate levels in schizophrenia.

Chapter 5

Discussion

5.1 Summary of findings

The objective of this dissertation was to determine how the EAATs are expressed and potentially dysregulated in schizophrenia. EAATs may be regulated at the level of transcript expression, alternative splicing, protein expression, post-translational modification, and protein-protein interactions. We attempted to determine whether there were changes in EAAT expression or regulation at each of these levels. First, we measured transcript expression of the EAATs and four EAAT interacting proteins using *in situ* hybridization. We found increased expression of transcripts for EAAT1, EAAT3, the EAAT4 interacting molecule KIAA0302, and the EAAT1-EAAT4 interacting molecule JWA. Then, we measured protein expression of the EAATs and two EAAT interacting proteins through Western blot analysis. We found increased protein expression of EAAT3, the EAAT2-EAAT4 interacting protein GPS1, and KIAA0302, and decreased protein expression of EAAT1. Next, we studied glycosylation of the EAATs using enzymatic deglycosylation.

We found decreased glycosylation of EAAT1 and EAAT2. Finally, we examined splice variant expression of the EAATs using QPCR. We found increased transcript expression of the dominant negative splice variant of EAAT2 EAAT2Δ9. Below is a summary of all changes we found in schizophrenia compared to comparison groups (Table 5.1).

Technique	Molecule	Region	
		ACC	DLPFC
ISH	EAAT1	↑	
	EAAT3	↑	
	KIAA	↑	
	JWA	↑	
Westerns	EAAT1		↓
	EAAT3	↑	
	GPS1	↑	
	KIAA		↑
Glycosylation	EAAT1	↓	
	EAAT2		↓
QPCR	EAAT2Δ9	↑	↑

Table 5.1: Summary of changes EAAT expression and regulation in schizophrenia. Arrows indicate changes in schizophrenia relative to comparison groups. Abbreviations: in situ hybridization (ISH), quantitative polymerase chain reaction (QPCR), anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), excitatory amino acid transporter (EAAT), KIAA0302 (KIAA), g-protein pathway suppressor 1 (GPS1), excitatory amino acid transporter 2 exon 9 skipping (EAAT2Δ9).

5.1.1 EAAT protein expression

Using Western blot analysis, we measured protein expression of EAAT1, EAAT2, and EAAT3 in prefrontal cortex. We found decreased protein levels of EAAT1, no changes in EAAT2, and increased protein levels of EAAT3. These data are consistent with decreased glutamate uptake into glia, which comprises the major component of synaptic glutamate buffering and reuptake, and increased uptake into neurons which comprise a smaller component of glutamate buffering and reuptake.

5.1.2 Alternative splicing of EAATs

Using QPCR, we found increased transcript expression of EAAT2Δ9 in two regions of cortex. This exon 9 skipping variant is retained in the ER and also causes retention of full length EAAT2 by heterodimerization[230]. Removal of exon 9 also results in decreased glycosylation of EAAT2[230]. These data suggest that EAAT2 exit from the ER may be decreased in schizophrenia. Based on these data, we would also predict less glycosylation of EAAT2 in schizophrenia.

5.1.3 EAAT Glycosylation

Using enzymatic deglycosylation, we found less N-linked glycosylation of EAAT1 and EAAT2 in schizophrenia. Because proper glycosylation is necessary for exit from the ER, these data point to ER trafficking deficits of these transporters.

5.1.4 EAAT Interacting Proteins

Using *in situ* hybridization, we found increased transcript expression of JWA. JWA downregulates EAAT1, EAAT2, EAAT3, and EAAT4 function by interacting with the exon 9 ER exit motif shared by these transporters[301]. Using Western blot analysis, we found increased expression of GPS1. GPS1 downregulates EAAT2 function by interacting with the exon 9 ER exit motif of EAAT2[50], and likely downregulates the function of EAAT3 and EAAT4 through the same mechanism[50]. These data also suggest that EAAT exit from the ER may be decreased in schizophrenia.

5.2 Schizophrenia as a disorder of ER retention

We have shown in schizophrenia decreased EAAT protein levels, increased expression of splice variants associated with ER retention, decreased levels of glycosylation, and increased levels of molecules that promote ER retention. Put together, these data demonstrate several levels of support for a hypothesis that the astrocytic EAATs are expressed in lower levels in the plasma membrane due to relative retention in the ER. This is illustrated in Figure 5.1.

Previous studies in this and other labs point to abnormalities in ER trafficking of glutamatergic molecules. For example, abnormal expression of splice variants affecting ER trafficking have previously been demonstrated in schizophrenia. One group found an increase in the ratio of the flip: flop

isoforms of the GluR2 AMPA subunit in hippocampus in schizophrenia[107]. Because the flop variant contains an ER retention sequence and the flip variant does not, these data suggest AMPA receptors may be processed too quickly in schizophrenia. Another study found increased expression of the alternatively spliced NR1 NMDA receptor subunit NR1C2' in ACC in schizophrenia[104]. This variant confers accelerated trafficking of the NMDA receptor through the ER. Decreased expression of exon 22 containing splice variants of the NR1 NMDA receptor subunit was found in the thalamus in schizophrenia[33]. This finding also points to accelerated forward trafficking of the NMDA receptor because the long C-terminal tails of these variants block release from the ER. In addition to these splice variant studies, our group has detected a decrease in the ratio of Endo H sensitive to Endo H insensitive GluR2 in DLPFC[302]. This finding suggests that GluR2 is processed more quickly in schizophrenia because glycoproteins become Endo H insensitive once they have been trafficked through the ER. Taken together, these ER trafficking implications point to faster forward trafficking of receptors and slower trafficking of transporters. This would lead to increased synaptic glutamate accompanied by increased number of receptors to which glutamate may bind.

This idea that regulation of protein trafficking through the ER may be disrupted in schizophrenia is consistent with the notion that oxidative stress may play a role in the pathophysiology of schizophrenia. Oxidative stress has long been implicated in schizophrenia[303]. Dysregulation of protein processing

in the ER could be primary or secondary to increased oxidative stress. On the one hand, the oxidative environment in the ER must be tightly regulated in order to allow for disulfide bond formation and ensure proper protein folding. Increased presence of free radicals in the ER could offset this balance and put additional stress on the machinery required to ensure proper protein folding and modification. On the other hand, disulfide bond formation in the ER leads to the formation of reactive oxygen species[304]. Thus, problems with ER functioning could lead to creation of more reactive oxygen species and additional oxidative stress in the cell.

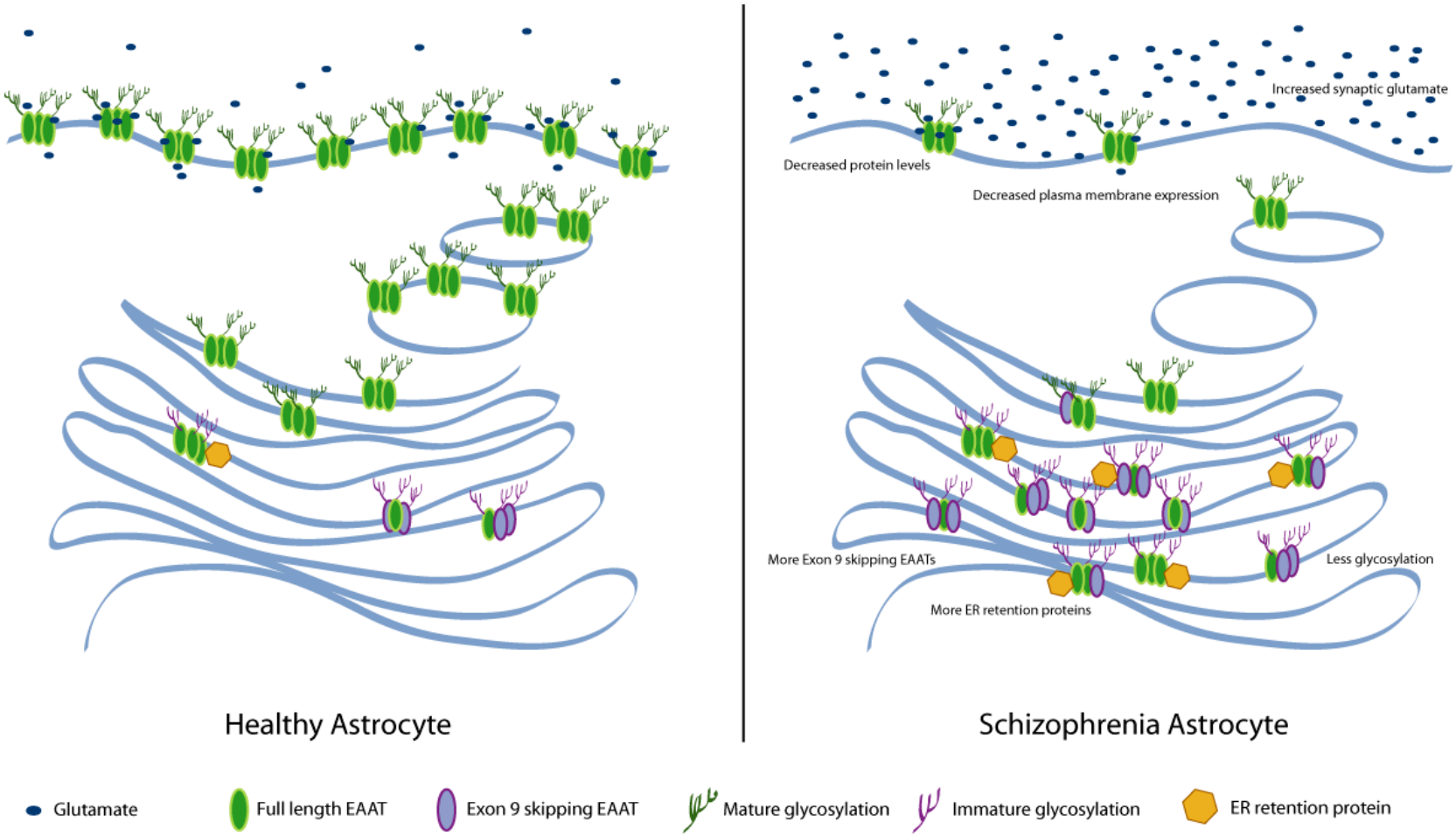

Figure 5.1: Schematic of EAAT abnormalities in schizophrenia.

5.3 Potential effects of glutamate transporter abnormalities on neurotransmission

I found decreased EAAT1 expression, and I found several factors that indicate decreased localization of EAATs at the synapse including decreased glycosylation, increased expression of an ER retention variant, and increased expression of molecules that retain EAATs in the ER. These data suggest that there are fewer EAATs expressed at the cell surface, and thus a decrease in the capacity for glutamate buffering and transport. This decreased capacity would likely lead to increased synaptic glutamate levels.

Increased synaptic glutamate can have a number of effects. First, it could lead to increased activation of receptors at postsynaptic sites. Second, glutamate that is not removed from the synapse could also spillover into perisynaptic regions and activate perisynaptic receptors. Finally, extrasynaptic glutamate may also spillover into adjacent synapses and activate receptors in those synapses, leading to a loss of input specificity[299]. LTP is associated with a translocation of the EAAT3 from the cytosol to the plasma membrane[234], and inhibition of glutamate transporters is sufficient to reduce input specificity of LTP[287].

5.3.1 Literature support for this hypothesis

Based on my data suggesting decreased glutamate reuptake, I propose a hypothesis of increased synaptic glutamate levels and/or glutamate spillover in schizophrenia. This hypothesis is supported by the fact that a

subject with schizophrenia has a partial deletion of the EAAT1 gene [176]. Also consistent with this hypothesis, EAAT1 deficient mice exhibit schizophrenia-like endophenotypes including self-neglect, social withdrawal, and impaired learning[20]. In addition, increased prefrontal cortical glutamate levels in with schizophrenia has been shown through spectroscopy[305, 306]. Further, glutaminase deficient mice, which cannot efficiently convert glutamine into glutamate, are resistant to pharmacological models of schizophrenia[22]. A recent study has indicated that a member of the family of phosphatidylinositol-phosphate kinases phosphatidylinositol-4-phosphate 5-kinase II alpha (PIP5K2A), results in increased EAAT3 mediated glutamate reuptake when coexpressed in oocytes, whereas a mutant version that has been associated with schizophrenia, ^{N251S}PIP5K2A, downregulates EAAT3 mediated glutamate uptake[307]. Additionally, GFAP, a protein that helps increase EAAT1 function by anchoring it at the plasma membrane[308] is decreased in ACC in schizophrenia[189].

Finally, our data suggest that reducing synaptic glutamate could be a useful strategy in the treatment of schizophrenia. Cognitive and behavioral abnormalities caused by NMDA antagonist administration in rodents can be rescued by glutamate release inhibitors such as lamotrigine[309] and mGluR 2 agonists[310]. One study using an mGluR2/3 agonist, which decreases glutamate release, had antipsychotic effects in schizophrenia [177]. Taken together, these data support a role for diminished glutamate reuptake in the pathophysiology of schizophrenia.

5.4 Primary vs. compensatory changes

I propose that any number of changes that lead to increased synaptic glutamate contribute to the pathophysiology of schizophrenia. This is supported by the fact that a patient with schizophrenia was identified with a genetic disruption of the EAAT1 gene SLC1A3[176]. It is unlikely that there is any one primary deficit that causes schizophrenia, as evidenced by the fact that many genes have been identified as risk factors, each contributing a very small percentage of risk. However, many factors can lead to increased synaptic glutamate levels.

Perhaps the reason why NMDA receptor blockade models many aspects of schizophrenia is because it can result in increased synaptic glutamate release[311]. Administration of PCP also results in increased EAAT expression[312] which may be compensatory to the increase in glutamate release. Therefore, if changes in EAAT expression were secondary to changes in synaptic glutamate levels, one might expect to find increased EAAT expression. However, I found decreased EAAT1 expression, increased EAAT3 expression, and other changes that may lead to decreased surface expression of the astrocytic EAATs.

This hypothesis is further supported by the fact that the drug memantine, which is an uncompetitive NMDA receptor antagonist used to treat Alzheimer's disease, does not appear to affect schizophrenia symptoms[313]. If schizophrenia symptoms were induced purely through NMDA receptor signaling and not through increased glutamate release, one would

expect memantine to induce these symptoms as well. This suggests that the noncompetitive NMDA receptor antagonists might exert their psychomimetic effects through increased glutamate release.

Alternatively, the changes I found could be secondary to alterations in dopamanergic signaling. The original hypothesis behind the pathogenesis of schizophrenia is that it is a disorder of too much dopamine. Monoaminergic signaling cascades can converge to affect NMDA and AMPA receptor functioning[314]. Dopaminergic neurons act to modulate glutamatergic function by terminating on presynaptic glutamate terminals and inhibiting presynaptic release[315, 316]. Thus, increased dopaminergic tone could act to cause decreased glutamatergic signalling. Therefore, glutamate reuptake may be downregulated in schizophrenia to compensate for decreased gluatamate release.

We hypothesize that the changes in EAAT3 expression are secondary to decreases in plasma membrane activity of EAAT1 and EAAT2. Decreased plasma membrane expression of EAAT1 and EAAT2 may cause decreased glutamate reuptake in glial cells, leading to increased basal synaptic glutamate levels and synaptic spillover. EAAT3 is largely localized in perisynaptic regions and contributes to clearance of glutamate spillover [272, 273]. Thus, EAAT3 expression may be increased in neurons to compensate for these changes. Consistent with this notion, EAAT3 protein is localized to regulatable cytosolic pools that may be rapidly mobilized to the plasma membrane, suggesting a biological process that is highly responsive to

changes in glutamate levels [164]. It is also possible that EAAT3 expression is compensatory to increased glutamatergic signaling through a different mechanism. Perhaps the increased EAAT3 expression is localized to presynaptic GABA terminals. This would supply more glutamate to GABAergic neurons for GABA synthesis, allowing those cells to inhibit overactive pyramidal neurons.

Alternatively, the changes in EAAT3 expression may have more to do with its ability to transport cysteine than its ability to transport glutamate. In animal models, knockdown of EAAC1 does not result in changes in extracellular glutamate, but it does lead to 20-40% decreases in cysteine and glutathione, as well as increased oxidant levels and increased vulnerability to oxidative stress[317]. Transport of cysteine by EAAT3 is the rate-limiting step of glutathione synthesis in neurons[317]. Glutathione is the major free-radical scavenger present in neurons and plays an important role in maintaining oxidative balance. Many posit schizophrenia to be a disorder of increased oxidative stress[303]. Increases in EAAT3 expression may be compensatory in order to increase the amount of cysteine available in neurons to synthesize glutathione and combat increases in oxidative stress.

5.5 Region specific changes

It is important to note that the changes we have described are region specific. With the exception of increased transcript of EAAT2Δ9, all changes were detected in either DLPFC or ACC, but not both. Even within one of

these areas, there can be considerable heterogeneity. For example, we found differences in EAAT1 transcript expression in the ACC between the *in situ* hybridization and QPCR studies. This may be attributable to the fact that the ACC samples used in the QPCR study were dissected from a portion of the cingulate that is located more caudally than the samples from the *in situ* hybridization study. Therefore, it is important to consider the areas being examined when comparing different studies, and to understand the capacity for heterogeneity even within a defined area such as prefrontal cortex

In this dissertation, we described abnormalities in EAAT expression and regulation in ACC and DLPFC, but there are many brain areas that are also affected in schizophrenia. Abnormal mRNA expression of the EAATs and EAAT interacting proteins has also been described in other regions of the brain in schizophrenia. EAAT3 transcript was decreased in striatum in schizophrenia, using brain samples from another brain collection[173]. Using samples from the same brain collection used in this dissertation, several changes in EAAT and EAAT interacting molecules have also been found. EAAT1 and EAAT2 transcript expression was increased in thalamus[36], and JWA and KIAA transcripts were increased in thalamus[34]. A decrease in EAAT2 mRNA was described in the parahippocampal gyrus[184]. Thus, it appears that abnormalities in EAAT expression are not unique to the prefrontal cortex.

5.6 Cell specific changes

The cellular expression profiles of the EAATs have been well described in the literature. For example, EAAT1 and EAAT2 are generally localized to astrocytes and EAAT3 is generally localized to neurons[137-140]. However, several exceptions to these expression profiles have been demonstrated. For example, EAAT1 expression has been demonstrated in other types of glia including oligodendrocytes and microglia[283]. EAAT2 mRNA has been detected in Bergmann glia and some neurons[318]. Some expression of EAAT3 has been identified in astrocytes[158].

Splice variants of the EAATs are often expressed in different cell types than their full-length counterparts[154, 182, 183]. Therefore, changes in splice variant expression can result in altered localization of the EAATs to different cell types. Increased expression of EAAT2b, and decreased expression of full length EAAT2, resulting in reduced astrocytic expression and increased neuronal expression has been demonstrated in ALS[183]. One group hypothesizes that mRNA for the "glial" EAATs is constitutively expressed in neurons, but that they are not translated into protein unless the cells are stressed[224]. EAAT1 was strongly expressed in cortical pyramidal neurons, co-localizing with tau in patients with Alzheimer's dementia[144]. It is unknown whether the EAATs are abnormally expressed in different cell types in schizophrenia.

5.7 Transcript vs. protein

One phenomenon that we observed is that changes in transcript expression do not necessarily correspond to similar changes in protein expression. For example, we found increased EAAT1 mRNA expression in ACC cortex with no changes in protein expression, and decreased EAAT1 protein expression in DLPFC with no changes in mRNA expression. No change in protein expression with increased mRNA expression may be due to a number of inter-related causes including improper translation and folding, abnormal post-translational modifications, or increased protein turnover.

In some cases, differences in transcript and protein expression can be attributable to cell type. For example, a protein that is expressed postsynaptically may exist in a projection that is in another region of the brain from the cell body where the transcripts are expressed. Therefore, a change in protein expression in the DLPFC could be the result of a change in mRNA expression in the thalamus. This however is an unlikely explanation for proteins that are generally expressed in astrocytes such as EAAT1 and EAAT2.

Another phenomenon is that the differences in direction of change can be observed across regions. The most striking example of this is EAAT1, which is increased at the transcript level in the ACC and decreased at the protein level in the DLPFC. This could be attributed to two possible factors: methodology or pathophysiology.

Methodologically, the reason why we do not detect consistency between the two regions could simply be due to the fact that these studies are underpowered. In general, we find that changes seem to occur in the same direction across regions, but that only one region reaches statistical significance. Given a larger number of subjects, it is possible we would detect more changes that are consistent across both regions. Another methodological explanation is that there is limited overlap in the subjects used for transcript and protein studies. If a subset of subjects is responsible for a given finding, they may not be present for both types of studies. However, because we don't have the same set of subjects for all studies, it would be difficult to mine for differences in subgroups.

Another explanation is that there are complex pathophysiological differences between these regions that could account for the seeming anatomical mismatches. Many studies show opposite changes between ACC and DLPFC, whereas studies examining closely related areas of DLPFC (Brodmann areas 9 and 46) are relatively consistent[319]. For example, decreases in neuropil and number of synapses as well as decreases in astrocytic processes have been demonstrated in DLPFC, whereas the ACC appears to be relatively spared[319, 320]. Thus, EAAT1, which is expressed on astrocytic processes may be decreased at the protein level in DLPFC due to changes in astrocyte morphology, even though transcripts that may be present closer to the nucleus may be spared. Changes in cell density, neuropil, astrocytic processes, or synapse number have not been

demonstrated in the ACC, although metabolic changes have been demonstrated[319]. Perhaps the ACC is challenged in schizophrenia similarly to the DLPFC, but is able to quickly repair sick parts of cells through active mechanisms that require energy. Thus, it is possible that in the ACC, EAAT1 is degraded more quickly in schizophrenia compared to healthy individuals, but that by increased synthesis (requiring increased EAAT1 transcription), cells are able to maintain a steady level of EAAT1 protein.

Given that this phenomenon is observed in many postmortem studies of schizophrenia brain[319], it appears more likely that these patterns do exist as part of the pathology and not just as artifacts. Careful examination of these patterns across multiple studies could yield important information about what kinds of cells and circuits are most vulnerable in this illness.

Given that protein and transcript studies can yield opposite results, one might question the usefulness of transcript studies, since protein studies provide a more direct measure of what is occurring in the cell. One advantage of *in situ* hybridization is that it yields quantitative anatomical information. For example, we were able to examine layer specific expression of the EAAT interacting proteins using *in situ* hybridization, whereas we could only measure regional changes using Western blot analysis. On a practical level, sometimes there are proteins for which specific antibodies have not been designed. For example, there are currently no commercially available antibodies for the alternative splice forms of the EAATs. Also, much less material is required to perform transcript studies, so protein studies may not

be feasible when studying small quantities of material such as laser capture microdissected cells.

5.8 Antipsychotic effects

A limitation of these studies is the potential effects of treatment with antipsychotic medications [278, 279]. We generally did not detect any differences between medicated and unmedicated patients with a few exceptions. These analyses, however, are relatively underpowered given that the vast majority of patients with schizophrenia received treatment up until the time of death. It should also be noted that the unmedicated group was defined as individuals not on medication within the 6 weeks prior to death. Therefore, medications taken prior to this period could still have potentially affected our dependent variables.

We also measured transcripts of the EAATs and EAAT interacting molecules in rats treated with haloperidol, clozapine, or vehicle. In general, there were few changes in these molecules in the haloperidol group. There tended to be more changes with clozapine. However, most of the patients in these studies were treated with typical antipsychotics, so the haloperidol treatment group is better for comparison to the patients with schizophrenia that were studied in this dissertation work. It should be noted that the treatment study in rats was acute, whereas patient antipsychotic treatment was chronic. It would therefore be useful to treat rats with antipsychotic medications chronically in order to better determine the effects of long-term

antipsychotic treatment on our dependent variables. It would also be useful to study the effects of antipsychotic treatment on protein expression, glycosylation, and splice variant expression, as these studies have not yet been done. It is also important to interpret these findings with the understanding that the effects of antipsychotic treatment in rats may be different than in patients with schizophrenia, due to differences both in species and the fact that they may act differently in healthy brain versus schizophrenia brain.

Another consideration is that the patients used in these studies were elderly. This means that not only could their brains have adjusted to a lifetime of protein alterations due to illness, but that they could also have adjusted to long-term antipsychotic drug treatment. In fact, schizophrenia studies involving younger patients have often found different results than studies involving older patients[104]. Despite these confounds, there are also advantages to studying an elderly cohort. For example, the patients are generally well cared for, don't have access to drugs of abuse or alcohol, and tend to die of natural causes, whereas younger cohorts have high comorbidities with drug abuse and suicide. Given these confounds, it is important to interpret our findings with the understanding that the changes found may be due to long term antipsychotic exposure and/or adjustment to lifelong changes in brain function/composition. However, even if the changes we found are purely attributable to these confounds, that does not render them unimportant. This would merely reflect that the data must be interpreted

as changes secondary to age and medication related factors rather than primary to the onset of illness itself.

5.9 Future directions

The current work has yielded many testable hypotheses. The following future directions attempt to address questions that arise from this dissertation work. First, we describe how we might attempt to address subcellular localization of the EAATs. Second, we describe other transcript and protein studies that might more completely describe the abnormalities in EAAT and EAAT interacting protein expression in schizophrenia. Third, we propose additional studies of EAAT posttranslational modifications that could shed additional light on how the EAATs may be abnormally regulated in schizophrenia. Finally, we describe animal studies that investigate the mechanisms of EAAT dysregulation in this illness.

5.9.1 Subcellular localization

We hypothesize that there is a decrease in EAAT mediated buffering and transport attributable to increased ER retention and decreased plasma membrane expression of the EAATs. It would therefore be important to directly test this hypothesis by examining the subcellular localization of the EAATs. This could be accomplished through subcellular fractionation or by using electron microscopy. Additionally, surface activity could be measured using reuptake assays in synaptosomal preparations. These studies would

yield a more complete picture of how EAAT mediated buffering and reuptake are altered in schizophrenia.

5.9.1.a Subcellular fractions

Given that we suspect changes in ER retention and plasma membrane expression of the EAATs, it would be useful to examine EAAT expression in individual subcellular compartments. Traditional methods of subcellular fractionation involve high speed centrifugation through a sucrose gradient. However, while these methods are excellent for separating organelles in freshly prepared tissue, they do not generally produce good results in postmortem tissue, likely due to rupture of cell membranes upon freezing of the tissue. However, immunoisolation has been used in our lab to successfully isolate subcellular compartments. Using sepharose beads attached to antibodies against the ER specific protein calnexin, we could isolate ER fractions from patients with schizophrenia and comparison subjects and probe for differences in EAAT expression using Western blot analysis. Specificity of this immunoisolation technique could be confirmed through electron microscopy of the beads and any attached organelles, as well as probing our Western blots for markers of other subcellular compartments from postmortem human brain. In addition to isolation of the ER, we could study other subcellular compartments such as plasma membranes, Golgi bodies, and endosomes. Antibodies directed against Na^+/K^+ ATPase or cadherin could be used to immunoisolate the plasma

membrane, antibodies directed against golgin-97 could be used to immunoisolate the Golgi, antibodies directed against EEA1, or rab5 could be used to immunoisolate early endosomes, antibodies directed against rab7 could be used to immunoisolate late endosomes, and antibodies directed against rab11 could be used to immunoisolate recycling endosomes.

5.9.1b Electron microscopy

Another way to measure subcellular localization of the EAATs is through electron microscopy. We could immunogold label EAAT1, EAAT2, and EAAT3 in the prefrontal cortex of patients with schizophrenia and comparison subjects. We could then examine expression of the EAATs at perisynaptic locations on the plasma membrane as well as expression in the ER. Immunogold labeling coupled to electron microscopy is a technically challenging technique, but it has been done in human postmortem brain[321-325].

5.9.1c Uptake assays

The functional endpoint of EAAT trafficking is glutamate reuptake. Measuring glutamate uptake in tissue from patients and comparison subjects provides a more direct measure of EAAT function than measuring expression alone. Although technically challenging, a few groups have measured reuptake of glutamate and other neurotransmitters in synaptosomes prepared from postmortem tissue[326-329]. Although synaptosomes are thought to be comprised of the terminals of neurons, knockdown of GLT-1 results in

dramatic reductions in synaptosomal glutamate uptake[18, 23]. Therefore, it appears as though synaptosomes are a good model system for uptake mediated by both neuronal and astrocytic EAATs. We could prepare synaptosomes from prefrontal cortical tissue of patients with schizophrenia and comparison subjects, and measure uptake of radioactive glutamate to test the hypothesis that glutamate reuptake is diminished in schizophrenia.

5.9.2 Expanded protein and transcript studies

There are several EAAT interacting molecules that we did not examine in this dissertation due to the fact they had not yet been identified or antibodies were not yet available at the time that those studies were performed. Sept2 is a molecule that interacts with EAAT1 and downregulates its function[188]. JM4 is a homologue to JWA that downregulates function of EAAT1, EAAT2, EAAT3, and EAAT4 by interacting with exon9 and preventing ER exit[197, 301]. CNTF is a molecule that increases expression of EAAT1 at the plasma membrane[284]. Analysis of these molecules would give a more complete picture of the abnormalities in EAAT regulation in schizophrenia. We could perform *in situ* hybridization to measure transcript expression of sept2, JM4, and CNTF. We could perform Western blot analysis to measure protein expression of CNTF, sept2, JWA, JM4, and ARHGEF11.

There are also several EAAT splice variants that we did not measure in this dissertation because either they were too low in abundance to detect with our methods, the functional effect on transport was unknown, or there were

technical problems with the assays. There exists an exon 3 skipping variant of EAAT1 that is unglycosylated and likely has an opposite orientation in the cell membrane[210, 224, 227]. Several additional splice variants exist for EAAT2. About 5-15% of EAAT2 can exist as an exon 7 skipping [219]. There also exists an exon skipping variant of EAAT2 that contains a partial deletion of exons 6 and 7[219]. 5'UTR intron retention variants of EAAT2 include HBGTII [218, 220], HBGTIIA [220], HBGTIIB [220], HBGTIIC [218, 220], EAAT2/3[218], and EAAT2/31[218]. 3'UTR variants of EAAT2 include GLT1 c which retains part of intron 10 and exists at very low levels in brain[221, 231], I7R which retains intron 7 and is marked for decay[222], and a variant that retains an intron from the 3' end to exon 7 and skips exons 8-11[219]. EAAT3 also can exist as an exon 9 skipping variant which is likely retained in the ER[224]. These splice variants could be measured through QPCR.

Given that we found increases in EAAT2Δ9 in schizophrenia, it would be interesting to see if this change in transcript results in a change in protein. This could be done by western blot with splice variant specific antibodies. An antibody against this variant exists and has been used for immunocytochemical localization of EAAT2Δ9, although it is not yet commercially available[223]. If we could obtain this antibody from the lab that has produced the antibody, we could glean important information on the functional effects of increased EAAT2Δ9 transcript expression. Additionally, we could perform western blot analysis for any additional variants for which we find changes in transcript expression.

Given the regional increases in transcript expression of these molecules, we are also interested in determining which cells express these changes. Cell level studies will allow us to determine if EAATs are being abnormally expressed in a different cell type and if there is a particular cell type that is more affected by the disease. Using laser capture microscopy and quantitative real-time PCR, we could measure cell-level transcript expression of the EAATs, EAAT splice variants, and EAAT interacting proteins. Specifically, we could analyze pyramidal neurons in layers II, III, and V, parvalbumin-containing GABAergic interneurons in layers II, III, and V, and astrocytes in all 6 layers of the DLPFC and ACC. Multiple subtypes of GABAergic interneurons exist in each cortical layer, and thus we have chosen a subset of these neurons that have been previously implicated in the pathology of schizophrenia[330-332]. These include chandelier cells, small GABAergic interneurons present in cortical layers II-VI with axo-axonic projections to nearby pyramidal cells within the same layer, and basket cells, large GABAergic interneurons present in cortical layers III-VI with projections to the soma and proximal dendrites of pyramidal cells within the same layer[333]. While this type of study is difficult to perform, the techniques have already been established in the lab.

5.9.3 Expanded post-translational regulation studies

Because EAAT activity is regulated through protein-protein interactions with specific proteins, it is important not only to probe for abnormalities in

expression, but also to detect any changes in the interactions between these proteins. We could use co-immunoprecipitation to probe for abnormalities in interactions between transporters and EAAT interacting proteins. Specifically, we could immunoprecipitate EAAT1, EAAT2, and EAAT3, and then probe immunologically for the proteins associated with each. For EAAT1, we would like to probe for interactions with mGluR2/3, CNTF, GFAP, Sept2, JWA, and JM4. For EAAT2, we would like to probe for interactions with GPS1, JWA, JM4, NR1, and PSD-95. We have developed a successful protocol for immunoprecipitation of EAAT1, which could be further developed and expanded to the other EAATs. However, this technique is very tissue intensive, so it is important to probe each immunoprecipitation for as many interacting partners as possible in a thoughtful manner to minimize tissue use.

Following gene expression of transporters and transporter interacting proteins, glutamate reuptake can also be modulated through transporter post-translational modification. The EAATs are known to be regulated by phosphorylation, ubiquitination, and glycosylation. We could develop phosphospecific antibodies to measure changes in EAAT phosphorylation. With an immunoisolation protocol, one can study additional posttranslational modifications of the EAATs. Ubiquitination can be studied by pulling down an EAAT and probing with an anti-ubiquitin antibody. Preliminary data from our lab suggests that ubiquitination of PSD95 is detectable in this tissue. One potential problem, however, is that we may not be able to detect changes in ubiquitination in our samples, because ubiquitinated transporters may be

degraded too quickly to be detected.

Lectins are proteins that recognize and bind to specific glycosyl residues. Biotinylated lectins, coupled with IR labeled streptavidin, can be used to detect specific sugar groups from an immunoprecipitated protein. Wheat germ agglutinin (WGA) recognizes n-acetylglucosamine, and is a marker for maturely glycosylated protiens. Lens culinaris agglutinin (LCA) binds to mannose and glucose and is a marker for immaturely glycosylated proteins. There also exist many other lectins that can be used to detect subtle differences in the branching patterns of glycosyl residues. We could determine what glycosyl residues are responsible for the changes in EAAT1 and EAAT2 glycosylation through lectin blot analysis of immunoprecipitated EAATs. We have performed preliminary experiments using this technique and it appears to be a viable approach to study glycosylation in human brain.

5.9.4 Animal models

Animal models provide a mechanistic way to study the changes we have described in postmortem tissue. We could mimic the expression of the transporters and their interacting partners in rodents using a viral expression vector or RNAi to overexpress or underexpress proteins of interest. For example, we could decrease EAAT1 expression or increase GPS1 or EAAT2Δ9 expression in rats. We could then perform behavioral tests to determine the effects of this manipulation on tasks implicated in schizophrenia such as working memory and prepulse inhibition. We could

also dissect the brains of these animals and determine the electrophysiological and neurochemical effects of altering these molecules.

5.10 Conclusions

In this dissertation work, I used *in situ* hybridization, Western Blot analysis, enzymatic deglycosylation, and QPCR to study EAAT expression and regulation in the prefrontal cortex in schizophrenia. I found decreased EAAT1 protein expression, and we found several factors that indicate decreased localization of EAATs at the synapse including decreased glycosylation, increased expression of an ER retention variant, and increased expression of molecules that retain EAATs in the ER. These data suggest decreased overall EAAT expression, and abnormal localization of EAATs that are expressed. Therefore, there are likely fewer EAATs expressed at the cell surface, and thus a decrease in the capacity for glutamate buffering and transport. I have found several levels of abnormal EAAT expression, thus demonstrating novel sites of disruption in the glutamate synapse in schizophrenia. This work could therefore provide alternative targets for developing new treatments for schizophrenia.

Appendix

Expression of Four Housekeeping Proteins in Elderly Patients with Schizophrenia

A.1 Abstract

We compared protein expression by Western blot analysis in four areas of postmortem brain from patients with schizophrenia and control subjects for several proteins that are often used as controls for Western blot studies: β-tubulin, actin, glyceraldehyde-3-phosphate dehydrogenase, and valosin containing protein. We did not detect any differences in expression between subjects with schizophrenia and a comparison group. These results suggest that all four proteins are suitable loading controls for postmortem studies of schizophrenia.

A.2 Introduction

Western blotting is a common technique used to measure protein levels. To ensure even loading, blotting, and processing between samples, an internal control is utilized. These loading controls are usually so-called housekeeping proteins measured in the same lane of the gel for each sample, and are assumed to have constant between-sample expression relative to the amount of sample loaded. Commonly used loading controls include the cytoskeletal structural molecules β-tubulin and actin, and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Valosin containing protein (VCP) is a 97kDa protein used as a loading control in one postmortem schizophrenia study [266]. VCP is an ATPase involved in the ubiquitin-proteasome degradation pathway, membrane fusion, transcription activation, cell cycle control, apoptosis, and molecular chaperoning [334]. Although it is not predicted to be affected in this disease, it has not been determined whether VCP is abnormally regulated in schizophrenia. In this study, we examined expression of VCP, β-tubulin, actin, and GAPDH in patients with schizophrenia and control subjects.

A.3 Materials and Methods

A.3.1 Subjects

Subjects from the Mount Sinai Medical Center Department of Psychiatry Schizophrenia Brain Bank were studied (Table 1), including 39 individuals diagnosed with schizophrenia and 41 comparison subjects.

Subjects were diagnosed with schizophrenia if the presence of schizophrenic symptoms were documented before age 40, the medical records contained evidence of psychotic symptoms and at least 10 years of psychiatric hospitalization with diagnosis of schizophrenia, and a DSM-III-R diagnosis of schizophrenia was agreed upon by two experienced clinicians. Diagnostic groups were matched for age, postmortem interval, and tissue pH. Upon neuropathological examination, no evidence of Alzheimer or other neurodegenerative disease was found. The brain banking procedures were approved by the Mount Sinai School of Medicine Institutional Review Board.

Table A.1: Subject Characteristics for Housekeeping Protein Study

	Comparison Group	Schizophrenia
N	41	39
Sex	18 m / 23 f	27 m / 12 f
Tissue pH	6.4 ± 0.3	6.4 ± 0.3
PMI (hours)	8.1 ± 6.3	13.7 ± 7.8
Age (years)	78 ± 14	74 ± 12
On / Off Rx	0 / 41	28 / 11
Values presented as mean ± standard deviation Abbreviations: male (m), female (f), antipsychotic medication (Rx), postmortem interval (PMI).		

A.3.2 Tissue preparation

Brains were obtained after autopsy and one hemisphere was cut coronally into ~0.8 - 1 cm^3 slabs and flash frozen. Tissue was dissected from anterior cingulate cortex (ACC) (n = 68), dorsolateral prefrontal cortex (DLPFC) (n = 66), hippocampus (n = 49), and primary visual cortex (PVC) (n = 46). Approximately 1 cm^3 of frozen tissue was first pulverized, then homogenized (10% wt/vol) in 5 mM Tris-HCl (pH 7.4) with 320 mM sucrose and 1 protease inhibitor tablet per 10 mL (Complete mini, Roche Diagnostics, Manheim, Germany) for 30 sec with a polytron homogenizer (Fisher Scientific, Pittsburgh, Pennsylvania) and stored at -80^oC in 0.5 mL aliquots. For our loading control, tissue from the frontal cortex was dissected from a 9.8 year old female pig-tail macaque (*Macaca nemestrina*) provided by the University of Washington Regional Primate Research Center. The animal was sacrificed as part of a protocol (which did not require brain tissue) unrelated to the present study. Protocols involving this animal were reviewed by the Washington Primate Research Center Research Review Committee and the University of Washington IACUC. Frozen macaque cortex was thawed on ice, cut into pieces of approximately 1 cm^3, and homogenized as described above and stored at -80^oC in 1.2 mL aliquots. To determine protein concentrations, assay by the Bradford method [290] was performed on these homogenates.

A.3.3 Western blot analysis

For gel electrophoresis, tissue samples were adjusted to a concentration of 0.8 µg/ul with sterile water, reducing buffer (Invitrogen), and denaturing buffer (Invitrogen). Samples were then incubated at 70^{o}C for 10 minutes.

The Novex Mini Cell NuPAGE system (Invitrogen) with 4-12% Bis-Tris gradient polyacrylamide gels (Invitrogen) was used. 8 µg of denatured protein homogenate was run in each lane. Samples were loaded in duplicate in adjacent lanes, and a molecular weight standard was run on each gel. A lane containing 8µg of homogenized macaque cortex was also loaded onto each gel to control for interblot variability. Gels were suspended in a bath of NuPAGE MES SDS running buffer (Invitrogen) with 500µl NuPAGE antioxidant (Invitrogen) during electrophoresis.

Following electrophoresis, proteins were transferred onto Immobilon-FL PVDF membranes (Millipore) using a semi-dry transfer apparatus (Bio-Rad). After electroblot transfer of the protein, membranes were washed twice and incubated with Odyssey Blocking Buffer (Li-Cor Biosciences) for 1 hour at room temperature with rocking to block nonspecific antibody binding. Membranes were exposed to the primary polyclonal antibody diluted 1:10,000 for actin (Chemicon MAB150R), VCP (Abcam ab11433), and β-tubulin (Upstate 05-661), and 1:20,000 for GAPDH (Sigma G9545) in blocking buffer with 0.1% tween overnight at 4^{o}C with rocking. Next, the membranes were washed three times for ten minutes in tris-buffered saline with 0.1% tween

(TBST), then rocked for 1 hour with anti-mouse IR-Dye 680 or 800CW secondary antibody (Li-Cor Biosciences) diluted 1:10,000 in blocking buffer with 0.1% tween. Membranes were washed three times for 10 minutes in TBST then washed 5 times in high purity water and allowed to dry for 3-5 minutes before scanning (infrared imaging system; Li-Cor Biosciences). We pre-tested the β-tubulin, actin, GAPDH, and VCP Western blot assays using varying concentrations of protein from a human cortical tissue homogenate sample. These experiments demonstrated that each assay was linear with protein concentrations found in this study (VCP: $R = 0.99$, $p < 0.01$; β-tubulin: $R = 0.99$, $p < 0.01$; actin: $R = 0.95$, $p = 0.01$; GAPDH: $R = 0.98$, $p < 0.01$).

A.3.4 Data Analysis

Membranes were scanned using a Li-Cor Odyssey scanner, and the intensity value for each protein band was measured using the Odyssey 2.1 software package. Specific protein values were corrected for lane background and divided by intensity values for the macaque cortex homogenate lane used as an internal loading control. Because all of the proteins studied are typically used as loading controls, we did not divide these values by another within-lane control value. Finally, adjusted intensity values from duplicate lanes were averaged for each subject.

A.3.5 Statistical Analysis

All statistical analyses were performed using Statistica (StatSoft, Tulsa,

Oklahoma). Outliers more than 6 standard deviations from the mean were excluded. Correlation analysis was performed to analyze associations between protein expression and age, postmortem interval, and pH. We analyzed protein expression using analysis of variance (ANOVA) or with analysis of covariance (ANCOVA) when significant correlations were detected.

A.4 Results

We detected bands for all four proteins at the predicted molecular masses of 97 kDa (VCP), 55 kDa (β-tubulin), 42 kDa (actin), and 36 kDa (GAPDH) (Figure A.1). After exclusion of outliers, the following numbers of subjects remained for analysis (VCP in ACC: 33 control / 33 schizophrenia; β-tubulin in ACC: 34 control / 33 schizophrenia; actin and GAPDH in ACC: 33 control / 32 schizophrenia; VCP, β-tubulin, and actin in hippocampus: 26 control / 22 schizophrenia; GAPDH in hippocampus: 27 control / 22 schizophrenia; VCP, β-tubulin, and GAPDH in PVC: 24 control / 22 schizophrenia; actin in PVC: 23 control / 22 schizophrenia; VCP in DLPFC: 31 control / 33 schizophrenia; β-tubulin in DLPFC: 30 control / 30 schizophrenia; actin in DLPFC: 31 control / 30 schizophrenia; GAPDH in DLPFC: 30 control / 32 schizophrenia). Regression analysis revealed a correlation between tissue pH and actin expression in PVC ($r = 0.33$, $p < 0.05$). No other correlations were detected between age (VCP in ACC: $r = 0.10$, $p = 0.43$; VCP in hippocampus: $r = 0.03$, $p = 0.29$; VCP in PVC: $r = 0.04$, $p = 0.78$; VCP

in DLPFC: r = 0.08, p = 0.52; β-tubulin in ACC: r = 0.14, p = 0.24; β-tubulin in hippocampus: r = 0.10, p = 0.51; β-tubulin in PVC: r = 0.13, p = 0.40; β-tubulin in DLPFC: r = 0.09, p = 0.50; actin in ACC: r = 0.07, p = 0.58; actin in hippocampus: r = 0.01, p = 0.95; actin in PVC: r = 0.09, p = 0.54; actin in DLPFC: r = 0.09, p = 0.48; GAPDH in ACC: r = 0.17, p = 0.18; GAPDH in hippocampus: r = 0.03, p = 0.86; GAPDH in PVC: r = 0.07, p = 0.63; GAPDH in DLPFC: r = 0.05, p = 0.69) or pH (VCP in ACC: r = 0.08, p = 0.52; VCP in hippocampus: r = 0.24, p = 0.10; VCP in PVC: r = 0.15, p = 0.32; VCP in DLPFC: r = 0.04, p = 0.77; β-tubulin in ACC: r = 0.06, p = 0.64; β-tubulin in hippocampus: r = 0.11, p = 0.44; β-tubulin in PVC: r = 0.02, p = 0.87; β-tubulin in DLPFC: r = 0.01, p = 0.93; actin in ACC: r = 0.09, p = 0.45; actin in hippocampus: r = 0.27, p = 0.06; actin in DLPFC: r = 0.01, p = 0.94; GAPDH in ACC: r = 0.14, p = 0.27; GAPDH in hippocampus: r = 0.15, p = 0.31; GAPDH in PVC: r = 0.15, p = 0.32; GAPDH in DLPFC: r = 0.20, p = 0.11) for any of the proteins studied. Because sex is a non-continuous variable, we performed ANOVA with sex as the independent variable, and protein expression as the dependent variable to test for effects of sex on protein expression. There was an increase in GAPDH expression in PVC in males compared to females (F = 7.35, p = 0.01), but this effect was not robust enough to withstand a correction for multiple comparisons. No other associations between sex and protein expression were detected (VCP in ACC: F = 0.21, p = 0.65; VCP in hippocampus: F = 0.00, p = 0.99; VCP in PVC: F = 2.37, p = 0.13; VCP in DLPFC: F = 3.03, p = 0.09; β-tubulin in ACC:

F = 0.10, p = 0.75; β-tubulin in hippocampus: F = 0.09, p = 0.77; β-tubulin in PVC: F = 2.22, p = 0.14; β-tubulin in DLPFC: F = 0.32, p = 0.57; actin in ACC: F = 0.16, p = 0.67; actin in hippocampus: F = 0.18, p = 0.68; actin in PVC: F = 1038, p = 0.25; actin in DLPFC: F = 0.02, p = 0.90; GAPDH in ACC: F = 0.91, p = 0.34; GAPDH in hippocampus: F = 0.02, p = 0.89; GAPDH in DLPFC: F = 2064, p = 0.11). We did not detect any differences in expression for any of the proteins studied between subjects with schizophrenia and the comparison group in any of the four regions studied (Figure A.1).

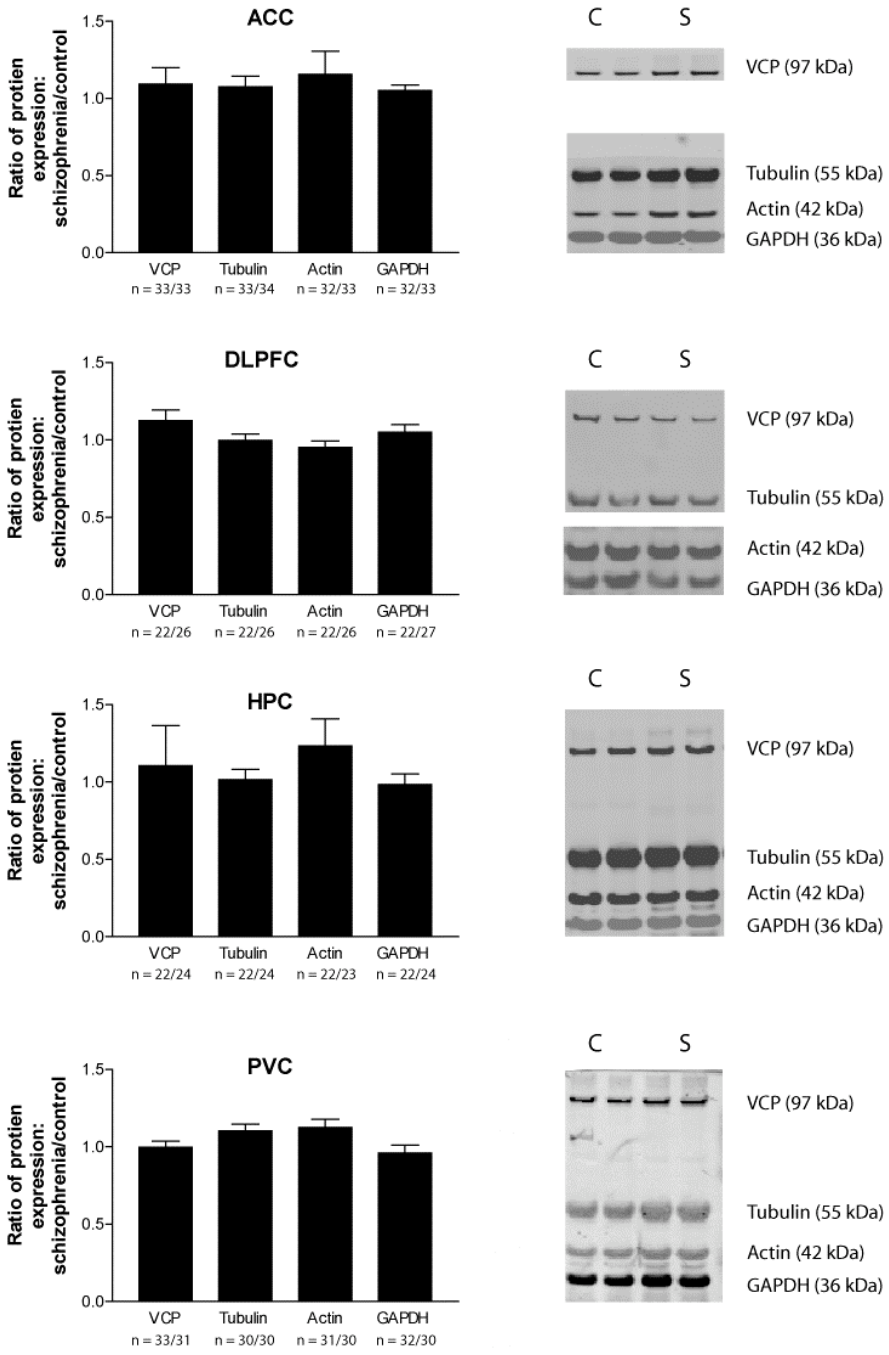

Figure A.1: Housekeeping protein expression in multiple brain regions. Data are expressed as ratio of signal intensity for subjects with schizophrenia divided by signal intensity for comparison subjects. None of these proteins differed between diagnostic groups in any region studied. On the right are representative Western blots for the 4 proteins studied. Abbreviations: anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (DLPFC), hippocampus (HPC), primary visual cortex (PVC), comparison subject (C), schizophrenia (S), valosin-containing protein (VCP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), kilodaltons (kDa).

A.5 Discussion

Several studies have shown variations in expression of housekeeping proteins in experimental or disease conditions. GAPDH protein levels in cultured human keratinocytes increased with 2,3,7,8-Tetrachlorodibenzo-p-dioxin treatment [335], spinal cord injury increased actin expression in rats [336], and actin, β-tubulin and GAPDH were expressed at higher levels in renal tumors compared to normal kidney tissue [337]. Sex differences were found in GAPDH expression in the developing rat brain [338]. These findings highlight the importance of confirming that the loading controls used for Western blot analyses are not affected by the condition being studied. We did not find differences in the expression of the proteins studied in this cohort in schizophrenia. It unlikely that the mismatch in sex distribution between groups influenced our results given that we did not find any significant influence of sex on protein expression. Because all of the proteins studied are putative loading controls, we did not normalize to a within-lane comparison protein. However, we normalized to a constant amount of protein loaded on each gel as a control for interblot variability. Our results are consistent with one other study that also found no changes in actin expression in schizophrenia [339].

While actin, β-tubulin, and GAPDH have traditionally been used as loading controls, VCP has a higher molecular weight (97 kDa) which makes it useful on blots probed for smaller proteins. In summary, we found no

changes in four different brain regions of expression of 3 housekeeping proteins that are commonly used to normalize protein data, and a fourth protein, VCP, that has not previously been characterized in schizophrenia. Based on these findings, VCP appears to be an acceptable loading control to use for low molecular weight proteins in postmortem protein studies of schizophrenia.

References

1. Association AP. *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision*. Fourth Edition ed. Washington, D.C.: American Psychiatric Association; 2000.

2. Bhugra D. The global prevalence of schizophrenia. *PLoS Med* May 2005;2(5):e151; quiz e175.

3. Tandon R, Keshavan MS, Nasrallah HA. Schizophrenia, "just the facts" what we know in 2008. 2. Epidemiology and etiology. *Schizophr Res* Jul 2008;102(1-3):1-18.

4. Sharif Z. Side effects as influencers of treatment outcome. *J Clin Psychiatry* 2008;69 Suppl 3:38-43.

5. Tandon R, Keshavan MS, Nasrallah HA. Schizophrenia, "Just the Facts": what we know in 2008 part 1: overview. *Schizophr Res* Mar 2008;100(1-3):4-19.

6. Laruelle M, Abi-Dargham A, Gil R, Kegeles L, Innis R. Increased dopamine transmission in schizophrenia: relationship to illness phases. *Biol Psychiatry* Jul 1 1999;46(1):56-72.

7. Joyce JN, Meador-Woodruff JH. Linking the family of D2 receptors to neuronal circuits in human brain: insights into schizophrenia. *Neuropsychopharmacology* Jun 1997;16(6):375-384.

8. Javitt DC, Zukin SR. Recent advances in the phencyclidine model of schizophrenia. *Am J Psychiatry* 1991;148(10):1301-1308.

9. Tamminga C. Glutamatergic aspects of schizophrenia. *Br J Psychiatry Suppl* 1999(37):12-15.

10. Lahti AC, Holcomb HH, Medoff DR, Tamminga CA. Ketamine activates psychosis and alters limbic blood flow in schizophrenia. *Neuroreport* 1995;6(6):869-872.

11. Hertzmann M, Reba RC, Kotlyarov EV. Single photon emission computed tomography in phencyclidine and related drug abuse. *Am J Psychiatry* Feb 1990;147(2):255-256.

12. Jones LB. Recent cytoarchitechtonic changes in the prefrontal cortex of schizophrenics. *Front Biosci* Nov 1 2001;6:E148-153.

13. Lewis DA, Glantz LA, Pierri JN, Sweet RA. Altered cortical glutamate neurotransmission in schizophrenia: evidence from morphological studies of pyramidal neurons. *Ann N Y Acad Sci* Nov 2003;1003:102-112.

14. McCullumsmith RE, Clinton SM, Meador-Woodruff JH. Schizophrenia as a disorder of neuroplasticity. *Int Rev Neurobiol* 2004;59:19-45.

15. Meador-Woodruff JH, Hogg AJ, Smith RE. Striatal ionotropic glutamate receptor expression in schizophrenia, bipolar disorder, and major depressive disorder. *Brain Res Bull* 2001;55(5):631-640.

16. Ohrmann P, Siegmund A, Suslow T, Spitzberg K, Kersting A, Arolt V, Heindel W, Pfleiderer B. Evidence for glutamatergic neuronal dysfunction in the prefrontal cortex in chronic but not in first-episode patients with schizophrenia: a proton magnetic resonance spectroscopy study. *Schizophr Res* Mar 1 2005;73(2-3):153-157.

17. Quintana J, Wong T, Ortiz-Portillo E, Marder SR, Mazziotta JC. Anterior cingulate dysfunction during choice anticipation in schizophrenia. *Psychiatry Res* Dec 15 2004;132(2):117-130.

18. Rothstein JD, Dykes-Hoberg M, Pardo CA, et al. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 1996;16(3):675-686.

19. Karlsson RM, Tanaka K, Heilig M, Holmes A. Loss of glial glutamate and aspartate transporter (excitatory amino acid transporter 1) causes locomotor hyperactivity and exaggerated responses to psychotomimetics: rescue by haloperidol and metabotropic glutamate 2/3 agonist. *Biol Psychiatry* Nov 1 2008;64(9):810-814.

20. Karlsson RM, Tanaka K, Saksida LM, Bussey TJ, Heilig M, Holmes A. Assessment of glutamate transporter GLAST (EAAT1)-deficient mice for phenotypes relevant to the negative and executive/cognitive symptoms of schizophrenia. *Neuropsychopharmacology* May 2009;34(6):1578-1589.

21. Mohn AR, Gainetdinov RR, Caron MG, Koller BH. Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. *Cell* 1999;98(4):427-436.

22. Gaisler-Salomon I, Miller GM, Chuhma N, et al. Glutaminase-Deficient Mice Display Hippocampal Hypoactivity, Insensitivity To Pro-Psychotic Drugs And Potentiated Latent Inhibition: Relevance To Schizophrenia. *Neuropsychopharmacology* Jun 10 2009.

23. Tanaka K, Watase K, Manabe T, et al. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* 1997;276(5319):1699-1702.

24. Watase K, Hashimoto K, Kano M, et al. Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. *Eur J Neurosci* 1998;10(3):976-988.

25. Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, Nicoll RA. Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* Dec 21-28 2000;408(6815):936-943.

26. Douglas R, Martin KAC. *Neocortex*. Third edition ed. New York: Oxford University Press; 1990.

27. Siwek DF, Pandya DN. Prefrontal projections to the mediodorsal nucleus of the thalamus in the rhesus monkey. *J Comp Neurol* Oct 22 1991;312(4):509-524.

28. Ray JP, Price JL. The organization of projections from the mediodorsal nucleus of the thalamus to orbital and medial prefrontal cortex in macaque monkeys. *J Comp Neurol* Nov 1 1993;337(1):1-31.

29. Goldman-Rakic PS, Porrino LJ. The primate mediodorsal (MD) nucleus and its projection to the frontal lobe. *J Comp Neurol* Dec 22 1985;242(4):535-560.

30. Vogt BA, Pandya DN, Rosene DL. Cingulate cortex of the rhesus monkey: I. Cytoarchitecture and thalamic afferents. *J Comp Neurol* Aug 8 1987;262(2):256-270.

31. Auer DP, Wilke M, Grabner A, Heidenreich JO, Bronisch T, Wetter TC. Reduced NAA in the thalamus and altered membrane and glial metabolism in schizophrenic patients detected by 1H-MRS and tissue segmentation. *Schizophr Res* Oct 1 2001;52(1-2):87-99.

32. Bruneau EG, McCullumsmith RE, Haroutunian V, Davis KL, Meador-Woodruff JH. Increased expression of glutaminase and glutamine synthetase mRNA in the thalamus in schizophrenia. *Schizophr Res* Jun 1 2005;75(1):27-34.

33. Clinton SM, Haroutunian V, Davis KL, Meador-Woodruff JH. Altered Transcript Expression of NMDA Receptor-Associated Postsynaptic Proteins in the Thalamus of Subjects With Schizophrenia. *Am J Psychiatry* Jun 2003;160(6):1100-1109.

34. Huerta I, McCullumsmith RE, Haroutunian V, Gimenez-Amaya JM, Meador-Woodruff JH. Expression of excitatory amino acid transporter interacting protein transcripts in the thalamus in schizophrenia. *Synapse* Jun 1 2006;59(7):394-402.

35. Meador-Woodruff JH, Clinton SM, Beneyto M, McCullumsmith RE. Molecular abnormalities of the glutamate synapse in the thalamus in schizophrenia. *Ann N Y Acad Sci* Nov 2003;1003:75-93.

36. Smith RE, Haroutunian V, Davis KL, Meador-Woodruff JH. Expression of excitatory amino acid transporter transcripts in the thalamus of subjects with schizophrenia. *Am J Psychiatry* 2001;158(9):1393-1399.

37. Davidkova G, McCullumsmith RE, Meador-Woodruff JH. Expression of ARHGEF11 mRNA in schizophrenic thalamus. *Ann N Y Acad Sci* Nov 2003;1003:375-377.

38. Smith RE, Haroutunian V, Davis KL, Meador-Woodruff JH. Vesicular glutamate transporter transcript expression in the thalamus in schizophrenia. *Neuroreport* 2001;12(13):2885-2887.

39. Salt TE, Eaton SA. Functions of ionotropic and metabotropic glutamate receptors in sensory transmission in the mammalian thalamus. *Prog Neurobiol* Jan 1996;48(1):55-72.

40. Bellocchio EE, Reimer RJ, Fremeau RT, Jr., Edwards RH. Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science* 2000;289(5481):957-960.

41. Takamori S, Rhee JS, Rosenmund C, Jahn R. Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* 2000;407(6801):189-194.

42. Ghose S, Weickert CS, Colvin SM, Coyle JT, Herman MM, Hyde TM, Kleinman JE. Glutamate carboxypeptidase II gene expression in the human frontal and temporal lobe in schizophrenia. *Neuropsychopharmacology* Jan 2004;29(1):117-125.

43. Hollmann M, Heinemann S. Cloned glutamate receptors. *Annu Rev Neurosci* 1994;17:31-108.

44. Masson J, Sagne C, Hamon M, El Mestikawy S. Neurotransmitter transporters in the central nervous system. *Pharmacol Rev* 1999;51(3):439-464.

45. Bassi MT, Gasol E, Manzoni M, et al. Identification and characterisation of human xCT that co-expresses, with 4F2 heavy

chain, the amino acid transport activity system xc. *Pflugers Arch* 2001;442(2):286-296.

46. Kim JY, Kanai Y, Chairoungdua A, et al. Human cystine/glutamate transporter: cDNA cloning and upregulation by oxidative stress in glioma cells. *Biochim Biophys Acta* 2001;1512(2):335-344.

47. Jackson M, Song W, Liu MY, et al. Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins. *Nature* Mar 1 2001;410(6824):89-93.

48. Lin CI, Orlov I, Ruggiero AM, Dykes-Hoberg M, Lee A, Jackson M, Rothstein JD. Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18. *Nature* Mar 1 2001;410(6824):84-88.

49. Marie H, Billups D, Bedford FK, Dumoulin A, Goyal RK, Longmore GD, Moss SJ, Attwell D. The amino terminus of the glial glutamate transporter GLT-1 interacts with the LIM protein Ajuba. *Mol Cell Neurosci* 2002;19(2):152-164.

50. Watanabe M, Robinson MB, Kalandadze A, Rothstein JD. GPS1, interacting protein with GLT-1. *Program No 37216 2003 SFN Abstract* 2003.

51. Danbolt NC. Glutamate uptake. *Prog Neurobiol* 2001;65(1):1-105.

52. Danbolt NC, Chaudhry FA, Dehnes Y, Lehre KP, Levy LM, Ullensvang K, Storm-Mathisen J. Properties and localization of glutamate transporters. *Prog Brain Res* 1998;116:23-43.

53. Wilson NR, Kang J, Hueske EV, Leung T, Varoqui H, Murnick JG, Erickson JD, Liu G. Presynaptic regulation of quantal size by the vesicular glutamate transporter VGLUT1. *Journal of Neuroscience* 2005;25(26):6221-6234, 2005 Jun 6229.

54. Hisano S. Vesicular glutamate transporters in the brain. *Anat Sci Int* Dec 2003;78(4):191-204.

55. Wheal HV, Thomson AM. Excitatory Amino Acids and Synaptic Transmission (2nd ed.). *Academis Press, New York* 1995.

56. Nakanishi S. Molecular diversity of glutamate receptors and implications for brain function. *Science* Oct 23 1992;258(5082):597-603.

57. Durand GM, Bennett MV, Zukin RS. Splice variants of the N-methyl-D-aspartate receptor NR1 identify domains involved in regulation by

polyamines and protein kinase C. *Proc Natl Acad Sci U S A* Jul 15 1993;90(14):6731-6735.

58. Corti C, Cavanni P, Caveggion E, Ferraguti F, Corsi M, Trist DG. Different levels of receptor expression as a new procedure to estimate agonist affinity constant. Application to the metabotropic receptors. *Ann N Y Acad Sci* May 30 1997;812:231-233.

59. Corti C, Restituito S, Rimland JM, Brabet I, Corsi M, Pin JP, Ferraguti F. Cloning and characterization of alternative mRNA forms for the rat metabotropic glutamate receptors mGluR7 and mGluR8. *Eur J Neurosci* Dec 1998;10(12):3629-3641.

60. Pin JP, Duvoisin R. The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* Jan 1995;34(1):1-26.

61. Prezeau L, Carrette J, Helpap B, Curry K, Pin JP, Bockaert J. Pharmacological characterization of metabotropic glutamate receptors in several types of brain cells in primary cultures. *Mol Pharmacol* Apr 1994;45(4):570-577.

62. Saugstad JA, Kinzie JM, Shinohara MM, Segerson TP, Westbrook GL. Cloning and expression of rat metabotropic glutamate receptor 8 reveals a distinct pharmacological profile. *Mol Pharmacol* Jan 1997;51(1):119-125.

63. Schoepp DD. Novel functions for subtypes of metabotropic glutamate receptors. *Neurochem Int* May 1994;24(5):439-449.

64. Pin JP, Waeber C, Prezeau L, Bockaert J, Heinemann SF. Alternative splicing generates metabotropic glutamate receptors inducing different patterns of calcium release in Xenopus oocytes. *Proc Natl Acad Sci U S A* Nov 1 1992;89(21):10331-10335.

65. Pickering DS, Thomsen C, Suzdak PD, et al. A comparison of two alternatively spliced forms of a metabotropic glutamate receptor coupled to phosphoinositide turnover. *J Neurochem* Jul 1993;61(1):85-92.

66. McCool BA, Pin JP, Brust PF, Harpold MM, Lovinger DM. Functional coupling of rat group II metabotropic glutamate receptors to an omega-conotoxin GVIA-sensitive calcium channel in human embryonic kidney 293 cells. *Mol Pharmacol* Oct 1996;50(4):912-922.

67. Joly C, Gomeza J, Brabet I, Curry K, Bockaert J, Pin JP. Molecular, functional, and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. *J Neurosci* May 1995;15(5 Pt 2):3970-3981.

68. Aramori I, Nakanishi S. Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron* Apr 1992;8(4):757-765.

69. Contractor A, Heinemann SF. Glutamate receptor trafficking in synaptic plasticity. *Sci STKE* Oct 29 2002;2002(156):RE14.

70. McGee AW, Bredt DS. Assembly and plasticity of the glutamatergic postsynaptic specialization. *Curr Opin Neurobiol* Feb 2003;13(1):111-118.

71. Lin DT, Makino Y, Sharma K, Hayashi T, Neve R, Takamiya K, Huganir RL. Regulation of AMPA receptor extrasynaptic insertion by 4.1N, phosphorylation and palmitoylation. *Nat Neurosci* Jul 2009;12(7):879-887.

72. Hayashi T, Rumbaugh G, Huganir RL. Differential regulation of AMPA receptor subunit trafficking by palmitoylation of two distinct sites. *Neuron* Sep 1 2005;47(5):709-723.

73. Thomas GM, Lin DT, Nuriya M, Huganir RL. Rapid and bi-directional regulation of AMPA receptor phosphorylation and trafficking by JNK. *Embo J* Jan 23 2008;27(2):361-372.

74. Zhu Y, Pak D, Qin Y, et al. Rap2-JNK removes synaptic AMPA receptors during depotentiation. *Neuron* Jun 16 2005;46(6):905-916.

75. Sheng M, Pak DT. Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. *Annu Rev Physiol* 2000;62:755-778.

76. Lin JW, Wyszynski M, Madhavan R, Sealock R, Kim JU, Sheng M. Yotiao, a novel protein of neuromuscular junction and brain that interacts with specific splice variants of NMDA receptor subunit NR1. *J Neurosci* Mar 15 1998;18(6):2017-2027.

77. Ehlers MD, Fung ET, O'Brien RJ, Huganir RL. Splice variant-specific interaction of the NMDA receptor subunit NR1 with neuronal intermediate filaments. *J Neurosci* Jan 15 1998;18(2):720-730.

78. Dracheva S, McGurk SR, Haroutunian V. mRNA expression of AMPA receptors and AMPA receptor binding proteins in the cerebral cortex of elderly schizophrenics. *J Neurosci Res* Mar 15 2005;79(6):868-878.

79. Toyooka K, Iritani S, Makifuchi T, et al. Selective reduction of a PDZ protein, SAP-97, in the prefrontal cortex of patients with chronic schizophrenia. *J Neurochem* Nov 2002;83(4):797-806.

80. Kozlovsky N, Scarr E, Dean B, Agam G. Postmortem brain calcineurin protein levels in schizophrenia patients are not different from controls. *Schizophr Res* Apr 2006;83(2-3):173-177.

81. Erdely HA, Tamminga CA, Roberts RC, Vogel MW. Regional alterations in RGS4 protein in schizophrenia. *Synapse* Jun 15 2006;59(8):472-479.

82. Lipska BK, Mitkus S, Caruso M, et al. RGS4 mRNA Expression in Postmortem Human Cortex Is Associated with COMT Val158Met Genotype and COMT Enzyme Activity. *Hum Mol Genet* Aug 11 2006.

83. Mirnics K, Middleton FA, Stanwood GD, Lewis DA, Levitt P. Disease-specific changes in regulator of G-protein signaling 4 (RGS4) expression in schizophrenia. *Mol Psychiatry* May 2001;6(3):293-301.

84. Xiao B, Tu JC, Petralia RS, et al. Homer regulates the association of group 1 metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. *Neuron* Oct 1998;21(4):707-716.

85. Tu JC, Xiao B, Yuan JP, Lanahan AA, Leoffert K, Li M, Linden DJ, Worley PF. Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron* Oct 1998;21(4):717-726.

86. Kammermeier PJ, Xiao B, Tu JC, Worley PF, Ikeda SR. Homer proteins regulate coupling of group I metabotropic glutamate receptors to N-type calcium and M-type potassium channels. *J Neurosci* Oct 1 2000;20(19):7238-7245.

87. Meador-Woodruff JH, Reyes, E., Haroutunian, V., and Kristiansen, L. V. Abnormal Expression of Transcripts for GKAP and Shank Suggest Alterations of Interactions Between Metabotropic and Ionotropic Glutamate Receptors in Schizophrenic Brain. Paper presented at: ACNP 44th Annual Meeting; December 11-15, 2005, 2005; Waikoloa, Hawaii.

88. Dracheva S, Marras SA, Elhakem SL, Kramer FR, Davis KL, Haroutunian V. N-methyl-D-aspartic acid receptor expression in the dorsolateral prefrontal cortex of elderly patients with schizophrenia. *Am J Psychiatry* Sep 2001;158(9):1400-1410.

89. Sokolov BP. Expression of NMDAR1, GluR1, GluR7, and KA1 glutamate receptor mRNAs is decreased in frontal cortex of "neuroleptic-free" schizophrenics: evidence on reversible up-regulation by typical neuroleptics. *J Neurochem* 1998;71(6):2454-2464.

90. Kornhuber J, Mack-Burkhardt F, Riederer P, Hebenstreit GF, Reynolds GP, Andrews HB, Beckmann H. [3H]MK-801 binding sites in postmortem brain regions of schizophrenic patients. *J Neural Transm* 1989;77(2-3):231-236.

91. Humphries C, Mortimer A, Hirsch S, de Belleroche J. NMDA receptor mRNA correlation with antemortem cognitive impairment in schizophrenia. *Neuroreport* 1996;7(12):2051-2055.

92. Akbarian S, Sucher NJ, Bradley D, et al. Selective alterations in gene expression for NMDA receptor subunits in prefrontal cortex of schizophrenics. *J Neurosci* 1996;16(1):19-30.

93. Grimwood S, Slater P, Deakin JF, Hutson PH. NR2B-containing NMDA receptors are up-regulated in temporal cortex in schizophrenia. *Neuroreport* 1999;10(3):461-465.

94. Nudmamud S, Reynolds GP. Increased density of glutamate/N-methyl-D-aspartate receptors in superior temporal cortex in schizophrenia. *Neurosci Lett* May 18 2001;304(1-2):9-12.

95. Ishimaru M, Kurumaji A, Toru M. Increases in strychnine-insensitive glycine binding sites in cerebral cortex of chronic schizophrenics: evidence for glutamate hypothesis. *Biol Psychiatry* Jan 15 1994;35(2):84-95.

96. Ishimaru M, Kurumaji A, Toru M. NMDA-associated glycine binding site increases in schizophrenic brains. *Biol Psychiatry* Aug 15 1992;32(4):379-381.

97. Zavitsanou K, Ward PB, Huang XF. Selective alterations in ionotropic glutamate receptors in the anterior cingulate cortex in schizophrenia. *Neuropsychopharmacology* Nov 2002;27(5):826-833.

98. Noga JT, Hyde TM, Bachus SE, Herman MM, Kleinman JE. AMPA receptor binding in the dorsolateral prefrontal cortex of schizophrenics and controls. *Schizophr Res* Mar 30 2001;48(2-3):361-363.

99. Newell KA, Zavitsanou K, Huang XF. Ionotropic glutamate receptor binding in the posterior cingulate cortex in schizophrenia patients. *Neuroreport* Aug 22 2005;16(12):1363-1367.

100. Scarr E, Beneyto M, Meador-Woodruff JH, Deans B. Cortical glutamatergic markers in schizophrenia. *Neuropsychopharmacology* Aug 2005;30(8):1521-1531.

101. Le Corre S, Harper CG, Lopez P, Ward P, Catts S. Increased levels of expression of an NMDARI splice variant in the superior temporal gyrus in schizophrenia. *Neuroreport* Apr 7 2000;11(5):983-986.

102. Mueller HT, Meador-Woodruff JH. NR3A NMDA receptor subunit mRNA expression in schizophrenia, depression and bipolar disorder. *Schizophr Res* Dec 1 2004;71(2-3):361-370.

103. Woo TU, Walsh JP, Benes FM. Density of glutamic acid decarboxylase 67 messenger RNA-containing neurons that express the N-methyl-D-aspartate receptor subunit NR2A in the anterior cingulate cortex in schizophrenia and bipolar disorder. *Arch Gen Psychiatry* Jul 2004;61(7):649-657.

104. Kristiansen LV, Beneyto M, Haroutunian V, Meador-Woodruff JH. Changes in NMDA receptor subunits and interacting PSD proteins in dorsolateral prefrontal and anterior cingulate cortex indicate abnormal regional expression in schizophrenia. *Mol Psychiatry* Aug 2006;11(8):737-747, 705.

105. Breese CR, Freedman R, Leonard SS. Glutamate receptor subtype expression in human postmortem brain tissue from schizophrenics and alcohol abusers. *Brain Res* 1995;674(1):82-90.

106. Eastwood SL, McDonald B, Burnet PW, Beckwith JP, Kerwin RW, Harrison PJ. Decreased expression of mRNAs encoding non-NMDA glutamate receptors GluR1 and GluR2 in medial temporal lobe neurons in schizophrenia. *Brain Res Mol Brain Res* 1995;29(2):211-223.

107. Eastwood SL, Burnet PW, Harrison PJ. GluR2 glutamate receptor subunit flip and flop isoforms are decreased in the hippocampal formation in schizophrenia: a reverse transcriptase- polymerase chain reaction (RT-PCR) study. *Brain Res Mol Brain Res* 1997;44(1):92-98.

108. Eastwood SL, Kerwin RW, Harrison PJ. Immunoautoradiographic evidence for a loss of alpha-amino-3-hydroxy-5- methyl-4-isoxazole propionate-preferring non-N-methyl-D-aspartate glutamate receptors within the medial temporal lobe in schizophrenia. *Biol Psychiatry* 1997;41(6):636-643.

109. Harrison PJ, McLaughlin D, Kerwin RW. Decreased hippocampal expression of a glutamate receptor gene in schizophrenia. *Lancet* 1991;337(8739):450-452.

110. Kerwin R, Patel S, Meldrum B. Quantitative autoradiographic analysis of glutamate binding sites in the hippocampal formation in normal and schizophrenic brain post mortem. *Neuroscience* 1990;39(1):25-32.

111. Freed WJ, Dillon-Carter O, Kleinman JE. Properties of [3H]AMPA binding in postmortem human brain from psychotic subjects and controls: increases in caudate nucleus associated with suicide. *Exp Neurol* 1993;121(1):48-56.

112. Beneyto M, Meador-Woodruff JH. Lamina-Specific abnormalities of AMPA receptor trafficking and signaling molecule transcripts in the prefrontal cortex in schizophrenia. *Synapse* 2006:In press.

113. Takamori S, Malherbe P, Broger C, Jahn R. Molecular cloning and functional characterization of human vesicular glutamate transporter 3. *EMBO Rep* Aug 2002;3(8):798-803.

114. Theberge J, Bartha R, Drost DJ, et al. Glutamate and glutamine measured with 4.0 T proton MRS in never-treated patients with schizophrenia and healthy volunteers. *Am J Psychiatry* Nov 2002;159(11):1944-1946.

115. Rutter AR, Freeman FM, Stephenson FA. Further characterization of the molecular interaction between PSD-95 and NMDA receptors: the effect of the NR1 splice variant and evidence for modulation of channel gating. *J Neurochem* Jun 2002;81(6):1298-1307.

116. Gupta DS, McCullumsmith RE, Beneyto M, Haroutunian V, Davis KL, Meador-Woodruff JH. Metabotropic glutamate receptor protein expression in the prefrontal cortex and striatum in schizophrenia. *Synapse* Sep 1 2005;57(3):123-131.

117. Ghose S, Gleason KA, Potts BW, Lewis-Amezcua K, Tamminga CA. Differential expression of metabotropic glutamate receptors 2 and 3 in schizophrenia: a mechanism for antipsychotic drug action? *Am J Psychiatry* Jul 2009;166(7):812-820.

118. Schluter K, Figiel M, Rozyczka J, Engele J. CNS region-specific regulation of glial glutamate transporter expression. *Eur J Neurosci* Sep 2002;16(5):836-842.

119. Kurumaji A, Ishimaru M, Toru M. Alpha-[3H]amino-3-hydroxy-5-methylisoxazole-4-propionic acid binding to human cerebral cortical membranes: minimal changes in postmortem brains of chronic schizophrenics. *J Neurochem* Sep 1992;59(3):829-837.

120. Dracheva S, McGurk SR, Haroutunian V. mRNA expression of AMPA receptors and AMPA receptor binding proteins in the cerebral cortex of elderly schizophrenics. *J Neurosci Res* Feb 4 2005;79(6):868-878.

121. Vawter MP, Crook JM, Hyde TM, Kleinman JE, Weinberger DR, Becker KG, Freed WJ. Microarray analysis of gene expression in the

prefrontal cortex in schizophrenia: a preliminary study. *Schizophr Res* Nov 1 2002;58(1):11-20.

122. Smith RE, Haroutunian V, Davis KL, Meador-Woodruff JH. Expression of glutaminase transcripts
in the thalamus in schizophrenia. *Biological Psychiatry* 2002;51:25.

123. Meador-Woodruff JH, Davis KL, Haroutunian V. Abnormal kainate receptor expression in prefrontal cortex in schizophrenia. *Neuropsychopharmacology* May 2001;24(5):545-552.

124. Tarsy D, Baldessarini RJ, Tarazi FI. Effects of newer antipsychotics on extrapyramidal function. *CNS Drugs* 2002;16(1):23-45.

125. Ohnuma T, Kato H, Arai H, Faull RL, McKenna PJ, Emson PC. Gene expression of PSD95 in prefrontal cortex and hippocampus in schizophrenia. *Neuroreport* Sep 28 2000;11(14):3133-3137.

126. Funk AJ, Rumbaugh G, Harotunian V, McCullumsmith RE, Meador-Woodruff JH. Decreased expression of NMDA receptor-associated proteins in frontal cortex of elderly patients with schizophrenia. *Neuroreport* Jul 15 2009;20(11):1019-1022.

127. Mirnics K, Middleton FA, Marquez A, Lewis DA, Levitt P. Molecular characterization of schizophrenia viewed by microarray analysis of gene expression in prefrontal cortex. *Neuron* Oct 2000;28(1):53-67.

128. Imai C, Sugai T, Iritani S, et al. A quantitative study on the expression of synapsin II and N-ethylmaleimide-sensitive fusion protein in schizophrenic patients. *Neurosci Lett* Jun 15 2001;305(3):185-188.

129. Gray L, Scarr E, Dean B. N-Ethylmaleimide sensitive factor in the cortex of subjects with schizophrenia and bipolar I disorder. *Neurosci Lett* Jan 2 2006;391(3):112-115.

130. Prabakaran S, Swatton JE, Ryan MM, et al. Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. *Mol Psychiatry* Jul 2004;9(7):684-697, 643.

131. Arriza JL, Kavanaugh MP, Fairman WA, Wu YN, Murdoch GH, North RA, Amara SG. Cloning and expression of a human neutral amino acid transporter with structural similarity to the glutamate transporter gene family. *J Biol Chem* 1993;268(21):15329-15332.

132. Utsunomiya-Tate N, Endou H, Kanai Y. Cloning and functional characterization of a system ASC-like Na+- dependent neutral amino acid transporter. *J Biol Chem* 1996;271(25):14883-14890.

133. Zerangue N, Kavanaugh MP. ASCT-1 is a neutral amino acid exchanger with chloride channel activity. *J Biol Chem* Nov 8 1996;271(45):27991-27994.

134. Levy LM, Warr O, Attwell D. Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a Chinese hamster ovary cell line selected for low endogenous Na+-dependent glutamate uptake. *J Neurosci* Dec 1 1998;18(23):9620-9628.

135. Gendreau S, Voswinkel S, Torres-Salazar D, et al. A trimeric quaternary structure is conserved in bacterial and human glutamate transporters. *J Biol Chem* Sep 17 2004;279(38):39505-39512.

136. Haugeto O, Ullensvang K, Levy LM, Chaudhry FA, Honore T, Nielsen M, Lehre KP, Danbolt NC. Brain glutamate transporter proteins form homomultimers. *J Biol Chem* Nov 1 1996;271(44):27715-27722.

137. Banner SJ, Fray AE, Ince PG, Steward M, Cookson MR, Shaw PJ. The expression of the glutamate re-uptake transporter excitatory amino acid transporter 1 (EAAT1) in the normal human CNS and in motor neurone disease: an immunohistochemical study. *Neuroscience* 2002;109(1):27-44.

138. Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW. Localization of neuronal and glial glutamate transporters. *Neuron* 1994;13(3):713-725.

139. Furuta A, Rothstein JD, Martin LJ. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. *J Neurosci* 1997;17(21):8363-8375.

140. Furuta A, Martin LJ, Lin CL, Dykes-Hoberg M, Rothstein JD. Cellular and synaptic localization of the neuronal glutamate transporters excitatory amino acid transporter 3 and 4. *Neuroscience* 1997;81(4):1031-1042.

141. Bar-Peled O, Ben-Hur H, Biegon A, Groner Y, Dewhurst S, Furuta A, Rothstein JD. Distribution of glutamate transporter subtypes during human brain development. *J Neurochem* 1997;69(6):2571-2580.

142. Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. *J Neurosci* Mar 1995;15(3 Pt 1):1835-1853.

143. Schmitt A, Asan E, Puschel B, Kugler P. Cellular and regional distribution of the glutamate transporter GLAST in the CNS of rats:

nonradioactive in situ hybridization and comparative immunocytochemistry. *J Neurosci* 1997;17(1):1-10.

144. Scott HL, Pow DV, Tannenberg AE, Dodd PR. Aberrant expression of the glutamate transporter excitatory amino acid transporter 1 (EAAT1) in Alzheimer's disease. *J Neurosci* Feb 1 2002;22(3):RC206.

145. Chaudhry FA, Lehre KP, van Lookeren Campagne M, Ottersen OP, Danbolt NC, Storm-Mathisen J. Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron* 1995;15(3):711-720.

146. Conradt M, Stoffel W. Inhibition of the high-affinity brain glutamate transporter GLAST-1 via direct phosphorylation. *J Neurochem* Mar 1997;68(3):1244-1251.

147. Figiel M, Maucher T, Rozyczka J, Bayatti N, Engele J. Regulation of glial glutamate transporter expression by growth factors. *Exp Neurol* Sep 2003;183(1):124-135.

148. Figiel M, Engele J. Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuron-derived peptide regulating glial glutamate transport and metabolism. *J Neurosci* May 15 2000;20(10):3596-3605.

149. Gamboa C, Ortega A. Insulin-like growth factor-1 increases activity and surface levels of the GLAST subtype of glutamate transporter. *Neurochem Int* Apr 2002;40(5):397-403.

150. Milton ID, Banner SJ, Ince PG, Piggott NH, Fray AE, Thatcher N, Horne CH, Shaw PJ. Expression of the glial glutamate transporter EAAT2 in the human CNS: an immunohistochemical study. *Brain Res Mol Brain Res* 1997;52(1):17-31.

151. Berger UV, Hediger MA. Comparative analysis of glutamate transporter expression in rat brain using differential double in situ hybridization. *Anat Embryol (Berl)* Jul 1998;198(1):13-30.

152. Rauen T, Kanner BI. Localization of the glutamate transporter GLT-1 in rat and macaque monkey retinae. *Neurosci Lett* Mar 14 1994;169(1-2):137-140.

153. Chen W, Mahadomrongkul V, Berger UV, et al. The glutamate transporter GLT1a is expressed in excitatory axon terminals of mature hippocampal neurons. *J Neurosci* Feb 4 2004;24(5):1136-1148.

154. Schmitt A, Asan E, Lesch KP, Kugler P. A splice variant of glutamate transporter GLT1/EAAT2 expressed in neurons: cloning and localization in rat nervous system. *Neuroscience* 2002;109(1):45-61.

155. Volterra A, Trotti D, Racagni G. Glutamate uptake is inhibited by arachidonic acid and oxygen radicals via two distinct and additive mechanisms. *Mol Pharmacol* 1994;46(5):986-992.

156. Hu S, Sheng WS, Ehrlich LC, Peterson PK, Chao CC. Cytokine effects on glutamate uptake by human astrocytes. *Neuroimmunomodulation* 2000;7(3):153-159.

157. Dunlop J, Lou Z, Zhang Y, McIlvain HB. Inducible expression and pharmacology of the human excitatory amino acid transporter 2 subtype of L-glutamate transporter. *Br J Pharmacol* 1999;128(7):1485-1490.

158. Conti F, DeBiasi S, Minelli A, Rothstein JD, Melone M. EAAC1, a high-affinity glutamate tranporter, is localized to astrocytes and gabaergic neurons besides pyramidal cells in the rat cerebral cortex. *Cereb Cortex* Mar 1998;8(2):108-116.

159. Kugler P, Schmitt A. Glutamate transporter EAAC1 is expressed in neurons and glial cells in the rat nervous system. *Glia* 1999;27(2):129-142.

160. He Y, Hof PR, Janssen WG, Rothstein JD, Morrison JH. Differential synaptic localization of GluR2 and EAAC1 in the macaque monkey entorhinal cortex: a postembedding immunogold study. *Neurosci Lett* Oct 5 2001;311(3):161-164.

161. He Y, Janssen WG, Rothstein JD, Morrison JH. Differential synaptic localization of the glutamate transporter EAAC1 and glutamate receptor subunit GluR2 in the rat hippocampus. *J Comp Neurol* Mar 13 2000;418(3):255-269.

162. Cheng C, Glover G, Banker G, Amara SG. A novel sorting motif in the glutamate transporter excitatory amino acid transporter 3 directs its targeting in Madin-Darby canine kidney cells and hippocampal neurons. *J Neurosci* Dec 15 2002;22(24):10643-10652.

163. Lortet S, Samuel D, Had-Aissouni L, Masmejean F, Kerkerian-Le Goff L, Pisano P. Effects of PKA and PKC modulators on high affinity glutamate uptake in primary neuronal cell cultures from rat cerebral cortex. *Neuropharmacology* Mar 1999;38(3):395-402.

164. Davis KE, Straff DJ, Weinstein EA, Bannerman PG, Correale DM, Rothstein JD, Robinson MB. Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma. *J Neurosci* Apr 1 1998;18(7):2475-2485.

165. Dowd LA, Robinson MB. Rapid stimulation of EAAC1-mediated Na+-dependent L-glutamate transport activity in C6 glioma cells by phorbol ester. *J Neurochem* Aug 1996;67(2):508-516.

166. Dowd LA, Coyle AJ, Rothstein JD, Pritchett DB, Robinson MB. Comparison of Na+-dependent glutamate transport activity in synaptosomes, C6 glioma, and Xenopus oocytes expressing excitatory amino acid carrier 1 (EAAC1). *Mol Pharmacol* Mar 1996;49(3):465-473.

167. Sims KD, Straff DJ, Robinson MB. Platelet-derived growth factor rapidly increases activity and cell surface expression of the EAAC1 subtype of glutamate transporter through activation of phosphatidylinositol 3-kinase. *J Biol Chem* Feb 18 2000;275(7):5228-5237.

168. Najimi M, Maloteaux JM, Hermans E. Cytoskeleton-related trafficking of the EAAC1 glutamate transporter after activation of the G(q/11)-coupled neurotensin receptor NTS1. *FEBS Lett* Jul 17 2002;523(1-3):224-228.

169. Ma K, Zheng S, Zuo Z. The transcription factor regulatory factor X1 increases the expression of neuronal glutamate transporter type 3. *J Biol Chem* Jul 28 2006;281(30):21250-21255.

170. Yamada K, Watanabe M, Shibata T, Tanaka K, Wada K, Inoue Y. EAAT4 is a post-synaptic glutamate transporter at Purkinje cell synapses. *Neuroreport* 1996;7(12):2013-2017.

171. Nagao S, Kwak S, Kanazawa I. EAAT4, a glutamate transporter with properties of a chloride channel, is predominantly localized in Purkinje cell dendrites, and forms parasagittal compartments in rat cerebellum. *Neuroscience* 1997;78(4):929-933.

172. Hu WH, Walters WM, Xia XM, Karmally SA, Bethea JR. Neuronal glutamate transporter EAAT4 is expressed in astrocytes. *Glia* Oct 2003;44(1):13-25.

173. McCullumsmith RE, Meador-Woodruff JH. Striatal excitatory amino acid transporter transcript expression in schizophrenia, bipolar disorder, and major depressive disorder. *Neuropsychopharmacology* Mar 2002;26(3):368-375.

174. Massie A, Vandesande F, Arckens L. Expression of the high-affinity glutamate transporter EAAT4 in mammalian cerebral cortex. *Neuroreport* Feb 12 2001;12(2):393-397.

175. Dehnes Y, Chaudhry FA, Ullensvang K, Lehre KP, Storm-Mathisen J, Danbolt NC. The glutamate transporter EAAT4 in rat cerebellar Purkinje cells: a glutamate-gated chloride channel concentrated near the synapse in parts of the dendritic membrane facing astroglia. *J Neurosci* 1998;18(10):3606-3619.

176. Walsh T, McClellan JM, McCarthy SE, et al. Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. *Science* Apr 25 2008;320(5875):539-543.

177. Patil ST, Zhang L, Martenyi F, et al. Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nat Med* Sep 2007;13(9):1102-1107.

178. Ohnuma T, Augood SJ, Arai H, McKenna PJ, Emson PC. Expression of the human excitatory amino acid transporter 2 and metabotropic glutamate receptors 3 and 5 in the prefrontal cortex from normal individuals and patients with schizophrenia. *Brain Res Mol Brain Res* 1998;56(1-2):207-217.

179. Matute C, Melone M, Vallejo-Illarramendi A, Conti F. Increased expression of the astrocytic glutamate transporter GLT-1 in the prefrontal cortex of schizophrenics. *Glia* Feb 2005;49(3):451-455.

180. Lauriat TL, Dracheva S, Chin B, Schmeidler J, McInnes LA, Haroutunian V. Quantitative analysis of glutamate transporter mRNA expression in prefrontal and primary visual cortex in normal and schizophrenic brain. *Neuroscience* Feb 2006;137(3):843-851.

181. Chaudhry FA, Lehre KP, van Lookeren Campagne M, Ottersen OP, Danbolt NC, Storm-Mathisen J. Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron* Sep 1995;15(3):711-720.

182. Kugler P, Schmitt A. Complementary neuronal and glial expression of two high-affinity glutamate transporter GLT1/EAAT2 forms in rat cerebral cortex. *Histochem Cell Biol* Jun 2003;119(6):425-435.

183. Maragakis NJ, Dykes-Hoberg M, Rothstein JD. Altered expression of the glutamate transporter EAAT2b in neurological disease. *Ann Neurol* Apr 2004;55(4):469-477.

184. Ohnuma T, Tessler S, Arai H, Faull RL, McKenna PJ, Emson PC. Gene expression of metabotropic glutamate receptor 5 and excitatory amino acid transporter 2 in the schizophrenic hippocampus. *Brain Res Mol Brain Res* Dec 28 2000;85(1-2):24-31.

185. Eastwood SL, Harrison PJ. Decreased expression of vesicular glutamate transporter 1 and complexin II mRNAs in schizophrenia: further evidence for a synaptic pathology affecting glutamate neurons. *Schizophr Res* Mar 1 2005;73(2-3):159-172.

186. Wilson NR, Kang J, Hueske EV, Leung T, Varoqui H, Murnick JG, Erickson JD, Liu G. Presynaptic regulation of quantal size by the vesicular glutamate transporter VGLUT1. *J Neurosci* Jun 29 2005;25(26):6221-6234.

187. Marie H, Attwell D. C-terminal interactions modulate the affinity of GLAST glutamate transporters in salamander retinal glial cells. *J Physiol* Oct 15 1999;520 Pt 2:393-397.

188. Kinoshita N, Kimura K, Matsumoto N, Watanabe M, Fukaya M, Ide C. Mammalian septin Sept2 modulates the activity of GLAST, a glutamate transporter in astrocytes. *Genes Cells* Jan 2004;9(1):1-14.

189. Sullivan SM, Lee A, Bjorkman ST, Miller SM, Sullivan RK, Poronnik P, Colditz PB, Pow DV. Cytoskeletal anchoring of GLAST determines susceptibility to brain damage: an identified role for GFAP. *J Biol Chem* Oct 5 2007;282(40):29414-29423.

190. Lee A, Rayfield A, Hryciw DH, et al. Na+-H+ exchanger regulatory factor 1 is a PDZ scaffold for the astroglial glutamate transporter GLAST. *Glia* Jan 15 2007;55(2):119-129.

191. Gonzalez MI, Susarla BT, Robinson MB. Evidence that protein kinase Calpha interacts with and regulates the glial glutamate transporter GLT-1. *J Neurochem* Sep 2005;94(5):1180-1188.

192. Marie H, Pratt SJ, Betson M, et al. The LIM protein Ajuba is recruited to cadherin-dependent cell junctions through an association with alpha-catenin. *J Biol Chem* Jan 10 2003;278(2):1220-1228.

193. Boston-Howes W, Gibb SL, Williams EO, Pasinelli P, Brown RH, Jr., Trotti D. Caspase-3 cleaves and inactivates the glutamate transporter EAAT2. *J Biol Chem* May 19 2006;281(20):14076-14084.

194. Gonzalez MI, Bannerman PG, Robinson MB. Phorbol myristate acetate-dependent interaction of protein kinase Calpha and the neuronal glutamate transporter EAAC1. *J Neurosci* Jul 2 2003;23(13):5589-5593.

195. Ruggiero A, Vidensky S, Rothstein JD. GTRAP3-18 protein is able to regulate the activity of excitatory amino acid transporters through alterations in ASN linked glycosyl processing. *Abstract viewer, Society for Neuroscience* 2003:Program No. 372.315.

196. Butchbach ME, Lai L, Lin CL. Molecular cloning, gene structure, expression profile and functional characterization of the mouse glutamate transporter (EAAT3) interacting protein GTRAP3-18. *Gene* Jun 12 2002;292(1-2):81-90.

197. Schweneker M, Bachmann AS, Moelling K. JM4 is a four-transmembrane protein binding to the CCR5 receptor. *FEBS Lett* Mar 14 2005;579(7):1751-1758.

198. Xia P, Pei G, Schwarz W. Regulation of the glutamate transporter EAAC1 by expression and activation of delta-opioid receptor. *Eur J Neurosci* Jul 2006;24(1):87-93.

199. Zhu Y, Fei J, Schwarz W. Expression and transport function of the glutamate transporter EAAC1 in Xenopus oocytes is regulated by syntaxin 1A. *J Neurosci Res* Feb 15 2005;79(4):503-508.

200. Yu YX, Shen L, Xia P, Tang YW, Bao L, Pei G. Syntaxin 1A promotes the endocytic sorting of EAAC1 leading to inhibition of glutamate transport. *J Cell Sci* Sep 15 2006;119(Pt 18):3776-3787.

201. Fournier KM, Robinson MB. A dominant-negative variant of SNAP-23 decreases the cell surface expression of the neuronal glutamate transporter EAAC1 by slowing constitutive delivery. *Neurochem Int* May-Jun 2006;48(6-7):596-603.

202. Kiryu-Seo S, Gamo K, Tachibana T, Tanaka K, Kiyama H. Unique anti-apoptotic activity of EAAC1 in injured motor neurons. *Embo J* Jul 26 2006;25(14):3411-3421.

203. Kalandadze A, Wu Y, Robinson MB. Protein kinase C activation decreases cell surface expression of the GLT-1 subtype of glutamate transporter. Requirement of a carboxyl-terminal domain and partial dependence on serine 486. *J Biol Chem* Nov 29 2002;277(48):45741-45750.

204. Casado M, Bendahan A, Zafra F, Danbolt NC, Aragon C, Gimenez C, Kanner BI. Phosphorylation and modulation of brain glutamate transporters by protein kinase C. *J Biol Chem* Dec 25 1993;268(36):27313-27317.

205. Fang H, Huang Y, Zuo Z. The different responses of rat glutamate transporter type 2 and its mutant (tyrosine 403 to histidine) activity to volatile anesthetics and activation of protein kinase C. *Brain Res* Oct 25 2002;953(1-2):255-264.

206. Daniels KK, Vickroy TW. Reversible activation of glutamate transport in rat brain glia by protein kinase C and an okadaic acid-sensitive phosphoprotein phosphatase. *Neurochem Res* 1999;24(8):1017-1025.

207. Sheldon AL, Gonzalez MI, Krizman-Genda EN, Susarla BT, Robinson MB. Ubiquitination-mediated internalization and degradation of the astroglial glutamate transporter, GLT-1. *Neurochem Int* Dec 2008;53(6-8):296-308.

208. Yang L, Wang S, Sung B, Lim G, Mao J. Morphine induces ubiquitin-proteasome activity and glutamate transporter degradation. *J Biol Chem* Aug 1 2008;283(31):21703-21713.

209. Conradt M, Storck T, Stoffel W. Localization of N-glycosylation sites and functional role of the carbohydrate units of GLAST-1, a cloned rat brain L-glutamate/L-aspartate transporter. *Eur J Biochem* May 1 1995;229(3):682-687.

210. Huggett J, Vaughan-Thomas A, Mason D. The open reading frame of the Na(+)-dependent glutamate transporter GLAST-1 is expressed in bone and a splice variant of this molecule is expressed in bone and brain. *FEBS Lett* Nov 17 2000;485(1):13-18.

211. Raunser S, Haase W, Bostina M, Parcej DN, Kuhlbrandt W. High-yield expression, reconstitution and structure of the recombinant, fully functional glutamate transporter GLT-1 from Rattus norvegicus. *J Mol Biol* Aug 19 2005;351(3):598-613.

212. Schulte S, Stoffel W. UDP galactose:ceramide galactosyltransferase and glutamate/aspartate transporter. Copurification, separation and characterization of the two glycoproteins. *Eur J Biochem* Nov 1 1995;233(3):947-953.

213. Yang W, Kilberg MS. Biosynthesis, intracellular targeting, and degradation of the EAAC1 glutamate/aspartate transporter in C6 glioma cells. *J Biol Chem* Oct 11 2002;277(41):38350-38357.

214. Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. *Cell* Sep 8 2006;126(5):855-867.

215. Narayan S, Head SR, Gilmartin TJ, Dean B, Thomas EA. Evidence for disruption of sphingolipid metabolism in schizophrenia. *J Neurosci Res* Aug 5 2008.

216. Trotti D, Aoki M, Pasinelli P, Berger UV, Danbolt NC, Brown RH, Jr., Hediger MA. Amyotrophic lateral sclerosis-linked glutamate transporter mutant has impaired glutamate clearance capacity. *J Biol Chem* Jan 5 2001;276(1):576-582.

217. Meyer T, Fromm A, Munch C, et al. The RNA of the glutamate transporter EAAT2 is variably spliced in amyotrophic lateral sclerosis and normal individuals. *J Neurol Sci* 1999;170(1):45-50.

218. Munch C, Penndorf A, Schwalenstocker B, Troost D, Ludolph AC, Ince P, Meyer T. Impaired RNA splicing of 5'-regulatory sequences of the astroglial glutamate transporter EAAT2 in human astrocytoma. *J Neurol Neurosurg Psychiatry* Nov 2001;71(5):675-678.

219. Honig LS, Chambliss DD, Bigio EH, Carroll SL, Elliott JL. Glutamate transporter EAAT2 splice variants occur not only in ALS, but also in AD and controls. *Neurology* Oct 24 2000;55(8):1082-1088.

220. Lauriat TL, Schmeidler J, McInnes LA. Early rapid rise in EAAT2 expression follows the period of maximal seizure susceptibility in human brain. *Neurosci Lett* Jan 22 2007;412(1):89-94.

221. Lauriat TL, Richler E, McInnes LA. A quantitative regional expression profile of EAAT2 known and novel splice variants reopens the question of aberrant EAAT2 splicing in disease. *Neurochem Int* Jan 2007;50(1):271-280.

222. Hoogland G, van Oort RJ, Proper EA, et al. Alternative splicing of glutamate transporter EAAT2 RNA in neocortex and hippocampus of temporal lobe epilepsy patients. *Epilepsy Res* Apr-May 2004;59(2-3):75-82.

223. Macnab LT, Pow DV. Expression of the exon 9-skipping form of EAAT2 in astrocytes of rats. *Neuroscience* Dec 12 2007;150(3):705-711.

224. Pow DV, Cook DG. Neuronal Expression of Splice Variants of "Glial" Glutamate Transporters in Brains Afflicted by Alzheimer's Disease: Unmasking an Intrinsic Neuronal Property. *Neurochem Res* Mar 25 2009.

225. Lauriat TL, McInnes LA. EAAT2 regulation and splicing: relevance to psychiatric and neurological disorders. *Mol Psychiatry* Dec 2007;12(12):1065-1078.

226. Sullivan SM, Macnab LT, Bjorkman ST, Colditz PB, Pow DV. GLAST1b, the exon-9 skipping form of the glutamate-aspartate transporter EAAT1 is a sensitive marker of neuronal dysfunction in the hypoxic brain. *Neuroscience* Oct 26 2007;149(2):434-445.

227. Macnab LT, Williams SM, Pow DV. Expression of the exon 3 skipping form of GLAST, GLAST1a, in brain and retina. *Neuroreport* Dec 18 2006;17(18):1867-1870.

228. Macnab LT, Pow DV. Central nervous system expression of the exon 9 skipping form of the glutamate transporter GLAST. *Neuroreport* May 28 2007;18(8):741-745.

229. Vallejo-Illarramendi A, Domercq M, Matute C. A novel alternative splicing form of excitatory amino acid transporter 1 is a negative regulator of glutamate uptake. *J Neurochem* Oct 2005;95(2):341-348.

230. Kalandadze A, Wu Y, Fournier K, Robinson MB. Identification of motifs involved in endoplasmic reticulum retention-forward trafficking of the GLT-1 subtype of glutamate transporter. *J Neurosci* Jun 2 2004;24(22):5183-5192.

231. Kindlundh-Hogberg AM, Blomqvist A, Malki R, Schioth HB. Extensive neuroadaptive changes in cortical gene-transcript expressions of the glutamate system in response to repeated intermittent MDMA administration in adolescent rats. *BMC Neurosci* 2008;9:39.

232. Arriza JL, Eliasof S, Kavanaugh MP, Amara SG. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc Natl Acad Sci U S A* 1997;94(8):4155-4160.

233. Williams SM, Sullivan RK, Scott HL, Finkelstein DI, Colditz PB, Lingwood BE, Dodd PR, Pow DV. Glial glutamate transporter expression patterns in brains from multiple mammalian species. *Glia* Mar 2005;49(4):520-541.

234. Levenson J, Weeber E, Selcher JC, Kategaya LS, Sweatt JD, Eskin A. Long-term potentiation and contextual fear conditioning increase neuronal glutamate uptake. *Nat Neurosci* Feb 2002;5(2):155-161.

235. Crino PB, Jin H, Shumate MD, Robinson MB, Coulter DA, Brooks-Kayal AR. Increased expression of the neuronal glutamate transporter (EAAT3/EAAC1) in hippocampal and neocortical epilepsy. *Epilepsia* Mar 2002;43(3):211-218.

236. Manoach DS. Prefrontal cortex dysfunction during working memory performance in schizophrenia: reconciling discrepant findings. *Schizophr Res* Apr 1 2003;60(2-3):285-298.

237. Tamminga CA, Thaker GK, Buchanan R, Kirkpatrick B, Alphs LD, Chase TN, Carpenter WT. Limbic system abnormalities identified in schizophrenia using positron emission tomography with fluorodeoxyglucose and neocortical alterations with deficit syndrome. *Arch Gen Psychiatry* Jul 1992;49(7):522-530.

238. Liu YL, Shen-Jang Fann C, Liu CM, et al. Evaluation of RGS4 as a candidate gene for schizophrenia. *Am J Med Genet B Neuropsychiatr Genet* Jun 5 2006;141(4):418-420.

239. Varoqui H, Schafer MK, Zhu H, Weihe E, Erickson JD. Identification of the differentiation-associated Na+/PI transporter as a novel vesicular glutamate transporter expressed in a distinct set of glutamatergic synapses. *J Neurosci* Jan 1 2002;22(1):142-155.

240. Saransaari P, Lillrank SM, Oja SS. Phencyclidine treatment in mice: effects on phencyclidine binding sites and glutamate uptake in cerebral cortex preparations. *J Neural Transm Gen Sect* 1993;93(1):47-59.

241. Goff DC, Wine L. Glutamate in schizophrenia: clinical and research implications. *Schizophr Res* Oct 30 1997;27(2-3):157-168.

242. Coyle JT. The glutamatergic dysfunction hypothesis for schizophrenia. *Harv Rev Psychiatry* Jan-Feb 1996;3(5):241-253.

243. Itil T, Keskiner A, Kiremitci N, Holden JM. Effect of phencyclidine in chronic schizophrenics. *Can Psychiatr Assoc J* 1967;12(2):209-212.

244. Aanonsen LM, Wilcox GL. Phencyclidine selectively blocks a spinal action of N-methyl-D- aspartate in mice. *Neurosci Lett* 1986;67(2):191-197.

245. Krystal JH, Karper LP, Seibyl JP, et al. Subanesthetic effects of the noncompetitive NMDA antagonist, ketamine, in humans. Psychotomimetic, perceptual, cognitive, and neuroendocrine responses. *Arch Gen Psychiatry* 1994;51(3):199-214.

246. Bjerkenstedt L, Edman G, Hagenfeldt L, Sedvall G, Wiesel FA. Plasma amino acids in relation to cerebrospinal fluid monoamine metabolites in schizophrenic patients and healthy controls. *Br J Psychiatry* Sep 1985;147:276-282.

247. Macciardi F, Lucca A, Catalano M, Marino C, Zanardi R, Smeraldi E. Amino acid patterns in schizophrenia: some new findings. *Psychiatry Res* Apr 1990;32(1):63-70.

248. Gattaz WF, Gasser T, Beckmann H. Multidimensional analysis of the concentrations of 17 substances in the CSF of schizophrenics and controls. *Biol Psychiatry* Apr 1985;20(4):360-366.

249. Perry TL. Normal cerebrospinal fluid and brain glutamate levels in schizophrenia do not support the hypothesis of glutamatergic neuronal dysfunction. *Neurosci Lett* Jan 22 1982;28(1):81-85.

250. Korpi ER, Kaufmann CA, Marnela KM, Weinberger DR. Cerebrospinal fluid amino acid concentrations in chronic schizophrenia. *Psychiatry Res* Apr 1987;20(4):337-345.

251. Alfredsson G, Wiesel FA. Monoamine metabolites and amino acids in serum from schizophrenic patients before and during sulpiride treatment. *Psychopharmacology (Berl)* 1989;99(3):322-327.

252. Deutsch SI, Mastropaolo J, Schwartz BL, Rosse RB, Morihisa JM. A "glutamatergic hypothesis" of schizophrenia. Rationale for pharmacotherapy with glycine. *Clin Neuropharmacol* Feb 1989;12(1):1-13.

253. Omori M, Pearce J, Komoroski RA, Griffin WS, Mrak RE, Husain MM, Karson CN. In vitro 1H-magnetic resonance spectroscopy of postmortem brains with schizophrenia. *Biol Psychiatry* Sep 1 1997;42(5):359-366.

254. Kegeles LS, Humaran TJ, Mann JJ. In vivo neurochemistry of the brain in schizophrenia as revealed by magnetic resonance spectroscopy. *Biol Psychiatry* Sep 15 1998;44(6):382-398.

255. Deicken RF, Zhou L, Schuff N, Weiner MW. Proton magnetic resonance spectroscopy of the anterior cingulate region in schizophrenia. *Schizophr Res* Oct 17 1997;27(1):65-71.

256. Steel RM, Bastin ME, McConnell S, Marshall I, Cunningham-Owens DG, Lawrie SM, Johnstone EC, Best JJ. Diffusion tensor imaging (DTI) and proton magnetic resonance spectroscopy (1H MRS) in schizophrenic subjects and normal controls. *Psychiatry Res* May 30 2001;106(3):161-170.

257. Burbaeva G, Boksha IS, Turishcheva MS, Vorobyeva EA, Savushkina OK, Tereshkina EB. Glutamine synthetase and glutamate dehydrogenase in the prefrontal cortex of patients with schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry* Jun 2003;27(4):675-680.

258. Callicott JH, Bertolino A, Egan MF, Mattay VS, Langheim FJ, Weinberger DR. Selective relationship between prefrontal N-acetylaspartate measures and negative symptoms in schizophrenia. *Am J Psychiatry* Oct 2000;157(10):1646-1651.

259. Cecil KM, Lenkinski RE, Gur RE, Gur RC. Proton magnetic resonance spectroscopy in the frontal and temporal lobes of neuroleptic naive patients with schizophrenia. *Neuropsychopharmacology* Feb 1999;20(2):131-140.

260. Bertolino A, Callicott JH, Nawroz S, Mattay VS, Duyn JH, Tedeschi G, Frank JA, Weinberger DR. Reproducibility of proton magnetic resonance spectroscopic imaging in patients with schizophrenia. *Neuropsychopharmacology* Jan 1998;18(1):1-9.

261. Choe BY, Kim KT, Suh TS, Lee C, Paik IH, Bahk YW, Shinn KS, Lenkinski RE. 1H magnetic resonance spectroscopy characterization of neuronal dysfunction in drug-naive, chronic schizophrenia. *Acad Radiol* Nov 1994;1(3):211-216.

262. Egan MF, Straub RE, Goldberg TE, et al. Variation in GRM3 affects cognition, prefrontal glutamate, and risk for schizophrenia. *Proc Natl Acad Sci U S A* Aug 24 2004;101(34):12604-12609.

263. Oni-Orisan A, Kristiansen LV, Haroutunian V, Meador-Woodruff JH, McCullumsmith RE. Altered Vesicular Glutamate Transporter Expression in the Anterior Cingulate Cortex in Schizophrenia. *Biol Psychiatry* Dec 21 2007.

264. Rajkowska G, Goldman-Rakic PS. Cytoarchitectonic definition of prefrontal areas in the normal human cortex: I. Remapping of areas 9 and 46 using quantitative criteria. *Cereb Cortex* Jul-Aug 1995;5(4):307-322.

265. Lauderback CM, Hackett JM, Huang FF, Keller JN, Szweda LI, Markesbery WR, Butterfield DA. The glial glutamate transporter, GLT-1, is oxidatively modified by 4-hydroxy-2-nonenal in the Alzheimer's disease brain: the role of Abeta1-42. *J Neurochem* Jul 2001;78(2):413-416.

266. Stan AD, Ghose S, Gao XM, Roberts RC, Lewis-Amezcua K, Hatanpaa KJ, Tamminga CA. Human postmortem tissue: what quality markers matter? *Brain Res* Dec 6 2006;1123(1):1-11.

267. Robinson MB. The family of sodium-dependent glutamate transporters: a focus on the GLT-1/EAAT2 subtype. *Neurochem Int* 1998;33(6):479-491.

268. Watanabe M, Ueki T, Jackson M, Ruggiero AM, Sawa A, Kalandadze A, Robinson MB, Rothstein JD. CSN1 Downregulates GLT-1 activity. Paper presented at: SFN Abstract, 2004.

269. Spain BH, Bowdish KS, Pacal AR, Staub SF, Koo D, Chang CY, Xie W, Colicelli J. Two human cDNAs, including a homolog of Arabidopsis FUS6 (COP11), suppress G-protein- and mitogen-activated protein kinase-mediated signal transduction in yeast and mammalian cells. *Mol Cell Biol* Dec 1996;16(12):6698-6706.

270. Cartmell J, Schoepp DD. Regulation of neurotransmitter release by metabotropic glutamate receptors. *J Neurochem* Sep 2000;75(3):889-907.

271. van Holde KE. *Physical Biochemistry*. 2nd ed. Englewood Cliffs, NJ: Prentice-Hall, Inc.; 1985.

272. Diamond JS. Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. *J Neurosci* Nov 1 2001;21(21):8328-8338.

273. Huang YH, Bergles DE. Glutamate transporters bring competition to the synapse. *Curr Opin Neurobiol* Jun 2004;14(3):346-352.

274. Lin CL, Tzingounis AV, Jin L, Furuta A, Kavanaugh MP, Rothstein JD. Molecular cloning and expression of the rat EAAT4 glutamate transporter subtype. *Brain Res Mol Brain Res* 1998;63(1):174-179.

275. Holleran EA, Ligon LA, Tokito M, Stankewich MC, Morrow JS, Holzbaur EL. beta III spectrin binds to the Arp1 subunit of dynactin. *J Biol Chem* Sep 28 2001;276(39):36598-36605.

276. Stankewich MC, Tse WT, Peters LL, et al. A widely expressed betaIII spectrin associated with Golgi and cytoplasmic vesicles. *Proc Natl Acad Sci U S A* Nov 24 1998;95(24):14158-14163.

277. Katsel P, Davis KL, Gorman JM, Haroutunian V. Variations in differential gene expression patterns across multiple brain regions in schizophrenia. *Schizophr Res* Sep 15 2005;77(2-3):241-252.

278. De Souza IE, McBean GJ, Meredith GE. Chronic haloperidol treatment impairs glutamate transport in the rat striatum. *Eur J Pharmacol* 1999;382(2):139-142.

279. Melone M, Vitellaro-Zuccarello L, Vallejo-Illarramendi A, et al. The expression of glutamate transporter GLT-1 in the rat cerebral cortex is down-regulated by the antipsychotic drug clozapine. *Mol Psychiatry* 2001;6(4):380-386.

280. Takeuchi M, Yamagishi S. Involvement of toxic AGEs (TAGE) in the pathogenesis of diabetic vascular complications and Alzheimer's disease. *J Alzheimers Dis* Apr 2009;16(4):845-858.

281. Hung WY, Mold DE, Tourian A. Huntington's-chorea fibroblasts. Cellular protein glycosylation. *Biochem J* Sep 15 1980;190(3):711-719.

282. Gegelashvili G, Civenni G, Racagni G, Danbolt NC, Schousboe I, Schousboe A. Glutamate receptor agonists up-regulate glutamate

transporter GLAST in astrocytes. *Neuroreport* Dec 20 1996;8(1):261-265.

283. Kondo K, Hashimoto H, Kitanaka J, Sawada M, Suzumura A, Marunouchi T, Baba A. Expression of glutamate transporters in cultured glial cells. *Neurosci Lett* Mar 24 1995;188(2):140-142.

284. Escartin C, Brouillet E, Gubellini P, et al. Ciliary neurotrophic factor activates astrocytes, redistributes their glutamate transporters GLAST and GLT-1 to raft microdomains, and improves glutamate handling in vivo. *J Neurosci* May 31 2006;26(22):5978-5989.

285. Shashidharan P, Huntley GW, Murray JM, Buku A, Moran T, Walsh MJ, Morrison JH, Plaitakis A. Immunohistochemical localization of the neuron-specific glutamate transporter EAAC1 (EAAT3) in rat brain and spinal cord revealed by a novel monoclonal antibody. *Brain Res* Oct 31 1997;773(1-2):139-148.

286. Bauer D, Gupta D, Harotunian V, Meador-Woodruff JH, McCullumsmith RE. Abnormal expression of glutamate transporter and transporter interacting molecules in prefrontal cortex in elderly patients with schizophrenia. *Schizophr Res* Sep 2008;104(1-3):108-120.

287. Tsvetkov E, Shin RM, Bolshakov VY. Glutamate uptake determines pathway specificity of long-term potentiation in the neural circuitry of fear conditioning. *Neuron* Jan 8 2004;41(1):139-151.

288. Overstreet LS, Kinney GA, Liu YB, Billups D, Slater NT. Glutamate transporters contribute to the time course of synaptic transmission in cerebellar granule cells. *J Neurosci* Nov 1 1999;19(21):9663-9673.

289. Marcaggi P, Attwell D. Short- and long-term depression of rat cerebellar parallel fibre synaptic transmission mediated by synaptic crosstalk. *J Physiol* Jan 15 2007;578(Pt 2):545-550.

290. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* May 7 1976;72:248-254.

291. Jarvie KR, Niznik HB, Seeman P. Dopamine D2 receptor binding subunits of Mr congruent to 140,000 and 94,000 in brain: deglycosylation yields a common unit of Mr congruent to 44,000. *Mol Pharmacol* Aug 1988;34(2):91-97.

292. Jimenez-Huete A, Lievens PM, Vidal R, Piccardo P, Ghetti B, Tagliavini F, Frangione B, Prelli F. Endogenous proteolytic cleavage of normal and disease-associated isoforms of the human prion protein in

neural and non-neural tissues. *Am J Pathol* Nov 1998;153(5):1561-1572.

293. Nielsen D, Gyllberg H, Ostlund P, Bergman T, Bedecs K. Increased levels of insulin and insulin-like growth factor-1 hybrid receptors and decreased glycosylation of the insulin receptor alpha- and beta-subunits in scrapie-infected neuroblastoma N2a cells. *Biochem J* Jun 1 2004;380(Pt 2):571-579.

294. Toledo JR, Sanchez O, Montesino Segui R, Fernandez Garcia Y, Rodriguez MP, Cremata JA. Differential in vitro and in vivo glycosylation of human erythropoietin expressed in adenovirally transduced mouse mammary epithelial cells. *Biochim Biophys Acta* Oct 30 2005;1726(1):48-56.

295. Maguire TM, Thakore J, Dinan TG, Hopwood S, Breen KC. Plasma sialyltransferase levels in psychiatric disorders as a possible indicator of HPA axis function. *Biol Psychiatry* Jun 1 1997;41(11):1131-1136.

296. Varma R, Hoshino AY. Serum glycoproteins in schizophrenia. *Carbohydr Res* Jul 1980;82(2):343-351.

297. Barbeau D, Liang JJ, Robitalille Y, Quirion R, Srivastava LK. Decreased expression of the embryonic form of the neural cell adhesion molecule in schizophrenic brains. *Proc Natl Acad Sci U S A* Mar 28 1995;92(7):2785-2789.

298. Holmseth S, Dehnes Y, Bjornsen LP, Boulland JL, Furness DN, Bergles D, Danbolt NC. Specificity of antibodies: unexpected cross-reactivity of antibodies directed against the excitatory amino acid transporter 3 (EAAT3). *Neuroscience* 2005;136(3):649-660.

299. Tzingounis AV, Wadiche JI. Glutamate transporters: confining runaway excitation by shaping synaptic transmission. *Nat Rev Neurosci* Dec 2007;8(12):935-947.

300. Tong G, Jahr CE. Block of glutamate transporters potentiates postsynaptic excitation. *Neuron* Nov 1994;13(5):1195-1203.

301. Ruggiero AM, Liu Y, Vidensky S, et al. The endoplasmic reticulum exit of glutamate transporter is regulated by the inducible mammalian Yip6b/GTRAP3-18 protein. *J Biol Chem* Mar 7 2008;283(10):6175-6183.

302. Tucholski J, Patel S, Bauer D, Haroutunian V, Meador-Woodruff JH. Decreased Protein Expression and Alteration of N-Glycosylation of the Glur2 Ampa Receptor Subunit in Schizophrenia. *Schizophrenia Bulletin* 2009;35:234-234.

303. Wood SJ, Yucel M, Pantelis C, Berk M. Neurobiology of schizophrenia spectrum disorders: the role of oxidative stress. *Ann Acad Med Singapore* May 2009;38(5):396-396.

304. Chakravarthi S, Jessop CE, Bulleid NJ. The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. *EMBO Rep* Mar 2006;7(3):271-275.

305. Olbrich HM, Valerius G, Rusch N, Buchert M, Thiel T, Hennig J, Ebert D, Van Elst LT. Frontolimbic glutamate alterations in first episode schizophrenia: evidence from a magnetic resonance spectroscopy study. *World J Biol Psychiatry* 2008;9(1):59-63.

306. van Elst LT, Valerius G, Buchert M, et al. Increased prefrontal and hippocampal glutamate concentration in schizophrenia: evidence from a magnetic resonance spectroscopy study. *Biol Psychiatry* Nov 1 2005;58(9):724-730.

307. Fedorenko O, Tang C, Sopjani M, et al. PIP5K2A-dependent regulation of excitatory amino acid transporter EAAT3. *Psychopharmacology (Berl)* Jul 31 2009.

308. Steffek AE, McCullumsmith RE, Haroutunian V, Meador-Woodruff JH. Cortical expression of glial fibrillary acidic protein and glutamine synthetase is decreased in schizophrenia. *Schizophr Res* Aug 2008;103(1-3):71-82.

309. Anand A, Charney DS, Oren DA, Berman RM, Hu XS, Cappiello A, Krystal JH. Attenuation of the neuropsychiatric effects of ketamine with lamotrigine: support for hyperglutamatergic effects of N-methyl-D-aspartate receptor antagonists. *Arch Gen Psychiatry* Mar 2000;57(3):270-276.

310. Moghaddam B, Adams BW. Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats. *Science* Aug 28 1998;281(5381):1349-1352.

311. Farber NB. The NMDA receptor hypofunction model of psychosis. *Ann N Y Acad Sci* Nov 2003;1003:119-130.

312. Fattorini G, Melone M, Bragina L, et al. GLT-1 expression and Glu uptake in rat cerebral cortex are increased by phencyclidine. *Glia* Sep 2008;56(12):1320-1327.

313. Lieberman JA, Papadakis K, Csernansky J, Litman R, Volavka J, Jia XD, Gage A. A randomized, placebo-controlled study of memantine as adjunctive treatment in patients with schizophrenia. *Neuropsychopharmacology* Apr 2009;34(5):1322-1329.

314. Sanacora G, Zarate CA, Krystal JH, Manji HK. Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. *Nat Rev Drug Discov* May 2008;7(5):426-437.

315. Kline DD, Hendricks G, Hermann G, Rogers RC, Kunze DL. Dopamine inhibits N-type channels in visceral afferents to reduce synaptic transmitter release under normoxic and chronic intermittent hypoxic conditions. *J Neurophysiol* May 2009;101(5):2270-2278.

316. Govindaiah G, Cox CL. Depression of retinogeniculate synaptic transmission by presynaptic D(2)-like dopamine receptors in rat lateral geniculate nucleus. *Eur J Neurosci* Jan 2006;23(2):423-434.

317. Aoyama K, Watabe M, Nakaki T. Regulation of neuronal glutathione synthesis. *J Pharmacol Sci* Nov 2008;108(3):227-238.

318. Schmitt A, Asan E, Puschel B, Jons T, Kugler P. Expression of the glutamate transporter GLT1 in neural cells of the rat central nervous system: non-radioactive in situ hybridization and comparative immunocytochemistry. *Neuroscience* 1996;71(4):989-1004.

319. Selemon LD. Regionally diverse cortical pathology in schizophrenia: clues to the etiology of the disease. *Schizophr Bull* 2001;27(3):349-377.

320. Bennett MR. Synapse formation and regression in the cortex during adolescence and in schizophrenia. *Med J Aust* Feb 16 2009;190(4 Suppl):S14-16.

321. Roberts RC, Roche JK, Conley RR, Lahti AC. Dopaminergic synapses in the caudate of subjects with schizophrenia: relationship to treatment response. *Synapse* Jun 2009;63(6):520-530.

322. Roberts RC, Roche JK, Conley RR. Differential synaptic changes in the striatum of subjects with undifferentiated versus paranoid schizophrenia. *Synapse* Aug 2008;62(8):616-627.

323. Hutcherson L, Roberts RC. The immunocytochemical localization of substance P in the human striatum: a postmortem ultrastructural study. *Synapse* Sep 15 2005;57(4):191-201.

324. Roberts RC, Xu L, Roche JK, Kirkpatrick B. Ultrastructural localization of reelin in the cortex in post-mortem human brain. *J Comp Neurol* Feb 14 2005;482(3):294-308.

325. Kung L, Force M, Chute DJ, Roberts RC. Immunocytochemical localization of tyrosine hydroxylase in the human striatum: a

postmortem ultrastructural study. *J Comp Neurol* Jan 5 1998;390(1):52-62.

326. Dodd PR, Watson WE, Morrison MM, Johnston GA, Bird ED, Cowburn RF, Hardy JA. Uptake of gamma-aminobutyric acid and L-glutamic acid by synaptosomes from postmortem human cerebral cortex: multiple sites, sodium dependence and effect of tissue preparation. *Brain Res* Jun 26 1989;490(2):320-331.

327. Hardy JA, Barton A, Lofdahl E, Cheetham SC, Johnston GA, Dodd PR. Uptake of gamma-aminobutyric acid and glycine by synaptosomes from postmortem human brain. *J Neurochem* Aug 1986;47(2):460-467.

328. Westphalen RI, Scott HL, Dodd PR. Synaptic vesicle transport and synaptic membrane transporter sites in excitatory amino acid nerve terminals in Alzheimer disease. *J Neural Transm* Sep 2003;110(9):1013-1027.

329. Hassel B, Tessler S, Faull RL, Emson PC. Glutamate uptake is reduced in prefrontal cortex in Huntington's disease. *Neurochem Res* Feb 2008;33(2):232-237.

330. Lewis DA, Cruz DA, Melchitzky DS, Pierri JN. Lamina-specific deficits in parvalbumin-immunoreactive varicosities in the prefrontal cortex of subjects with schizophrenia: evidence for fewer projections from the thalamus. *Am J Psychiatry* 2001;158(9):1411-1422.

331. Hashimoto T, Volk DW, Eggan SM, Mirnics K, Pierri JN, Sun Z, Sampson AR, Lewis DA. Gene expression deficits in a subclass of GABA neurons in the prefrontal cortex of subjects with schizophrenia. *J Neurosci* Jul 16 2003;23(15):6315-6326.

332. Lewis DA, Hashimoto T, Volk DW. Cortical inhibitory neurons and schizophrenia. *Nat Rev Neurosci* Apr 2005;6(4):312-324.

333. Lewis DA, Lund JS. Heterogeneity of chandelier neurons in monkey neocortex: corticotropin-releasing factor- and parvalbumin-immunoreactive populations. *J Comp Neurol* Mar 22 1990;293(4):599-615.

334. Wang Q, Song C, Li CC. Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions. *J Struct Biol* Apr-May 2004;146(1-2):44-57.

335. McNulty SE, Toscano WA, Jr. Transcriptional regulation of glyceraldehyde-3-phosphate dehydrogenase by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biochem Biophys Res Commun* Jul 6 1995;212(1):165-171.

336. Liu NK, Xu XM. beta-tubulin is a more suitable internal control than beta-actin in western blot analysis of spinal cord tissues after traumatic injury. *J Neurotrauma* Dec 2006;23(12):1794-1801.

337. Ferguson RE, Carroll HP, Harris A, Maher ER, Selby PJ, Banks RE. Housekeeping proteins: a preliminary study illustrating some limitations as useful references in protein expression studies. *Proteomics* Feb 2005;5(2):566-571.

338. Perrot-Sinal TS, Davis AM, McCarthy MM. Developmental sex differences in glutamic acid decarboxylase (GAD(65)) and the housekeeping gene, GAPDH. *Brain Res* Dec 20 2001;922(2):201-208.

339. Eastwood SL, Harrison PJ. Synaptic pathology in the anterior cingulate cortex in schizophrenia and mood disorders. A review and a Western blot study of synaptophysin, GAP-43 and the complexins. *Brain Res Bull* Jul 15 2001;55(5):569-578.