

physiologic properties [3]. Among these, EAAT1 and EAAT2, primarily expressed in the plasma membranes of astrocytes and oligodendrocytes, are responsible for the majority of glutamate reuptake. They are ubiquitously expressed with different patterns, EAAT1 being predominant in the cerebellum and EAAT2 in the forebrain. Abnormal expression of these two proteins is involved in various psychiatric and neurologic diseases, including epilepsy, Alzheimer's disease and major depression.

Also in schizophrenia expression of both EAAT1 and EAAT2 seems to be altered. Shan *et al.* recently found a decreased expression of EAAT1 and EAAT2 proteins in the superior temporal gyrus and decreased EAAT2 in the hippocampus in a sample of elderly patients with schizophrenia [7]. These data appear to be consistent with the previous observation made by the same group of a lower glycosylation of the two transporters that could lead to impaired trafficking to plasma membrane and therefore to a reduced glutamate reuptake [8].

The first evidence of EAAT2 alterations among subjects with schizophrenia was presented by Ohnuma *et al.*, that found a reduced expression in the parahippocampal region and in the dorsolateral prefrontal cortex (DLPFC) [9]. Similarly, Egan *et al.* found that subjects carrying the high-risk *GRM3* haplotype, which is linked to schizophrenia, had lower EAAT2 expression in the prefrontal cortex [10]. The authors also reported a significant effect of *GRM3* genotype on cognitive functions, in particular for measures of verbal list learning and verbal fluency, and deleterious functional MRI activation patterns in the prefrontal cortex and hippocampus during these cognitive tasks. The authors suggested that these results may be correlated with EAAT2 reduction, whose expression is regulated by *GRM3*, although the process through which it affects EAAT2 is unclear.

An involvement of EAAT2 alterations in the cognitive dysfunction associated with schizophrenia was further suggested by Featherstone *et al.*, who showed that subchronic treatment with ketamine in rodents leads to permanent changes in electroencephalography, cognition and to a decreased expression of the glial specific glutamate transporter, GLT-1 (EAAT2 murine homologous) [11]. Moreover, adequate glutamate reuptake seems to be required for normal cognitive development, since knockout mice lacking GLT-1 showed impairment in long-term potentiation, which is strictly dependent on NMDA receptor activity [12].

We recently evaluated possible effects of a functional polymorphism [5] of *EAAT2* (rs4354668; -181T/G) on prefrontal cognitive performances in a sample of patients with schizophrenia, reporting a dis-

advantageous effect on executive functions and working memory among patients carrying the G allele, resulting in lower activity and thus increased glutamate concentrations [13]. Moreover, consistent with the study of Egan *et al.* [10], in a subsequent study we also found that poor working memory performance of *EAAT2* G carriers is associated with a frontal gray matter reduction [14].

Similarly, is also modulated by a SNP rs2731880 (C/T), located in the gene promoter region, with the C allele coding for a consensus sequence for the transcription factor STAT. Interestingly, EAAT1 was recently linked to schizophrenia [15], and found to be reduced in the DLPFC of elderly schizophrenic patients [16]. It also seems to be implicated in the pathogenesis of cognitive impairment; Karlsson *et al.* [17,18]. showed that GLAST (EAAT1 murine homologous) knockout mice exhibit novelty induced locomotor hyperactivity, abnormal social behavior, poor nesting and impaired pairwise visual discrimination learning, which represent measures considered relevant to the positive, negative and attentional/cognitive symptoms of schizophrenia.

Given this evidence, we hypothesize that genetic variability in both EAAT1 and EAAT2 could partially contribute to the widespread cognitive impairment associated to schizophrenia. In the present study we evaluate the effect of the *EAAT1* SNP rs2731880 (C/T) and *EAAT2* SNP rs4354668 (-181T/G) on several neuropsychological measures in a sample of clinically stabilized patients with schizophrenia.

Materials & methods

Sample

The study group included 192 biologically unrelated outpatients. Inclusion criteria were diagnosis of schizophrenia meeting Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR) criteria, aged between 18 and 65 years, IQ >70, treatment with a stable dose of the same antipsychotic in monotherapy for at least 3 months, and good response to treatment (defined as a reduction of 30% or more in Positive and Negative Syndrome Scale total score after 3 months of treatment). Exclusion criteria were psychiatric comorbidities, concomitant psychiatric treatments except benzodiazepines, substance abuse, neurological disorders and brain injury. All subjects provided informed consent to a protocol approved by the local ethical committee following the principles of the Declaration of Helsinki.

Genotyping

DNA was extracted from whole blood by a manual extraction, using the Illustra blood genomic Prep Midi Flow kit (GE Healthcare, Milan, Italy).

To identify the *SLCIA2* (*EAAT2*) polymorphism rs4354668 T/G (DNA forward strand), a standard PCR was carried out with the following primers: 5'-GCC ACC TGT GCT TTG CTG-3' and 5'-TGA TGT CAG CTC TCG ACG AA-3'. The PCR was carried out in a 10 µl volume containing 150 ng genomic DNA, 1 µl of 1× Hot Master Taq Buffer with Mg²⁺ (Eppendorf, Milan, Italy), 0.1 µl of each primer (50 µM), 1 µl of deaza-dNTPs (10 mM), 0.5 µl of dimethyl sulfoxide (DMSO) solution (Sigma-Aldrich, Milan, Italy) and 0.1 µl of Hot Master Taq (5U/µl; Eppendorf). After an initial step of 5 min at 94°C, 35 cycles of amplification (35 s at 94°C, 35 s at 58°C, 45 s at 70°C) and a final extension step of 10 min at 70°C were performed. An aliquot of PCR product was digested using *MspI* (20 U/µl; New England Biolabs, England, UK) and incubated at 37°C for 8 h; fragments were separated in agarose gels. Depending on the presence of two or three restriction *MspI* sites, either three fragments (allele T) or four fragments (allele G) were produced.

To identify the *SLCIA3* (*EAAT1*) polymorphism rs2731880 C/T (DNA forward strand), a standard PCR was performed with the following primers: 5'-TGT AAA TTC GGC CCC TAC TG-3' and 5'-GGC CCA ACA GAA GGT TAT GA-3'. The PCR was carried out in a 10 µl volume containing 150 ng genomic DNA, 1 µl of 1× Hot Master Taq Buffer with Mg⁺⁺ (Eppendorf), 0.1 µl of each primer (50 µM), 1 µl of dNTPs (200 µM) and 0.1 µl of Hot Master Taq (5U/µl; Eppendorf). After an initial step of 3 min at 94°C, 35 cycles of amplification (30 s at 94°C, 20 s at 58°C, 30 s at 70°C) and a final extension step of 10 min at 70°C were performed. The amplified fragment was then purified by Multi-Screen Colum Loader (Millipore, Milan, Italy), filled up and packaged with Sephadex G-50 (Sigma-Aldrich) to remove residual PCR reagents.

An aliquot of purified PCR product was then used to perform sequencing reaction, using DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Milan, Italy). In its turn, sequencing reaction product was purified following the abovementioned protocol, to remove excess fluorescent dyes not incorporated into the DNA fragment and then loaded onto a 48 capillaries genetic analyzer (MegaBace 500, GE Healthcare, Milan, Italy).

Assessment

Basic clinical and demographic data were collected from clinical records.

Psychopathology was assessed by means of the Positive and Negative Syndrome Scale for Schizophrenia [19], administered by a trained psychiatrist. Neuropsychological

performances were assessed by means of the Wechsler Adult Intelligence Scale-Revised (WAIS-R) for estimation of overall intellectual functioning; Wisconsin Card Sorting Test (WCST) for evaluation of executive functions; Continuous Performance Test (CPT), for the evaluation of attention; N-back test, for evaluation of working memory; Brief Assessment of Cognition in Schizophrenia (BACS), a broad battery evaluating several domains of cognition (verbal memory, working memory, psychomotor speed and coordination, selective attention, semantic fluency, letter fluency and executive functions). Normative Italian adjusted scores [20] were used for the BACS subtests. All test were administered by a trained psychologist.

Data analysis

Demographic and clinical characteristics were analyzed for group differences with Analysis of Variance (ANOVA) and χ^2 test (for dichotomic variables). ANOVA was also performed in order to evaluate differences in cognitive performances among genotype groups considering the scores of neuropsychological evaluations as dependent variables and *EAAT1-2* genotypes as categorical factors.

Results

The sample was composed of 192 patients, 129 males and 63 females. There were no differences in gender distribution between genotype groups (χ^2 not significant [ns]). DNA analysis showed the following allelic distributions: for *EAAT2*, 73 patients T/T, 79 T/G and 40 G/G, in Hardy-Weinberg equilibrium; for *EAAT1*, 52 patients T/T, 95 T/C and 45 C/C, in Hardy-Weinberg equilibrium. For analysis we grouped *EAAT2* subjects homozygous for the T allele versus carriers of the G allele, as previously reported in other studies [13]. Similarly, *EAAT1* patients were divided into C carriers versus subjects homozygous for the T allele.

Table 1 shows demographic and clinical variables of the sample. No significant differences were found for any of the variables considered between groups. The antipsychotic treatment was distributed as follows: 46% of patients was taking clozapine, 29% risperidone, 17% haloperidol and 8% other treatments (olanzapine, thioridazine, aripiprazole and fluphenazine). The antipsychotic treatments were homogeneously distributed, without significant differences between genotype groups (χ^2 ns).

As previously observed in an independent sample [5], ANOVA showed significant differences among the *EAAT2* genotype groups at WCST for the number of categories ($F = 5.46$, $p = 0.021$), a measure of executive functions more specifically related to abstract thinking, and at N-Back for measures of 1-back ($F = 7.79$,

Table 1. Demographic and clinical variables of the sample.

	Mean	Standard deviation
Age	40.9	9.42
Years of education	11.27	2.69
Onset	23.01	5.57
Duration of illness	18.26	7.63
PANSS total score	71.84	20.28
PANSS positive	15.77	5.84
PANSS negative	20.86	7.29
PANSS general	35.19	10.26
WAIS-R Total IQ	82.46	11.44

PANSS: Positive and Negative Syndrome Scale; WAIS-R: Wechsler Adult Intelligence Scale-Revised.

$p = 0.006$) and 2-back ($F = 5.71$, $p = 0.018$). Results showed that patients carrying the G allele performed significantly worse than T/T subjects at WCST and N-Back.

ANOVA also evidenced significant differences among *EAAT1* genotype groups at CPT (number of hits task) ($F = 10.45$, $p = 0.002$), WCST preservative errors ($F = 3.91$, $p = 0.049$) and BACS measures of verbal fluency ($F = 5.25$, $p = 0.024$) and verbal memory ($F = 5.35$, $p = 0.022$), in favor of the C carriers. Significant results for *EAAT1* and *EAAT2* genotypes are represented in Figure 1 & Figure 2, respectively.

Discussion

In this study we investigated possible influences of *EAAT1* SNP rs2731880 (C/T) and of *EAAT2* SNP rs4354668 (-181 T/G) on cognitive functions in a sample of patients affected by schizophrenia, a disease characterized by wide and heterogeneous cognitive deficits that are recognized as cardinal features of the illness and critical determinants of quality of life and functioning [21].

We observed worst executive functions (WCST, preservative errors), verbal fluency and verbal memory (BACS) performances in subjects homozygous for the *EAAT1* T allele, compared with the C carrier group. Moreover, we confirmed our previous report of better executive functions (WCST, number of categories) and working memory (N-back: 1-back, 2-back) performances among patients homozygous for the *EAAT2* T allele, compared with G carriers.

Since both *EAAT1* T allele and *EAAT2* G allele are related to a lower expression [7–9,16], these results suggest that impaired activity and expression of glutamate transporters is associated with a disadvantageous effect on core cognitive functions that mainly depend on prefrontal cortex activity, therefore contributing to the emergence of individual differences among patients

with schizophrenia. The observation of an involvement of separate cognitive tasks by the two polymorphisms may be owing to the different cortical expression of the transporters.

We could hypothesize that the presence of the disadvantageous alleles could determine a threefold effect on cognitive functions, probably acting in synergy. Indeed, a lower EAAT expression could lead on one side to an inefficient energy metabolism due to an impaired glutamate recycling and the consequent increased glucose consumption. As a matter of fact, a disruption of this pathway is suggested by the finding of greater activity of phosphate-activated glutaminase, the major enzyme responsible for the conversion of glutamine to glutamate, in DLPFC and thalamus of patients with schizophrenia [22]. This hypothesis is also consistent with the study of Egan *et al.*, who previously found a relatively deleterious activation functional MRI patterns in the prefrontal cortex among subjects carrying the high-risk *GRM3* haplotype [10].

On the other hand, a lower transporter activity could also contribute to a disturbance of the buffering control mechanisms necessary to prevent glutamate excitotoxicity [6], leading therefore to neuronal damage with a consequent disadvantageous effect on cognitive functions, as suggested by our recent data of association between poor working memory performance and gray matter reduction among *EAAT2* G carriers [14]. Moreover, besides the excitotoxic activity, an excess of glutamate in the synaptic cleft would cause an extrasynaptic glutamate release with redistribution of glutamate transporters and loss of input specificity [23].

Even though these hypotheses are only speculative and based on preliminary data, these results suggest that EAATs inefficiency may represent a target of interest for development of pharmacological strategies aimed to improve cognitive performances by compensating the impaired glutamate reuptake. To date selective enhancers of EAAT1–2 activity and/or expression haven't yet been developed but different substances were found to collaterally raise glutamate reuptake by increasing EAATs expression, such as riluzole, the group II mGluR agonist 4-aminopyrrolidine-2,4-dicarboxylate (APDC) and ceftriaxone [24]. Interestingly, Beller *et al.* recently evaluated the rate of glutamate uptake and neuroprotection in cell culture models of glutamate excitotoxicity treated with ceftriaxone and APDC [25]. They reported significant increase of glutamate uptake and decreased neuronal death associated with an elevation of EAAT1–2 protein levels, therefore supporting the hypothesis of a potential neuroprotective effect of EAAT enhancers that may also have posi-

tive repercussions on cognitive functioning. Thus, a thorough study of EAAT pathways and interactions appears to be crucial in order to better understand the mechanisms underlying these processes and identify potential new molecules and targets for more selective pharmacological approaches.

The results of this study are preliminary and need to be replicated by future and larger studies that may also include a group of healthy controls in order to observe if the polymorphisms only affect cognitive functioning of patients with schizophrenia or can also influence neurocognitive processes among unaffected subjects. Moreover, brain imaging studies should be helpful in order to individuate possible neuroanatomical and neurofunctional correlates of EAAT polymorphisms. Another interesting development is represented by the analysis of interactions of glutamate transporters with pharmacological treatments. In particular clozapine was found to decrease expression of EAAT interacting proteins with possible neurofunctional implications [26].

Finally, an evaluation of the environmental influences on genotype effect could represent an interesting development of this study. It is important to remember that cognitive functions are based on the interaction between several neurobiological pathways and other occult variables, therefore a thorough study of EAAT1–2 pathways and interactions is necessary to obtain a more precise estimate of association between genotype and specific neurocognitive abilities.

Conclusion

In conclusion, in this study we investigated the effect of *EAAT1* SNP rs2731880 (C/T) and of *EAAT2* SNP rs4354668 (-181 T/G) on cognitive performances in a sample of clinically stabilized schizophrenic patients. We reported a disadvantageous effect among patients carrying the *EAAT1* T allele and *EAAT2* G allele, which are associated with lower transporter expression and higher glutamate levels. These results are preliminary and need to be replicated; however, they suggest that EAAT inefficiency may represent a tar-

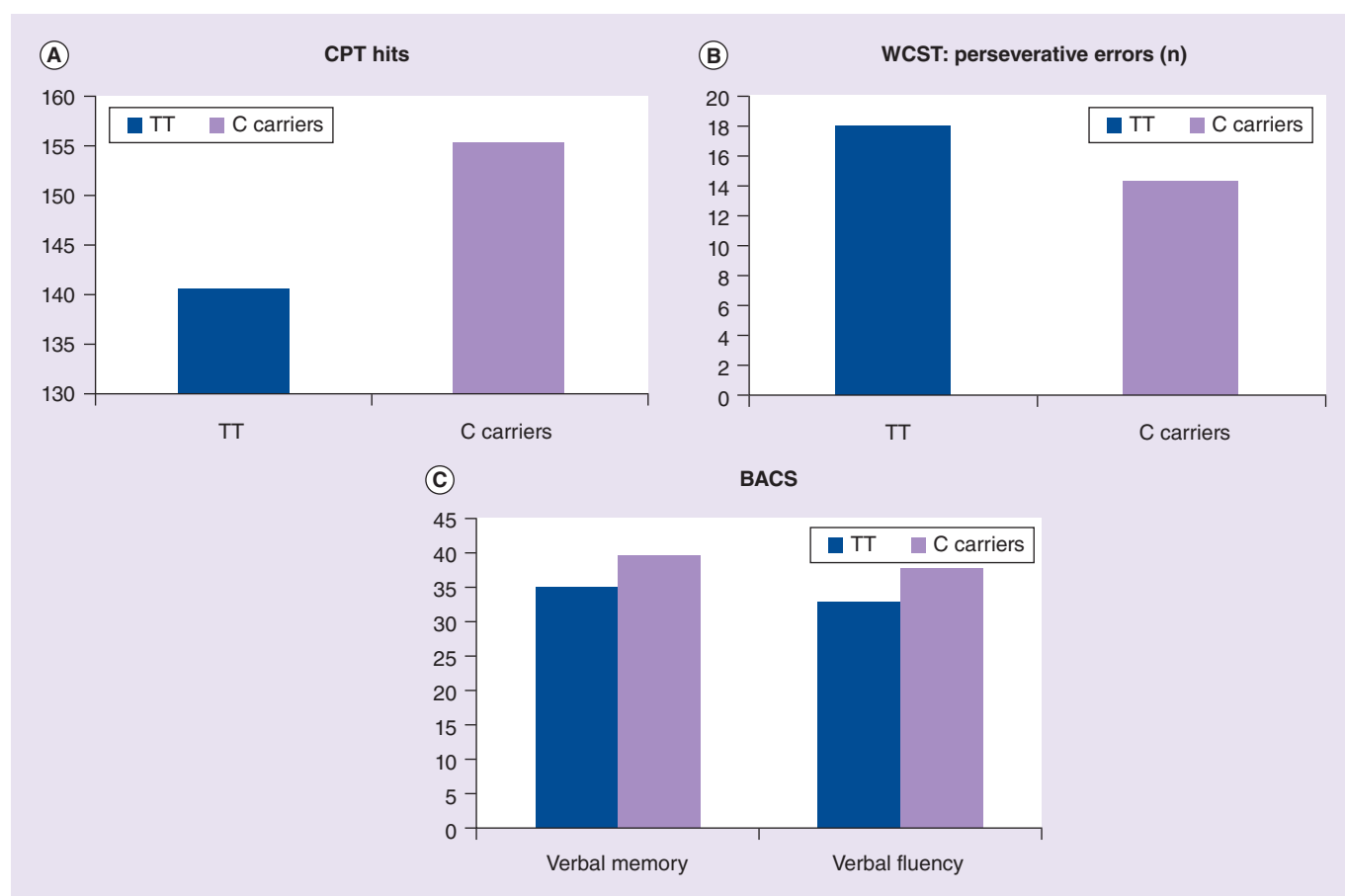


Figure 1. EAAT1: significant results. (A) CPT number of hits ($F = 10.45$, $p = 0.002$), (B) WCST perseverative errors ($F = 3.91$, $p = 0.049$) and (C) BACS measures of verbal fluency ($F = 5.25$, $p = 0.024$) and verbal memory ($F = 5.35$, $p = 0.022$) for *EAAT1* T/T and C carrier groups. Subjects homozygous for the *EAAT1* T allele performed significantly worse than C carriers. BACS: Brief Assessment of Cognition in Schizophrenia; CPT: Continuous Performance Test; WCST: Wisconsin Card Sorting Test.

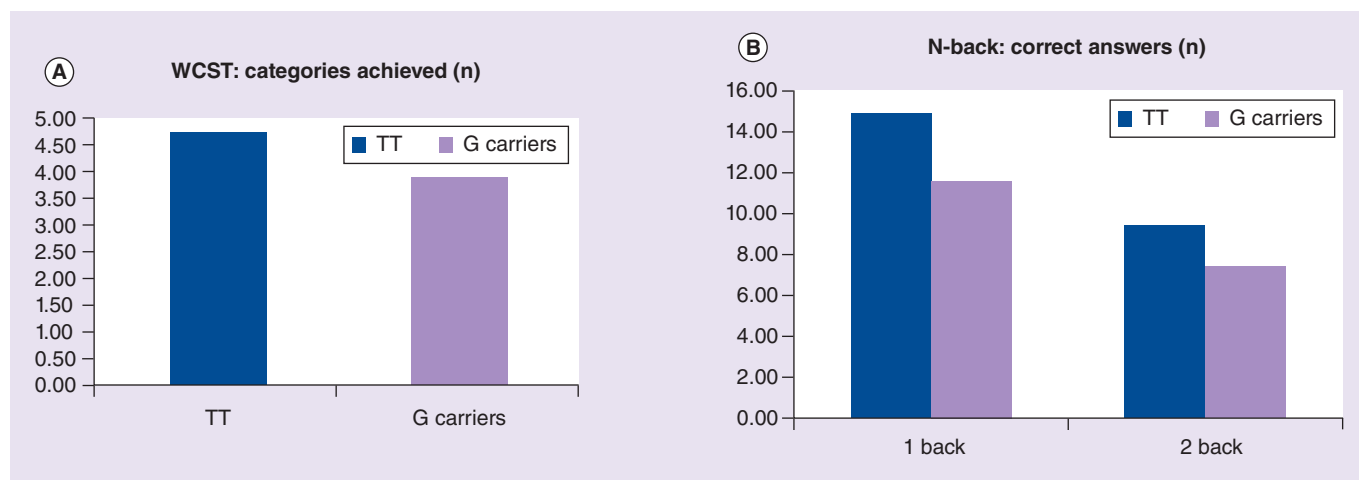


Figure 2. EAAT2: significant results. (A) WCST number of categories completed (ANOVA: $F = 5.46$, $p = 0.021$) and (B) N-back performances (ANOVA: 1-back: $F = 7.79$, $p = 0.006$; 2-back: $F = 5.71$, $p = 0.018$) for EAAT2 T/T and G carrier groups. Subjects homozygous for the EAAT2 T allele performed significantly better than G carriers. WCST: Wisconsin Card Sorting Test.

get of interest for the development of pharmacological strategies aimed to improve cognitive performances by compensating the impaired glutamate reuptake.

Future perspective

A confirmation of an association between cognitive functions and impaired glutamate reuptake in separate and larger sample groups could find different future applications.

Indeed, it would contribute to the development of individualized treatments for patients that would most benefit from cognitive enhancements, such as neuro-cognitive remediation programs, transcranial direct-current stimulations or adjunctive pharmacological treatments, taking into account the specific cognitive deficits related to the different genotypes of interest. Patients with impaired EAAT activity may benefit from treatments with EAAT enhancers such as ceftriaxone, which has shown encouraging results among murine

studies, assessable through double blind clinical trials. In this view, the identification of predictive factors of treatment efficacy would also be of great interest.

Moreover, besides schizophrenia, a thorough study of EAAT pathways and interactions would also be helpful in order to better analyze the cognitive impairment that involves other neuropsychiatric disorders such as bipolar disorder, dementia and neurodegenerative diseases.

Finally, literature suggests that EAAT inefficiency may represent a feature of schizophrenia at large. If supported by consistent results, this could represent the starting input for the development of selective pharmacological EAAT enhancers aimed to remediate the cognitive deficit of schizophrenia.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial

Executive summary

Background

- The excitatory amino acid transporters, EAAT1 and EAAT2, are responsible for the majority of glutamate uptake in the CNS.
- EAATs are crucial for cerebral energy metabolism and prevent glutamate excitotoxicity and neuronal cell death.
- EAAT1–2 are downregulated in schizophrenia.
- Cognitive deficit is a core symptom of schizophrenia and a critical determinant of quality of life and functioning.

Results

- EAAT2 rs4354668 and EAAT1 rs2731880 affect cognitive functions in schizophrenia.
- EAAT1 T allele and EAAT2 G allele are associated with lower expression and impaired cognitive functions.

Discussion

- Enhancement of EAAT1–2 expression leads to increased glutamate uptake and decreased neuronal death.
- EAAT1–2 inefficiency may represent a target for development of pharmacological agents aimed to improve cognitive performances by compensating for the impaired glutamate reuptake.

interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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