

Striatal Excitatory Amino Acid Transporter Transcript Expression in Schizophrenia, Bipolar Disorder, and Major Depressive Disorder

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Because abnormalities of glutamatergic neurotransmission in psychiatric illness are likely not limited to glutamate receptor expression, we investigated expression of excitatory amino acid transporters (EAATs) in the striatum. The EAATs, normally expressed in both glia (EAAT1 and EAAT2) and neurons (EAAT3 and EAAT4), have previously been implicated in Huntington's disease, amyotrophic lateral sclerosis, and schizophrenia. In this study, we investigated striatal expression of transcripts encoding EAATs in tissue from mood disordered and schizophrenic subjects. With probes designed for the human EAAT1, EAAT2, EAAT3, and EAAT4 transcripts, we performed in situ hybridization

and detected decreased expression of EAAT3 and EAAT4 transcripts in the striatum in bipolar disorder. We also detected decreased EAAT3 transcript expression in schizophrenia and decreased EAAT4 transcript expression in major depressive disorder. These results suggest that changes in striatal transporter mRNA expression are restricted to neuronal EAATs and extend the body of evidence implicating abnormal glutamatergic neurotransmission in schizophrenia and mood disorders.

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A growing literature suggests abnormal expression of ionotropic glutamate receptors in the brain in schizophrenia. The preponderance of these studies has detected complex and regionally specific alterations in α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kain-

ate, and N-methyl-D-aspartate (NMDA) receptor expression, especially in cingulate cortex and medial temporal lobe structures (Akbarian et al. 1996; Aparicio-Legarza et al. 1998; Deakin et al. 1989; Eastwood et al. 1997a, 1997b, 1995; Grimwood et al. 1999; Harrison et al. 1991; Healy et al. 1998; Humphries et al. 1996; Ibrahim et al. 2000; Kerwin et al. 1988, 1990; Kornhuber et al. 1989; Meador-Woodruff and Healy 2000; Nishikawa et al. 1983; Noga et al. 1997; Ohnuma et al. 1998; Porter et al. 1997; Sokolov 1998). Recently, such studies have been extended to other brain regions associated with limbic circuitry felt to be disturbed in psychiatric illnesses, including the striatum. Increased NMDA receptor binding at both the glycine/D-serine coagonist site and the intrachannel MK801 site was detected in the putamen, and increased CNQX (an AMPA/kainate antagonist) binding was detected in the caudate in schizophrenia (Aparicio-Legarza et al.

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1998; Kornhuber et al. 1989). In bipolar disorder, decreased GluR1 mRNA expression was detected in the striatum (Meador-Woodruff et al. 2001). Based on the finding of alterations in glutamate receptor expression in the striatum, we hypothesize that other components of the glutamate synapse may be abnormal as well. Candidates for such an abnormality include the family of excitatory amino acid transporters (EAATs), a group of molecules essential for normal glutamatergic neurotransmission (Sims and Robinson 1999).

The family of glutamate/aspartate transporters has five members (EAAT1, EAAT2, EAAT3, EAAT4, and EAAT5) that are similar to the neutral amino acid transporters ASCT1 and ASCT2 (Arriza et al. 1993; Utsunomiya-Tate et al. 1996). The transporters have specific patterns of cellular localization: EAAT1 and EAAT2 have been localized to astroglia, whereas EAAT3 and EAAT4 are localized to neurons (Bar-Peled et al. 1997; Chaudhry et al. 1995; Furuta et al. 1997; Lehre et al. 1995; Milton et al. 1997; Rothstein et al. 1994; Sims and Robinson 1999; Yamada et al. 1996, 1997). The best studied of the glutamate transporters, EAAT2 (called GLT-1 in the rat) accounts for ~90% of forebrain glutamate reuptake in the rodent (Rothstein et al. 1996; Tanaka et al. 1997). The predominately glial expression of EAAT2 mRNA and protein, observed throughout the human brain, is highest in the forebrain (Bar-Peled et al. 1997; Milton et al. 1997). Similar to EAAT2, EAAT3 (called EAAC1 in the rat) protein expression in the human brain is detected in the frontal cortex and the hippocampus (Bar-Peled et al. 1997). EAAT3 is localized to both post- and presynaptic neuronal soma and is associated with ~40% of rodent hippocampal glutamate transport (Rothstein et al. 1994). In contrast, levels of EAAT1 (called GLAST in the rat) and EAAT4 protein expression in the rodent central nervous system (CNS) are highest in the cerebellum in the Bergmann glia and Purkinje cell types, respectively, whereas human studies of EAAT1 protein expression indicate high levels in the frontal cortex (Rothstein et al. 1994; Yamada et al. 1996, 1997). CNS EAAT5 mRNA expression is limited to the retina (Arriza et al. 1997).

Studies of EAAT knockout mice demonstrate the potential clinical significance of abnormalities of EAAT expression. GLT-1 (EAAT2) knockout animals develop lethal seizures, whereas GLAST (EAAT1) knockout animals have subtler defects, including difficulties with coordination (Rothstein et al. 1996; Tanaka et al. 1997; Watase et al. 1998). Not surprisingly, alterations in EAAT expression have been detected in epilepsy, Huntington's disease, and amyotrophic lateral sclerosis (Arzberger et al. 1997; Fray et al. 1998; Lin et al. 1998; Meyer et al. 1998, 1999; Ohnuma et al. 1998). Thus, we hypothesize that an abnormality in striatal EAAT expression may contribute to glutamatergic dysfunction in psychiatric diseases. The aim of this study was to in-

vestigate striatal expression of members of the family of EAATs (EAAT1, EAAT2, EAAT3, and EAAT4) in bipolar disorder, schizophrenia, and major depressive disorder (MDD).

MATERIALS AND METHODS

Subjects

Postmortem brains from 60 subjects from the Stanley Foundation Neuropathology Consortium were studied, comprising four groups of 15 subjects each with diagnoses of schizophrenia, bipolar disorder, MDD, and a comparison group. A summary of a recently published detailed description of this collection is shown in Table 1 (Torrey et al. 2000). Brain specimens from the four treatment groups were processed simultaneously, and sections were matched for anatomical level by gross anatomical landmarks. Cryostat-sectioned (14- μ m) slides from the fresh frozen half of these brains were stored at -80°C until used.

In Situ Hybridization

The design of this study was to examine striatal expression of the transcripts encoding EAAT1, EAAT2, EAAT3, and EAAT4. To generate subclones for riboprobe synthesis, we amplified unique regions of EAAT1 (NCBI Genebank accession # U03504, nucleotide coding region 526–825), EAAT2 (NM004171, 601–1026), EAAT3 (NM004170, 156–979), and EAAT4 (NM005071, 541–900) from a human cDNA brain library (EdgeBiosystems, Gaithersburg, MD) using polymerase chain reaction (PCR). For EAAT2, the region selected is common to all known splice variants. Amplified cDNA segments were extracted (QIAquick Gel Extraction kit; Qiagen, Valencia, CA), subcloned (Zero Blunt TOPO PCR cloning kit; Invitrogen, Carlsbad, CA), and confirmed by nucleotide sequencing (Thermo Sequenase Radiolabeled Termination Cycle Sequencing kit; USB, Cleveland, OH).

Riboprobes were synthesized using 100 μCi of dried [^{35}S]-UTP, 2.0 μl 5 \times transcription buffer, 1.0 μl 0.1M DTT, 1.0 μl each of 10 mM ATP, CTP, and GTP, 2.0 μl linearized plasmid DNA, 0.5 μl RNase inhibitor, and 1.5 μl SP6 or T7 RNA polymerase and incubated for 2 h at 37°C . After this incubation, 1.0 μl DNase (RNase-free) was added and incubated for 15 min at room temperature. The reaction mixture was then sieved through a 1-ml syringe containing G-50 Sephadex equilibrated in Tris buffer (100 mM Tris-HCl, pH 7.5, 12.5 mM EDTA, pH 8.0, 150 mM NaCl, and 0.2% SDS), and 100- μl fractions were eluted. DTT was added to each fraction to a final concentration of 0.01 M.

Two slides per subject for each probe were removed from -80°C storage and placed in 4% (wt/vol) formaldehyde at room temperature for 1 h. The slides were

Table 1. Summary of Subject Characteristics

	Schizophrenia	Bipolar Disorder	Major Depression	Normal Controls
<i>n</i>	15	15	15	15
Age (years)	44.2 (25–62)	42.3 (25–61)	46.4 (30–65)	48.1 (29–68)
Sex	9 males, 6 females	9 males, 6 females	9 males, 6 females	9 males, 6 females
Race	13C, 2A	14C, 1AA	15C	14C, 1AA
PMI (hours)	33.7 (12–61)	32.5 (13–62)	27.5 (7–47)	23.7 (8–42)
Tissue pH	6.1 (5.8–6.6)	6.2 (5.8–6.5)	6.2 (5.6–6.5)	6.3 (5.8–6.6)
Side of brain	6 right, 9 left	8 right, 7 left	6 right, 9 left	7 right, 8 left
Suicide	4/15	9/15	7/15	0/15
ETOH use ^a	5/15	5/15	3/15	1/15
Medication ^b	12/15	12/15	14/15	0/15
SSRI	2/15	2/15	4/15	0/15
Lithium	2/15	4/15	2/15	0/15
Antipsychotic	12/15	6/15	0/15	0/15
Other MS ^c	1/15	8/15	0/15	0/15

C, Caucasian; AA, African-American; A, Asian; ETOH, ethanol; SSRI, selective serotonin reuptake inhibitor.

^aHistory of active ETOH abuse or dependence at time of death.

^bTreatment with psychotropic medication within 6 weeks of time of death.

^cOther mood stabilizers: carbamazepine and valproic acid.

briefly washed in $2 \times \text{SSC}$ (300 mM NaCl/30 mM sodium citrate, pH 7.2) three times. The slides were placed in 0.1M triethanolamine, pH 8.0/acetic anhydride, 400:1 (vol/vol) on a stir plate for 10 min. The final wash was in $2 \times \text{SSC}$ buffer for 5 min followed by dehydration through graded alcohols and air drying. A coverslip with 0.5 ml radiolabeled riboprobe (5×10^6 dpm), 50% formamide buffer (50% formamide, 10% dextran sulfate, $3 \times \text{SSC}$, 50 mM Na_2HPO_4 , pH 7.4, $1 \times \text{Denhardt's}$ solution, 100 $\mu\text{g/ml}$ yeast tRNA, 10 mM dithiothreitol), and 0.01 M DTT was placed on each slide. Slides were placed in a covered tray with filter paper saturated with 50% formamide buffer and incubated at 55°C overnight.

The next day, the coverslips were removed and the slides were placed in $2 \times \text{SSC}$ for 5 min, followed by RNase (200 $\mu\text{g/ml}$ in 10 mM Tris-HCl, pH 8.0/0.5 M NaCl) at 37°C for 30 min. The slides then underwent the following washes: $2 \times \text{SSC}$ at room temperature for 10 min; $1 \times \text{SSC}$ for 10 min at room temperature; $0.5 \times \text{SSC}$ for 10 min at room temperature; $0.1 \times \text{SSC}$ at 55°C for 60 min; a second wash in $0.1 \times \text{SSC}$ at 55°C for 60 min; and $0.5 \times \text{SSC}$ for 10 min at room temperature. Sections were dehydrated in graded alcohols and exposed to Kodak BIOMAX MR film for up to 4 weeks.

For each probe, all subjects were run in the same assay to eliminate interassay variability. Sense and antisense probes for EAAT1, EAAT2, EAAT3, and EAAT4 transcripts were tested in sectioned human brain to confirm probe specificity, as previously described (Ibrahim et al. 2000; Meador-Woodruff et al. 1993, 1994). Sections incubated with sense riboprobe or sections pretreated with RNase before incubation had no significant labeling, whereas sections incubated with antisense probe demonstrated specific labeling.

Data Analysis

The three striatal regions analyzed in this study (caudate, putamen, and ventral striatum) were identified based on gross morphological patterns. Images were acquired from digitized X-ray films and analyzed with a Macintosh-based imaging system. For digitized images, background-corrected gray scale values for each region were converted to optical density. Background values were obtained for each section from adjacent white matter. Values from two sections for each subject for each probe were averaged and used for statistical analysis. Statistical analysis was performed for each probe by two-way analysis of variance (ANOVA), with diagnosis and striatal region as the independent variables. Post-hoc analyses were by Tukey's HSD test for equal N. For all tests, $\alpha = 0.05$.

RESULTS

We performed in situ hybridization for EAAT mRNA expression on tissue from control subjects and from subjects with schizophrenia, bipolar disorder, and MDD. EAAT1, EAAT2, EAAT3, and EAAT4 transcripts were expressed in all striatal regions studied (Figure 1). There was a main effect for diagnosis for expression of EAAT1 ($F = 2.88$, df 3, 162, $p = .038$), EAAT3 ($F = 4.62$, df 3, 149, $p = .004$), and EAAT4 ($F = 4.69$, df 3, 160, $p = .004$) but not EAAT2 ($F = 0.07$, df 3, 161, $p = .978$) (Figure 2). We did not detect significant diagnosis by region interactions for any of the four transporter transcripts. Post-hoc analyses revealed significantly lower levels of mRNA expression for EAAT1 in MDD versus schizophrenia ($p = .024$), for EAAT3 in bipolar disorder ($p = .002$) and schizophrenia ($p = .032$) versus controls, and

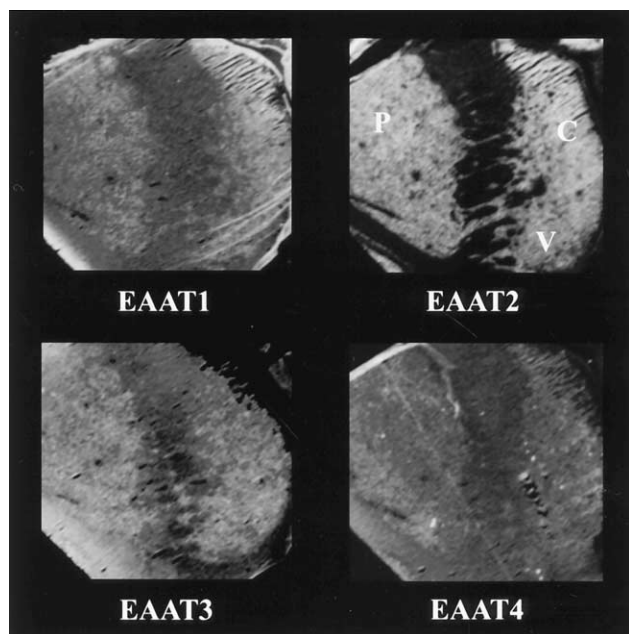


Figure 1. EAAT1, EAAT2, EAAT3, and EAAT4 transcript expression in human striatum. Locations of the caudate (C), putamen (P), and ventral striatum (V) are indicated.

for EAAT4 in bipolar disorder ($p = .010$) and major depressive disorder ($p = .006$) versus the control group. We detected a main effect for region for EAAT2 mRNA expression ($F = 18.4$, $df\ 2, 161$, $p < .0001$). Post-hoc analysis revealed significantly lower levels of EAAT2 mRNA expression in the ventral striatum versus the caudate ($p = .0002$) and putamen ($p = .001$) (Figure 2). Analysis of covariance with either pH or post-mortem interval (PMI) as covariates did not alter any of our primary findings of decreased EAAT3 and/or EAAT4 transcript expression in schizophrenia, bipolar disorder, or MDD.

DISCUSSION

Recently, glutamatergic dysfunction has been directly implicated in schizophrenia, whereas a small but growing body of literature has suggested a role for abnormal glutamatergic neurotransmission in mood disorders (Auer et al. 2000; Castillo et al. 2000; Gecz et al. 1999; Levine et al. 2000; Meador-Woodruff et al. 2001). Abnormalities in NMDA receptor binding and AMPA receptor subunit mRNA expression have been reported in some studies in the striatum in schizophrenia and bipolar disorder, supporting a hypothesis of glutamatergic dysfunction in subcortical structures (Aparicio-Legarza et al. 1998; Kornhuber et al. 1989; Meador-Woodruff et al. 2001; Noga et al. 1997). Recent work has directly implicated abnormalities of EAAT mRNA ex-

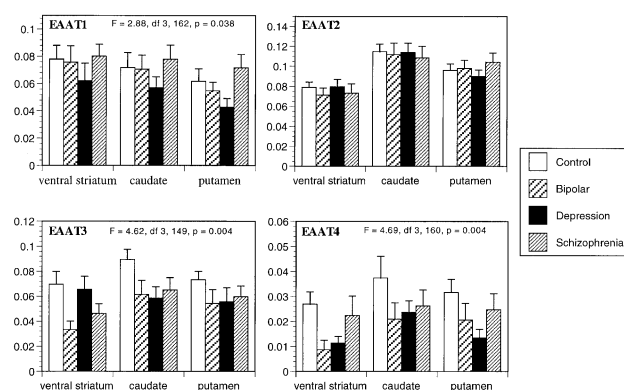


Figure 2. EAAT1, EAAT2, EAAT3, and EAAT4 mRNA expression in striatal regions in schizophrenia, bipolar disorder, MDD, and control subjects. Data are expressed as means \pm SEM. Post-hoc analyses for diagnosis are as follows: EAAT1, MDD versus schizophrenia ($p = .024$); EAAT3, bipolar disorder ($p = .002$) and schizophrenia ($p = .032$) versus control group; and EAAT4, bipolar disorder ($p = .010$) and MDD ($p = .006$) versus the control group.

pression in the prefrontal cortex and thalamus in schizophrenia (Ohnuma et al. 1998; Smith et al. 2001). Thus, we hypothesized that alterations in EAAT expression may be found in the striatum in schizophrenic and mood disordered subjects. In this study, we evaluated striatal expression of the EAATs, a family of molecules responsible for maintaining appropriate synaptic glutamate levels via transport of glutamate into neurons or glia. We detected significant alterations in EAAT mRNA expression in the striatum of subjects with mood disorders (EAAT3 and EAAT4) and schizophrenia (EAAT3)(Figure 2)

Recent work has implicated the glutamate neurotransmitter system in bipolar disorder. An increased glutamate plus glutamine level was detected in the basal ganglia of children with bipolar disorder by magnetic resonance spectroscopy (Castillo et al. 2000). Whereas this study does not indicate whether the increase in the glutamate metabolite level is in the pre-synaptic releasable neurotransmitter pool, the synapse, postsynaptic neurons or glia, or in intermediary metabolism, such an observation is consistent with our finding of decreased striatal EAAT mRNA expression in bipolar disorder. A decrease in EAAT3 and/or EAAT4 expression may diminish the capacity of the synapse to clear glutamate, resulting in increased levels of glutamate and its metabolites in the synapse.

We have recently reported increased expression of EAAT1 and EAAT2 mRNA and alterations in NMDA receptor subunit expression and binding in the thalamus in schizophrenia (Ibrahim et al. 2000; Smith et al. 2001). The observation of decreased striatal EAAT3 mRNA expression in schizophrenia extends the growing body of literature supporting a glutamatergic dys-

function hypothesis in schizophrenia and emphasizes that abnormalities of the glutamate system involve molecules other than the glutamate receptors.

Previously, several preclinical studies have implicated the NMDA receptor in the pathophysiology of affective disorders (Nowak et al. 1993; Skolnick et al. 1996; Trullas and Skolnick 1990; Trullas et al. 1991; Viu et al. 1998; Winslow et al. 1990). More recently, NMDA receptors were directly implicated in a clinical study that demonstrated an improvement in depressive symptomatology after treatment with an NMDA receptor antagonist (Berman et al. 2000). However, few studies have detailed abnormalities of glutamate physiology in MDD, and little is known regarding alterations in glutamate reuptake and metabolism in major depression (Auer et al. 2000; Levine et al. 2000). We have detected decreased EAAT4 mRNA expression in MDD, suggesting a role for abnormal postsynaptic glutamate reuptake in this illness.

We have detected significant alterations in EAAT3 and/or EAAT4 mRNA expression in the striatum in schizophrenia and mood disorders. Because EAAT3 and EAAT4 are expressed in neurons whereas EAAT1 and EAAT2 are expressed in astroglia, our results suggest an abnormality associated with neurons, but not astroglia, in the glutamate synapse (Bar-Peled et al. 1997; Chaudhry et al. 1995; Furuta et al. 1997; Lehre et al. 1995; Milton et al. 1997; Rothstein et al. 1994; Yamada et al. 1996, 1997). Interestingly, we have previously reported abnormalities in EAAT1 and EAAT2, but not EAAT3 or EAAT4, mRNA expression in the thalamus, a finding consistent with the notion of localization of glutamatergic abnormalities within the synapse to specific cell types in given brain regions (Smith et al. 2001).

One possible limitation of this study is the putative effects of psychotropic medications on striatal EAAT expression. Due to an inadequate number of subjects receiving a given medication, we were unable to perform statistical analysis to evaluate the influence of psychotropic medications on EAAT mRNA expression. Our findings in subjects with bipolar disorder are potentially confounded by the presence of mood stabilizer treatment, because valproic acid has been reported to increase synaptic glutamate levels in rodent cortical slices and glutamate reuptake is directly blocked by lithium in rodent cortical synaptosomes (Dixon and Hokin 1997, 1998). In addition, our finding of decreased EAAT3 transcript expression in schizophrenic subjects could be confounded by medication treatment, because striatal EAAT2 mRNA expression and uptake of D-[³H]aspartate in rat striatal synaptosomes are both decreased in rats treated with antipsychotics (De Souza et al. 1999; Melone et al. 2001; Schneider et al. 1998). In these studies of antipsychotic effects, EAAT3 and EAAT4 transcript expression was not evaluated. Effects of antidepressant

medication on EAAT expression have not previously been reported. Although there is no direct evidence that our findings of altered neuronal EAAT expression in the striatum are secondary to a medication effect, this remains a possibility.

In this study, we have detected decreased EAAT3 and EAAT4 mRNA expression in bipolar disorder and decreased EAAT4 mRNA expression in MDD, suggesting a role for glutamatergic dysfunction in the pathophysiology of mood disorders. In addition, we detected decreased EAAT3 mRNA expression in schizophrenia, extending previous findings of glutamatergic abnormalities in this illness. Based on the previously reported distribution of EAAT mRNA and protein expression, our findings of decreased EAAT3 and EAAT4 mRNA expression suggest a neuron-specific glutamatergic abnormality in the striatum, contrasted with our recent finding of abnormalities in glial glutamate transporters (EAAT1 and EAAT2) in the thalamus in schizophrenia (Smith et al. 2001). These results extend the body of evidence implicating abnormal glutamatergic neurotransmission in schizophrenia and provide novel evidence of putative alterations in glutamate neurotransmission in mood disorders.

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