by PANSS. Assessors also ensured that these patients had no symptomatic exacerbation for at least 1 month prior to the interview. All participants provided written informed consent, and study protocols were approved by the ethics committees of all participating institutions (Tokyo Metropolitan Matsuzawa Hospital and Tokyo Metropolitan Institute of Medical Science).

Statistical Analysis

All data were analyzed using SPSS version 20.0 statistical software (IBM). Simple comparisons of means and standard errors of data between group 1 and the other groups were performed using the unpaired *t* test (both 2 tailed). In comparisons of HbA1c, eGFR, GLO1 enzymatic activity, onset of disease, and duration of disease between groups 1 and 4, we conducted ANCOVA as age was considered a covariate. Likewise, ANCOVA was also performed when comparing GLO1 enzymatic activity between groups 1 and 3. Fisher's exact test was used for categorical variables and stepwise multiple regression analysis between psychological symptoms and each of the 2 measured biomarkers. Significance was defined as $P < .05$.

Results

Clinical Characterization

The mean (±SD) age of participants without carbonyl stress (group 1) and with enhanced carbonyl stress (group 4) were 46.9 ± 14.7 and 51.4 ± 11.7, respectively ($P = .07$). There were no significant differences linked to HbA1c, eGFR, smoking status, alcohol consumption, and male to female ratios in groups 1 and 4. In accordance with our prediction, the enhanced carbonyl stress group showed a more severe clinical course relative to the normal group (see table 1). The proportion of cases classified as inpatients were 23.9% (16 inpatients and 51 outpatients) in group 1 and 80.8% (21 inpatients and 5 outpatients) in group 4 ($P < .0001$). In addition, the mean (±SD) duration of hospitalization in group 1 was markedly shorter than that of group 4 (4.2 ± 9.2 years vs 17.4 ± 16.9 years, $P = .0002$). Furthermore, we noted higher daily doses of antipsychotics in group 4 compared with group 1 (773.8 ± 652.4 mg/day in group 1 and 1143.9 ± 743.6 mg/day in group 4, $P = .02$). We also observed lower educational status in group 4 compared with group 1 ($P = .02$). We were surprised to find that GLO1 enzymatic activity was significantly decreased in group 4, relative to group 1, despite group 4 containing a higher proportion of patients carrying the homozygous Glu111 allele compared with group 1 ($P = .0005$; see table 1). Significant differences in enzymatic activity remained, after excluding patients carrying frameshift mutations and homozygous Ala111 alleles from group 4. No significant differences were observed in other clinical parameters between groups 1 and 4. Intriguingly, groups 2 and 3 showed clinical features that were almost intermediate to those seen in groups 1 and 4 (see table 1).

Psychopathological Symptoms

Next, we evaluated clinical symptoms by analyzing the association between pentosidine and pyridoxal, biomarkers of enhanced carbonyl stress, and PANSS score (see table 2). We evaluated symptom severity of the 49 interviewed patients by PANSS. Of these 49 patients, 15 fell into group 1, 6 into group 2, 15 into group 3, and 13 into group 4. We conducted stepwise multiple regression analyses in which, age, in/outpatients status and antipsychotic medication were introduced into the model as confounding factors, as they are known to affect PANSS scores. We did not include other potential confound factors such as disease duration and hospitalizations because of

Table 2. Association Between PANSS Score and Pentosidine and Pyridoxal Levels

	Clinical Variables	Adjusted R^2	Standardized β	*t* Value	*P* Value
Total positive symptom score	Antipsychotics	.317	.405	3.362	.0016
	Pyridoxal		−.310	−2.577	.0133
	In/outpatients		.264	2.182	.0344
Total negative symptom score	Antipsychotics	.380	.394	3.160	.0028
	In/outpatients		.378	3.118	.0032
	Age		.332	2.586	.0130
Total general psychopathology score	Antipsychotics	.384	.481	3.867	.0004
	Pyridoxal		−.259	−2.194	.0336
	In/outpatients		.259	2.146	.0374
	Age		.224	1.694	.0973
Total PANSS score	Antipsychotics	.402	.398	3.533	.0010
	In/outpatients		.389	3.440	.0013
	Pyridoxal		−.285	−2.536	.0148

Note: PANSS, Positive and Negative Syndrome Scale. Pentosidine, pyridoxal, age, antipsychotic medication, and in/outpatient status were introduced into the model as independent variables. Stepwise multiple regression analysis was performed. Corrected *P* values less than .0125 (.05/4) were considered statistically significant.

high multicollinearity, defined as variance inflating factors, exceeding 2.0. Examining 4 subscales of PANSS, we found nominally significant, negative correlation between serum pyridoxal levels and items of the total positive symptom scale (standardized $\beta = -.31$, $P = .0133$), total general psychopathology score (standardized $\beta = -.26$, $P = .0336$), and the total PANSS score (standardized $\beta = -.29$, $P = .0148$). Pentosidine showed no association with PANSS scores (see table 2).

Discussion

This is the first study to focus on clinical features of schizophrenia observed in enhanced carbonyl stress. We found that a significantly higher proportion of subjects with carbonyl stress were inpatients, of low educational status, suffered longer durations of hospitalization, and were prescribed higher doses of antipsychotic medication, relative to subjects without carbonyl stress. Intriguingly, the 2 groups consisting of patients with either high pentosidine or low pyridoxal levels (group 2 or group 3) exhibited clinical features that were almost intermediate between groups 1 and 4. In addition, nominally significant negative association between serum pyridoxal levels and 3 subscales of PANSS—namely, the total positive symptom score, the total score for general psychopathology, and total PANSS scores—was observed. In contrast, plasma pentosidine levels showed no significant association with items of PANSS.

It should be noted that severe clinical features observed in patients with carbonyl stress, such as a higher inpatients status, a longer duration of hospitalization, and larger prescribed doses of antipsychotic medication, are very similar to those seen in treatment-resistant schizophrenia as defined by Kane et al.[9] In this cohort of patients, clozapine is the standard treatment, proving more effective than conventional or other atypical antipsychotics.[9 14] However, clozapine also induces serious and sometimes lethal adverse effects such as granulocytopenia.[15,16] Intriguingly, psychopathological symptoms tended towards association with low pyridoxal levels. Although, the precise mechanisms of decreased pyridoxal levels in patients with enhanced carbonyl stress are not fully understood, our observations strongly support the theory that supplementation of vitamin B6 for these patients may safely improve specific clinical symptoms associated with pyridoxal levels. So far, a number of clinical reports, including 4 randomized placebo-controlled studies, have tested the efficacy of vitamin B6 supplementation for schizophrenia, but the results are inconsistent.[17 24] A possible reason for the conflicting results could be that in these studies, no account was taken of the vitamin B6 levels in subjects. We believe that vitamin B6 supplementation may be most effective in schizophrenic patients with lower B6 levels associated with enhanced carbonyl stress. In terms of therapeutic benefits, this idea shares parallels with the use of clozapine in treatment-resistant schizophrenia. New clinical studies, utilizing vitamin B6 supplementation for schizophrenics with carbonyl stress are required.

In this study, we cannot exclude possibility that high plasma pentosidine levels were a consequence of high doses and long time exposure of antipsychotic medications. In addition, it is well known that some second-generation antipsychotic drugs induce significantly greater weight gain than conventional antipsychotics.[25,26] Indeed, simple regression models of statistical analysis revealed a significant positive correlation between plasma pentosidine and the daily dose of antipsychotic medication (Spearman's correlation coefficient = 0.301, $P < .0001$; see figure 1). The lack of association with serum pyridoxal levels (Spearman's correlation coefficient = −0.075, $P = .34$; see figure 1) appears to support this possibility. On the other hand, we have previously reported a case in which a drug-naive schizophrenic patient exhibited high plasma pentosidine.[27] Furthermore, Cannon et al[28] reported that DM in pregnant women increased the risk of schizophrenia in their offspring. These data endorse the hypothesis that exposure to enhanced carbonyl stress in the early stages of neural development may impact of physiological processes exemplified by the development of schizophrenia. Although we lacked information

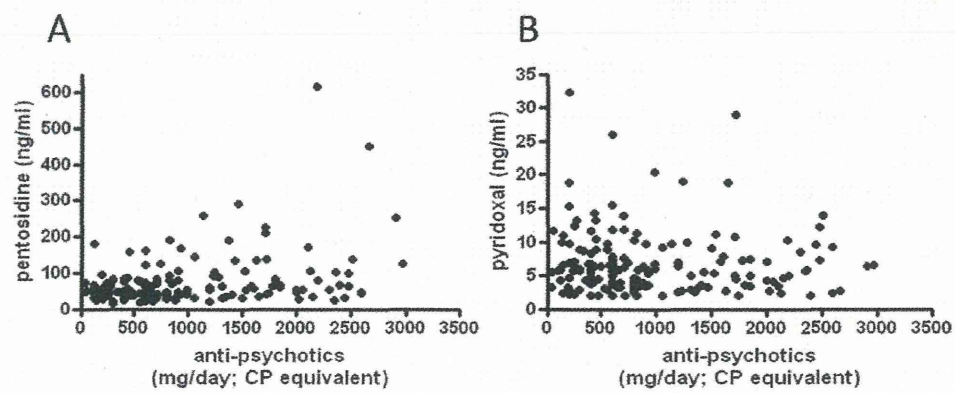


Figure 1. Correlation between daily dose of antipsychotic drugs and pentosidine (**A**) and pyridoxal (**B**).

on environment factors, such as socioeconomic status of origin, for a number of subjects we put forward, the possibility that early exposure of high pentosidine may be causative of the low educational status observed in patients with enhanced carbonyl stress. Further studies focused on drug-naive patients will be required to address these issues.

Psychopathological symptoms tended to correlate with serum pyridoxal, however, it remains unclear how decreased serum pyridoxal affects psychopathological symptoms. Pyridoxamine, one of the 3 forms of vitamin B6, is capable of scavenging pentosidine, and depletion of pyridoxamine during this process eventually leads to a decrease in serum pyridoxal levels. Given that pyridoxal is an important coenzyme in the synthesis of various neurotransmitters, such as dopamine, serotonin, and gamma-aminobutyric acid, the lack of pyridoxal in this situation raises the possibility that an imbalance of these neurotransmitters may influence severity of clinical features observed in these patients. Another possibility is that high pentosidine may itself affect clinical features by inducing an inflammatory response, through receptors for AGEs in the central nervous system (CNS). To examine this hypothesis, studies using animal models and human samples are necessary to reveal the precise molecular mechanisms of CNS involvement.

It was surprising to find that GLO1 activity was significantly decreased in group 4 compared with group 1 subjects, despite group 4 containing a higher proportion of homozygous Glu111 allele carriers (see table 1). The significant difference in activity between groups 1 and 4 persisted, even after excluding patients with frameshift mutations and homozygous Ala111 alleles. Although the exact mechanisms driving decreased GLO1 enzymatic activity in group 4 are unknown, preliminary data suggested that plasma zinc levels were significantly lower in schizophrenia relative to control samples (data not shown). So, one explanation for this paradoxical finding could be that enhanced carbonyl stress causes depletion of zinc ions, which are an essential trace element for maintaining GLO1 reactivity. In addition, other metabolic pathways, such as homocysteine metabolism, may be impaired under enhanced carbonyl stress conditions, because vitamin B6 plays a key role as a cofactor in this pathway. This lends plausible support to numerous recent reports investigating an association between elevated homocysteine levels and schizophrenia.[21,29] Thus, we propose that enhanced carbonyl stress causes not only abnormalities in pentosidine and pyridoxal metabolism but also in wider metabolic pathways associated with these biomarkers.

As with studies of this nature, our study has some limitations. First, because this research is of a retrograde cross-sectional design, we cannot fully evaluate whether association between enhanced carbonyl stress and severe clinical course is a causal relationship. Future studies, implementing prospective and longitudinal designs, will be needed to elucidate the exact clinical relationship between this disease and the 2 proposed biomarkers. Second, as sample sizes for each group were relatively small, our analysis had limited power to detect statistical significance. This was particularly relevant in the PANSS analysis, where because interview data were available for only 49 patients, while we could detect trends, we were unable to detect significant differences by a direct comparison of PANSS scores between the groups (data not shown). Third, we did not control for other possible confound factors, including body mass index (BMI) and less severe disturbances of glucose metabolism. For BMI, we had limited data on group 1 subjects and data on all subjects, except 1, in group 4. We found that mean (±SD) BMI in the carbonyl stress group was 21.6±4.8 for males and 21.3±2.2 for females. These values are lower than the Japanese standard for people aged in their fifties (24.2 in males and 22.8 in females, http://www.stat.go.jp/data/nihon/21.htm). Therefore, we think that the influence of BMI on pentosidine or pyridoxal levels may be weak. Finally, because mild glucose intolerance and inflammation may possibly enhance carbonyl stress, studies into more sensitive markers, such as the homeostasis model assessment, which is an index of insulin resistance and C-reactive protein will be required in the future.

Conclusion

Patients with enhanced carbonyl stress showed distinct clinical features such as a higher propensity to inpatient status, low educational status, and longer durations of hospitalization, and higher doses of antipsychotic medication. These features bear strong similarity to those seen in treatment-resistant schizophrenia. It was also clear that psychopathological symptoms showed a tendency towards negative correlation with serum pyridoxal levels. Given that clozapine with its serious adverse effects is the most effective agent for treatment-resistant schizophrenia, our results support the idea that simple treatments that reduce carbonyl stress, such as supplementation of pyridoxamine, may be of novel therapeutic benefit for this subset of patients. As mentioned before, larger and longitudinal clinical studies will be required to validate these novel findings.

Funding

Japan Society for the Promotion of Science KAKENHI (23791368).

Acknowledgments

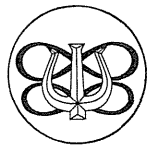
We especially thank Ms Hiroko Yuzawa at the Institute of Medical Sciences, Tokai University for the measurement of plasma pentosidine levels. We are also grateful for the

expert technical assistance of Izumi Nohara, Mayumi Arai, and Nanako Obata. We appreciate Ms Yuuki Yoshida and Ms Ikuyo Kito who made technical advice and assisted with the preparation of the manuscript. The authors have declared that there are no conflicts of interest in relation to the subject of this study.

References

1. Miyata T, van Ypersele de Strihou C, Kurokawa K, Baynes JW. Alterations in nonenzymatic biochemistry in uremia: origin and significance of "carbonyl stress" in long-term uremic complications. *Kidney Int*. 1999;55:389–399.
2. Nin JW, Jorsal A, Ferreira I, et al. Higher plasma levels of advanced glycation end products are associated with incident cardiovascular disease and all-cause mortality in type 1 diabetes: a 12-year follow-up study. *Diabetes Care*. 2011;34:442–447.
3. Koyama Y, Takeishi Y, Arimoto T, et al. High serum level of pentosidine, an advanced glycation end product (AGE), is a risk factor of patients with heart failure. *J Card Fail*. 2007;13:199–206.
4. Meli M, Perier C, Ferron C, et al. Serum pentosidine as an indicator of Alzheimer's disease. *J Alzheimers Dis*. 2002;4:93–96.
5. Park L, Raman KG, Lee KJ, et al. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med*. 1998;4:1025–1031.
6. Arai M, Yuzawa H, Nohara I, et al. Enhanced carbonyl stress in a subpopulation of schizophrenia. *Arch Gen Psychiatry*. 2010;67:589–597.
7. Miyata T, Taneda S, Kawai R, et al. Identification of pentosidine as a native structure for advanced glycation end products in beta-2-microglobulin-containing amyloid fibrils in patients with dialysis-related amyloidosis. *Proc Natl Acad Sci USA*. 1996;93:2353–2358.
8. Bisp MR, Bor MV, Heinsvig EM, Kall MA, Nexø E. Determination of vitamin B6 vitamers and pyridoxic acid in plasma: development and evaluation of a high-performance liquid chromatographic assay. *Anal Biochem*. 2002;305:82–89.
9. Kane J, Honigfeld G, Singer J, Meltzer H. Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. *Arch Gen Psychiatry*. 1988;45:789–796.
10. Azorin JM, Spiegel R, Remington G, et al. A double-blind comparative study of clozapine and risperidone in the management of severe chronic schizophrenia. *Am J Psychiatry*. 2001;158:1305–1313.
11. Buchanan RW, Breier A, Kirkpatrick B, Ball P, Carpenter WT Jr. Positive and negative symptom response to clozapine in schizophrenic patients with and without the deficit syndrome. *Am J Psychiatry*. 1998;155:751–760.
12. Kane JM, Marder SR, Schooler NR, et al. Clozapine and haloperidol in moderately refractory schizophrenia: a 6-month randomized and double-blind comparison. *Arch Gen Psychiatry*. 2001;58:965–972.
13. Kumra S, Kranzler H, Gerbino-Rosen G, et al. Clozapine and "high-dose" olanzapine in refractory early-onset schizophrenia: a 12-week randomized and double-blind comparison. *Biol Psychiatry*. 2008;63:524–529.
14. McEvoy JP, Lieberman JA, Stroup TS, et al. CATIE Investigators. Effectiveness of clozapine versus olanzapine, quetiapine, and risperidone in patients with chronic schizophrenia who did not respond to prior atypical antipsychotic treatment. *Am J Psychiatry*. 2006;163:600–610.
15. Alvir JM, Lieberman JA, Safferman AZ, Schwimmer JL, Schaaf JA. Clozapine-induced agranulocytosis. Incidence and risk factors in the United States. *N Engl J Med*. 1993;329:162–167.
16. Safferman A, Lieberman JA, Kane JM, Szymanski S, Kinon B. Update on the clinical efficacy and side effects of clozapine. *Schizophr Bull*. 1991;17:247–261.
17. Ananth JV, Ban TA, Lehmann HE. Potentiation of therapeutic effects of nicotinic acid by pyridoxine in chronic schizophrenics. *Can Psychiatr Assoc J*. 1973;18:377–383.
18. Bucci L. Pyridoxine and schizophrenia. *Br J Psychiatry*. 1973;122:240.
19. Lerner V, Kaptsan A, Miodownik C, Kotler M. Vitamin B6 in treatment of tardive dyskinesia: a preliminary case series study. *Clin Neuropharmacol*. 1999;22:241–243.
20. Lerner V, Liberman M. Movement disorders and psychotic symptoms treated with pyridoxine: a case report. *J Clin Psychiatry*. 1998;59:623–624.
21. Levine J, Stahl Z, Sela BA, et al. Homocysteine-reducing strategies improve symptoms in chronic schizophrenic patients with hyperhomocysteinemia. *Biol Psychiatry*. 2006;60:265–269.
22. Sandyk R, Pardeshi R. Pyridoxine improves drug-induced parkinsonism and psychosis in a schizophrenic patient. *Int J Neurosci*. 1990;52:225–232.
23. Ban TA, Lehmann HE, Deutsch M. Negative findings with megavitamins in schizophrenic patients: preliminary report. *Commun Psychopharmacol*. 1977;1:119–122.
24. Lerner V, Miodownik C, Kaptsan A, Cohen H, Loewenthal U, Kotler M. Vitamin B6 as add-on treatment in chronic schizophrenic and schizoaffective patients: a double-blind, placebo-controlled study. *J Clin Psychiatry*. 2002;63:54–58.
25. Leucht S, Corves C, Arbter D, Engel RR, Li C, Davis JM. Second-generation versus first-generation antipsychotic drugs for schizophrenia: a meta-analysis. *Lancet*. 2009;373:31–41.
26. Lieberman JA, Stroup TS, McEvoy JP, et al. Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) Investigators. Effectiveness of antipsychotic drugs in patients with chronic schizophrenia. *N Engl J Med*. 2005;353:1209–1223.
27. Arai M, Koike S, Oshima N, et al. Idiopathic carbonyl stress in a drug-naive case of at-risk mental state. *Psychiatry Clin Neurosci*. 2011;65:606–607.
28. Cannon M, Jones PB, Murray RM. Obstetric complications and schizophrenia: historical and meta-analytic review. *Am J Psychiatry*. 2002;159:1080–1092.
29. Brown AS, Bottiglieri T, Schaefer CA, et al. Elevated prenatal homocysteine levels as a risk factor for schizophrenia. *Arch Gen Psychiatry*. 2007;64:31–39.

doi:10.1111/pcn.12216



Psychiatry and Clinical Neurosciences

PCN Frontier Review

Carbonyl stress and schizophrenia

Makoto Arai, PhD,[1] Mitsuhiro Miyashita, MD, PhD,[2] Akiko Kobori,[1] Kazuya Toriumi, PhD,[1] Yasue Horiuchi, PhD[1] and Masanari Itokawa, MD, PhD[1]*

[1]*Department of Psychiatry and Behavioral Sciences, Tokyo Metropolitan Institute of Medical Science, Tokyo, and* [2]*Department of Psychiatry, Shinshu University School of Medicine, Nagano, Japan*

Appropriate biological treatment and psychosocial support are essential to achieve and maintain recovery for patients with schizophrenia. Despite extensive efforts to clarify the underlying disease mechanisms, the main cause and pathophysiology of schizophrenia remain unclear. This is due in large part to disease heterogeneity, which results in biochemical differences within a single disease entity. Other factors include variability across clinical symptoms and disease course, along with varied risk factors and treatment responses. Although schizophrenia's positive symptoms are largely managed through treatment with atypical antipsychotics, new classes of drugs are needed to address the unmet medical need for improving cognitive dysfunction and promoting recovery of negative symptoms in these patients. Accumulation of toxic reactive dicarbonyls, such as methylglyoxal, are typical indicators of carbonyl stress, and result in the modification of proteins and the formation of advanced glycation end products, such as pentosidine. In June 2010, we reported on idiopathic carbonyl stress in a subpopulation of schizophrenia patients, leading to a failure of metabolic systems with plasma pentosidine accumulation and serum pyridoxal depletion. Our findings suggest two markers, pentosidine and pyridoxal, as beneficial for distinguishing a specific subgroup of schizophrenics. We believe that this information, derived from *in vitro* and *in vivo* studies, is beneficial in the search for personalized and hopefully more effective treatment regimens in schizophrenia. Here, we define a subtype of schizophrenia based on carbonyl stress and the potential for using carbonyl stress as a biomarker in the challenge of overcoming heterogeneity in schizophrenia treatment.

Key words: advanced glycation end products, glyoxalase, pentosidine, pyridoxal, vitamin B_6.

SCHIZOPHRENIA IS A chronic and disabling brain disorder, thought to be a heterogeneous syndrome. The lifetime prevalence is approximately one percent, with onset of disease frequently occurring in early adulthood.[1,2] Numerous studies suggest oxidative stress and redox dysregulation as risk factors for the development of schizophrenia.[3–5] In addition to a genetic predisposition to disease, quantitative and qualitative resistance to stress interact with environmental elements and genetic factors, leading to a distorted metabolic balance in the body.[6] Although several potential candidate genes and chromosomal linkage loci for schizophrenia have been identified,[7–10] little information is available on their molecular and cellular intermediates.[11–15]

Dicarbonyls, such as methylglyoxal (MG), a potent protein-glycating agent, are formed from sugars, lipids and amino acids.[16–19] This dicarbonyl accumulation, which is referred to as carbonyl stress,[20] modi-

Correspondence: Masanari Itokawa, MD, PhD, Department of Psychiatry and Behavioral Sciences, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya, Tokyo 156-8506, Japan. Email: itokawa-ms@igakuken.or.jp

Accepted 26 June 2014.

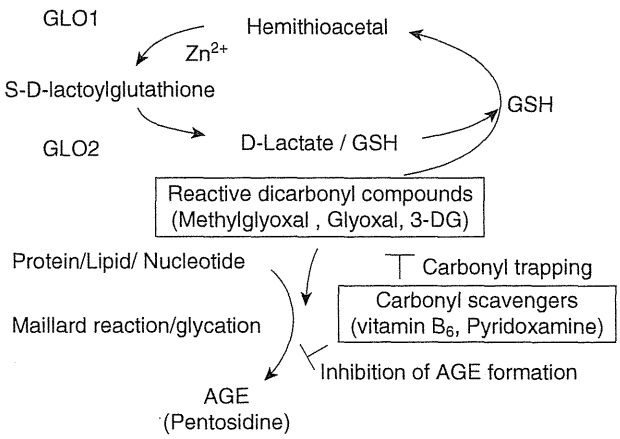


Figure 1. Glyoxalase detoxification system. Accumulation of reactive carbonyl compounds results in modification of proteins and the eventual formation of advanced glycation end products (AGE) and methylglyoxal-adducts. Glyoxalase proteins are ubiquitously expressed in various tissues, including the brain, and provide an effective defense against the accumulations of reactive dicarbonyl compounds. Vitamin B_6 also detoxifies reactive carbonyl compounds by trapping these products and/or by inhibiting the formation of AGE. GLO1, glyoxalase I; GLO2, glyoxalase II; GSH, glutathione.

fies proteins and leads to the eventual formation of advanced glycation end products (AGE). The formation of AGE is associated with three different pathways *in vivo*, namely the Maillard reaction, polyol pathway and lipid peroxidation.[21]

Some reports in humans implicate increased AGE in a variety of illnesses, including diabetes,[22,23] hemodialysis[24] and mental illness.[25–27] Cellular removal of dicarbonyls and AGE occurs via a glutathione-dependent glyoxalase, namely, glyoxalase I (Glo1) (Fig. 1). The glyoxalase detoxification system is found in all tissues, including the brain. It is also known that this system interacts with several metabolizing cascades, some of which have been reported as possible causative factors in the etiology of mental disorders.[28,29] Studies in humans have revealed reduced *GLO1* mRNA levels in peripheral leukocytes in patients with mood disorder,[30] genetic association of polymorphisms of *GLO1* with autism[31] and panic disorder,[32] altered frequencies of GLO1 enzyme activity phenotypes in alcoholism,[33] and altered *GLO1* mRNA levels in post-mortem brains of patients with Alzheimer's disease.[34]

In June 2010, we reported enhanced carbonyl stress as a disease feature in a subpopulation of schizophrenics.[35] We found a 1.7-fold increased concentration of plasma pentosidine, a marker for AGE, and significantly decreased levels of pyridoxal, one of the three forms of vitamin B_6, in schizophrenia compared with healthy control subjects. We detected heterozygous frameshift mutations with loss-of-function and moderate-effect polymorphisms in *GLO1*. These changes resulted in a 40–50% and 15–20% reduction in Glo1 activity in loss of function and moderate-effect mutants, respectively. Our results suggested involvement of accumulated dicarbonyls and/or pentosidine in the pathophysiology of schizophrenia patients carrying these genetic *GLO1*-deficits.

We also reported on a drug-naïve patient with at-risk mental state, who exhibited enhanced carbonyl stress, with high plasma pentosidine levels, suggesting the presence of stress before the onset of disease.[36] A follow-up study showed that decreased plasma pentosidine levels were accompanied by improved psychotic symptoms on follow up, compared with initial observations. Interestingly, plasma pentosidine levels are significantly lower in outpatients than in hospitalized cases, while Glo1 activity is significantly upregulated in outpatients (Arai *et al.*, unpublished findings). Schizophrenics with accumulated pentosidine and depleted pyridoxal showed distinct clinical features, such as a higher propensity to inpatient status, low educational status, higher frequency and longer durations of hospitalization and higher doses of anti-psychotic medication.[37]

Carbonyl stress as identified in patients with schizophrenia is a key concept for clarifying some of the pathogenic and pathological mechanisms in schizophrenia.

AGE

A robust susceptibility gene for schizophrenia has yet to be identified, although candidate polymorphisms and chromosomal regions associated with this disease have been reported from multi-center genome-wide association studies, conducted using large-scale samples.[7,8,38] A serious problem in the genetic research of schizophrenia is that of heterogeneity. Disease heterogeneity complicates the search for molecular patterns and subsequently hinders the identification of disease mechanisms.

Specific biomarkers would be useful for classifying heterogeneous psychotic syndromes into homogeneous subtypes, thereby improving disease diagnosis, as well as clinical staging and prognostic information on cognitive function.[39,40] In general, it is difficult to

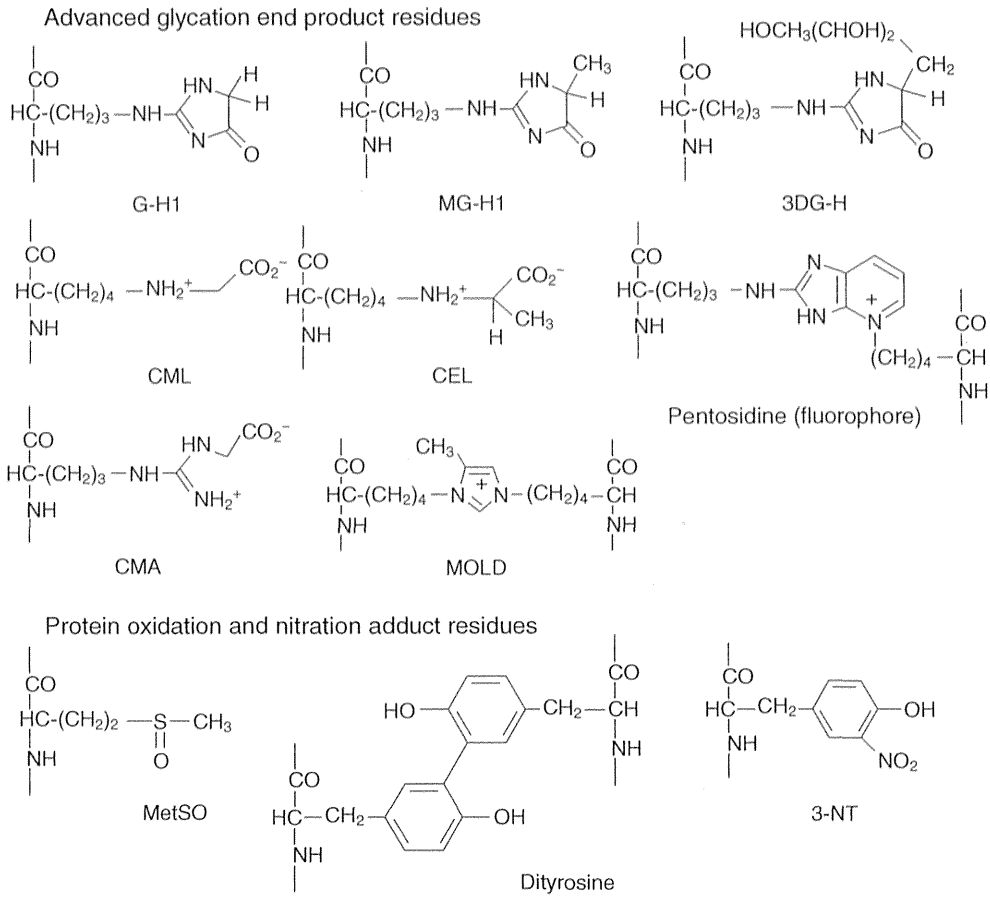


Figure 2. Molecular structure of protein glycation, oxidation, and nitration residues (modified and adapted from Rabbani *et al.*[45]). The extent of these types of protein modification is usually between 0.01% and 5%. The best technique for monitoring these adducts is through isotopic dilution analysis using liquid chromatography, with positive-ion electrospray-ionization tandem mass spectrometric detection (LC-MS/MS).[46] CEL, Nε-(1-carboxyethyl) lysine; CML, N-ε-(Carboxymethyl) lysine; MG-H1, N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MOLD, A1,3-di(Nε-lysino)-4-methyl-imidazolium.

capture information of the brain from peripheral blood. However, multiple omics research projects using peripheral blood and urine have proven to be very effective tools for identifying new causative genes, environmental factors and disease metabolites. The classification of schizophrenia into subgroups using biomarkers is the current research goal, ultimately leading to the discovery of new classes of therapeutic agents and the advent of personalized medicine.[41]

The condensation of carbonyl groups with amine groups leads to the formation of a reversible Schiff base. Subsequent reactions, such as oxidation and peroxidation, result in the irreversible final products known as AGE. These include N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), N-ε-(Carboxymethyl) lysine (CML), Nε-(1-carboxyethyl) lysine (CEL), 1,3-di(Nε-lysino)-4-methyl-imidazolium (MOLD) and pentosidine[42–44] (Fig. 2). Methylglyoxal (MG), glyoxal and 3-deoxyglucosone (3-DG) enhance AGE formation. Methionine sulfoxide, dityrosine and 3-nitrotyrosine are also markers for protein oxidation and nitration.[45] The structures of AGE were determined from peripheral blood, urine and other tissue samples. Typical AGE are primarily monitored using combinations of high-performance liquid chromatography (HPLC) and mass spectrometry (LC-MS/MS).[18,46] In our studies, pentosidine level was measured using HPLC techniques described in our previous publications.[35–37]

DISCOVERY OF ENHANCED CARBONYL STRESS IN SCHIZOPHRENIA

MG is a reactive glycating agent and a precursor of major quantitative adducts formed from proteins. It is a more reactive α-oxoaldehyde metabolite, formed after the degradation of triosephosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, by MG synthase in bacteria, the oxidation of acetone, ketone body metabolism, catabolism of threonine and degradation of glycated proteins.[47] Accumulation of MG induces activation and increased degradation of proteins, DNA mutagenesis and cytotoxicity.[48,49] Therefore, efficient metabolism and detoxification of MG is needed to prevent protein and DNA damage in cells. The glyoxalase pathway represents the major route for MG metabolism, as shown in Figure 1, and is supported by aldose reductase and aldehyde dehydrogenases.[50,51] In the glyoxalase pathway, Glo1 catalyzes the isomerization of hemithioacetals, formed non-enzymatically from MG and reduced glutathione, to S-D-lactoylglutathione. Next, Glo 2 catalyzes the hydrolysis of S-D-lactoylglutathione, to D-lactate and glutathione (GSH).

On 2 June 2006, we identified a novel schizophrenia case with a deficiency of *GLO1*, and published this report in *Archives of General Psychiatry* (AGP) (2010).[35] This patient was a 60-year-old man suffering from severe schizophrenia, with a brother affected by schizophrenia, a sibling who committed suicide and two maternal uncles who suffered from schizophrenia. We identified a novel frameshift mutation in this case (case #1). The c.79_80insA mutation causes a frameshift in *GLO1*. The adenine insertion at nucleotide 79, in exon 1 resulted in a frameshift at codon 27 and introduced a premature termination codon after aberrant translation of 15 amino acid residues (p.T27NfsX15). One year later, we found a new c.365delC mutation in another schizophrenic patient (case #2). The mutation generated a frameshift in codon 122 of exon 4 in *GLO1* and a premature termination after the addition of an aberrant 27 amino acid peptide (p. P122LfsX27) (Fig. 3).

Individuals with heterozygous frameshift mutations showed an approximately 40–50% reduction of Glo1 enzymatic activity in both erythrocytes and lymphocytes, supporting the belief that *GLO1* genetic mutations occur in one allele. We suspected that aberrant transcripts from the mutant allele were degraded by nonsense-mediated mRNA decay, as the predicted truncated proteins from p.T27NfsX15 and p.P122LfsX27 were not detected by immunoblotting. Lymphoblastoid cells derived from subjects carrying heterozygous frameshift mutations showed significantly decreased levels of *GLO1* mRNA, compared with those from both affected and unaffected individuals without heterozygous frameshift mutations. Glo1 enzymatic activity in heterozygous frameshift mutation carriers was lower than that of wild-type individuals (2.8 munit/10^6 red blood cells [RBC] in case #1, 3.0 munit/10^6 RBC in case #2, 6.2 munit/10^6 RBC in wild-type healthy control subjects).

As expected, heterozygous frameshift carriers with schizophrenia displayed increased concentrations of plasma pentosidine. Levels of plasma pentosidine detected by HPLC, a quantitatively accurate and stable technique, were 137 ng/mL in case #1 and 74.7 ng/mL in case #2. These high plasma pentosidine levels in the two patients carrying the mutation are most likely caused by reduced Glo1 function, as neither suffers from diabetes mellitus nor renal failure, two of the major causes of enhanced AGE formation.

Interestingly, these rises in pentosidine were concomitant with prominent decreases of pyridoxal, a major isoform of vitamin B_6. Vitamin B_6 plays an important role in various physiological functions and is converted to pyridoxine, pyridoxal and pyridoxamine *in vivo*. Pyridoxamine reacts with RCO by carbonyl-amine chemistry (Fig. 1). In the two *GLO1* frameshift mutation carriers, pyridoxal was markedly reduced, probably due to its depletion after detoxifying RCO. We found decreased pyridoxal levels in both cases compared with wild-type individuals (<2.0 ng/mL in case #1, 2.8 ng/mL in case #2, 11.9 ng/mL in wild-type healthy controls). The depletion of pyridoxal may reflect a state of carbonyl stress in these patients.

The *GLO1* gene also harbors a Glu111 (a major genotype)/Ala111 polymorphism. The frequency of the Ala111 allele exhibits high population diversity. We assayed the missense variants, Glu111/Glu111, Glu111/Ala111 and Ala111/Ala111, using 1586 schizophrenics. We identified nine homozygous Ala111 carriers, who showed approximately 20–30% lower Glo1 enzyme activity against the wild-type group. Mean values of enzymatic activity were approximately 4.7 munit/10^6 RBC in these patients. Patients carrying homozygous Ala111/Ala111 alleles

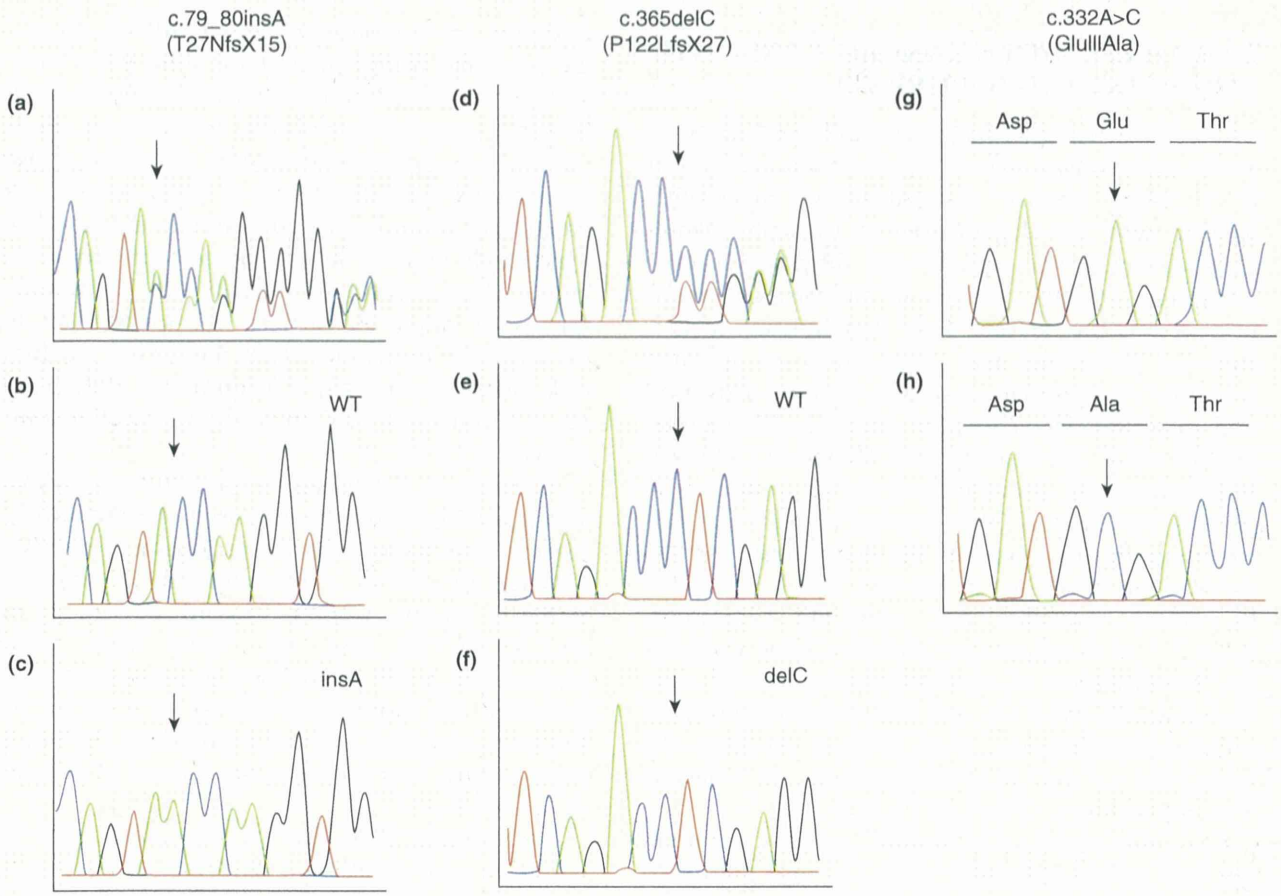


Figure 3. DNA sequence chromatograms showing frameshift and missense variants. The (a,b,c) left and (d,e,f) middle column chromatograms show heterozygous sequence traces derived from individuals carrying an adenine insertion within exon 1 and a cytosine deletion within exon 4, respectively. TA cloning and subsequent sequence analyses revealed (b,e) normal (denoted by WT) and (c,f) mutant (denoted by insA or delC) sequences. (g,h) Right chromatograms depict a Glu111Ala missense variant located within exon 4. The nucleotide positions of insertions, deletions and substitutions are indicated by arrows.

also showed higher plasma pentosidine levels (mean value of plasma pentosidine, 98.0 ng/mL), in the absence of diabetes mellitus or renal failure.

In order to investigate whether *GLO1* genotypes show modified enzymatic activity, we performed functional analyses of mutants, using recombinant GFP-tagged Glo1 proteins generated by site-directed mutagenesis. Our *in vitro* transfection study revealed an approximately 16% reduction in enzymatic activity for Ala111 carriers over Glu111 carriers, implying an intrinsically lower enzymatic activity for the Ala111/Ala111 genotype compared with the Glu111/Glu111 genotype. Recombinants expressing either GFP-T27NfsX15 or GFP-P122LfsX27 exhibited basal protein levels, indicating that the two frameshift mutations completely abolish enzymatic activity of Glo1 protein.

Our findings suggested that markedly low Glo1 activity and a concomitant increase in carbonyl stress elicited by heterozygous frameshift mutations and the homozygous Ala111 genotype in *GLO1* could be causative for schizophrenia in a small subset of patients. This provided the first evidence to support the multiple, rare variants–common disease hypothesis in schizophrenia, where the causative mechanism, in this case carbonyl stress, derived from rare large-effect mutations in a multiple affected pedigree could be replicated by a moderate-effect polymorphism in the general population.

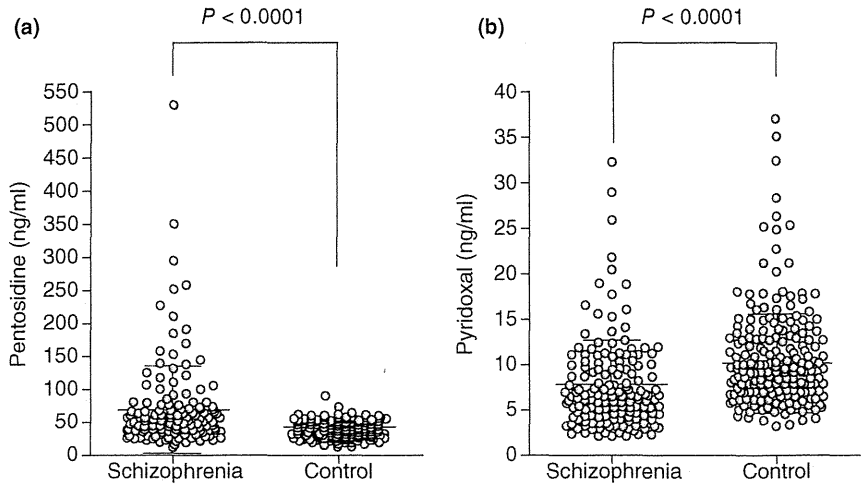


Figure 4. Replication of enhanced carbonyl stress in a subpopulation of schizophrenia. Data represent (a) plasma pentosidine accumulation and (b) serum pyridoxal depletion. The concentrations of pentosidine in patients and controls were 67.7 ± 64.9 ng/mL and 41.9 ± 11.1 ng/mL, respectively. Serum pyridoxal levels in schizophrenia were significantly lower than controls (7.7 ± 4.9 ng/mL and 10.2 ± 5.5 ng/mL, respectively).

REPLICATION OF IDIOPATHIC CARBONYL STRESS IN A SUBPOPULATION OF SCHIZOPHRENIA

Higher levels of plasma pentosidine were observed in 46.7% patients with schizophrenia (21 out of 45 cases), as reported in AGP (2010).[35] On the other hand, no healthy control subjects displayed increased pentosidine accumulation. The cut-off value of pentosidine was defined as the average value of healthy controls plus 2SD, 55.2 ng/mL. The mean pentosidine level of 68.4 ng/mL was 1.7-fold higher in schizophrenic patients compared with healthy controls. In addition to pentosidine accumulation, we found that pyridoxal concentrations were also significantly reduced in patients with schizophrenia. Roughly 24% ($n = 11$) of 45 cases showed marked reductions in pyridoxal levels, with four cases showing less than 2 ng/mL. Mean pyridoxal values were significantly reduced in schizophrenic patients (7.5 ng/mL in schizophrenia vs 11.9 ng/mL in healthy subjects). The mean pyridoxal level in three patients carrying heterozygous frameshift mutations was approximately 3.6 ng/mL. Our results showed that pentosidine accumulation (odds ratio [OR]: 25.8, 95% confidence interval [CI]: 5.6–118.8, $P < 0.0001$) as well as pyridoxal reduction (OR: 10.6, 95%CI: 3.9–28.3, $P < 0.0001$) increased the risks for developing schizophrenia.

In a *Psychiatry and Clinical Neurosciences* (PCN) (2014) publication, we increased the sample size and validated our association of enhanced carbonyl stress with schizophrenia.[52] We recruited 156 outpatients using DSM-IV criteria for schizophrenia or schizoaffective disorder, and 221 age-matched healthy control subjects. Plasma pentosidine concentrations in patients and healthy control subjects were 67.7 ng/mL and 41.9 ng/mL, respectively (Fig. 4). The mean pentosidine level from schizophrenic patients was 1.6-fold higher than that of healthy control subjects. Conversely, the mean pyridoxal value was significantly reduced in schizophrenic patients compared with controls (schizophrenia, 7.7 ng/mL; healthy control subjects, 10.2 ng/mL). We applied a cut-off point to differentiate between a carbonyl stress group and a non-stress group, set at the average value in healthy controls, plus 2SD. We found that 11.8% of patients with schizophrenia also displayed enhanced carbonyl stress. On the other hand, carbonyl stress was present in only 0.01% of healthy control subjects. These results are consistent with our previous report in AGP (2010),[35] replicating the presence of idiopathic carbonyl stress in a subpopulation of patients with schizophrenia.

CARBONYL STRESS AS A DISEASE STATE MARKER

We assayed alterations of pentosidine, Glo1 enzymatic activity and pyridoxal, using samples from both in- and outpatients with a follow-up period of 3–5 years. Reduced pentosidine and increased Glo1 enzymatic activity were observed in discharged cases. However, no significant changes in these parameters were observed in hospitalized patients.[53] These levels

of pentosidine and Glo1 enzymatic activity provide a suggestion that upregulation of Glo1 metabolism leads to inhibition of carbonyl stress and improves some symptoms for patients with schizophrenia. If we can find bioactives for improving dysfunctions of Glo1 metabolism, it may also prove to be of therapeutic value.

A drug-naïve patient with at-risk mental state (ARMS) who exhibited enhanced carbonyl stress with high plasma pentosidine levels highlighted the possibility that carbonyl stress existed before the onset of disease, as reported in *PCN* (2011).[36] The patient was a 21-year-old man who first developed obsessive thoughts at age 18 and sought medical help for communication difficulties and depression. He was diagnosed with ARMS, according to Structured Interview for Prodromal Syndromes criteria, 10 months after his initial consultation. At his initial visit, his symptoms and pentosidine levels were measured, and then rechecked after 16 months of treatment. Plasma pentosidine levels showed a remarkable decrease from 113.2 ng/mL to 44.1 ng/mL. His clinical score dropped from 84 to 58 on the total Positive and Negative Syndrome Scale (PANSS), a fall promoted to a great extent by biweekly counseling and psychotherapy. Negative subscale scores and general psychopathology subscale scores also improved from 22 to 9, and from 42 to 27, respectively. Global assessment of functioning changed from 55 to 65.

Unfortunately, his latest results show increased pentosidine levels of 300 ng/mL, and he has been admitted to hospital. This finding further supports our hypothesis that carbonyl stress status can help characterize the psychosis risk state for a subset of patients with schizophrenia. Pentosidine levels may constitute a genuine biomarker for the state of disease, if carbonyl stress is confirmed as being directly linked to schizophrenic signs and symptoms.

CLINICAL FEATURES OF SCHIZOPHRENIA EXHIBITING CARBONYL STRESS

Recently, we examined the clinical characteristics of patients with carbonyl stress, and psychopathological symptoms using PANSS.[37] In this study, patients were divided into four groups according to levels of pentosidine and pyridoxal: normal pentosidine and normal pyridoxal levels (group 1), normal pentosidine and low pyridoxal (group 2), high pentosidine and normal pyridoxal (group 3) and high pentosidine and low pyridoxal (group 4). Our hope was that this classification based on biomarkers would be able to distinguish homogenous patient subsets with specific clinical characteristics from the heterogeneous disease population. As mentioned earlier, more accurate disease subdivisions should pave the way for developing personalized medicine in schizophrenia.

We evaluated the symptom severity of 49 consenting patients. Examining 30 items of PANSS, we found significant negative correlations between serum pyridoxal levels and items of hostility, emotional withdrawal, passive/apathetic social withdrawal, tension, mannerism/posturing, disorientation and active social avoidance. Furthermore, we also found significant correlations between the total general psychopathological score and serum pyridoxal levels. These correlations would support the novel therapeutic idea of pyridoxamine supplementation for carbonyl stress-related schizophrenia.[54]

The clinical characteristics of schizophrenia with carbonyl stress suggest targeting this stress as a new therapy for psychiatric disorders. We regard pyridoxamine, a non-toxic, water-soluble vitamin B_6, as a novel medicine for schizophrenia with carbonyl stress, primarily because it inhibits the formation of AGE. Recently, Katsuta *et al.* (2014) also investigated the relation between carbonyl stress markers and clinical characteristics of acute-stage schizophrenic patients in a cross-sectional and longitudinal study.[26] The authors put forward vitamin B_6 supplementation as a candidate for augmentation therapy in a subpopulation of schizophrenics who showed vitamin B_6 depletion.

The enhanced carbonyl stress cohort, group 4, showed a more severe clinical course relative to the normal cohort, group 1 (Table 1). The proportions of subjects classified as inpatients were 23.9% in group 1 and 78.6% in group 4. Patients in group 4 showed a longer duration of hospitalization (4.2 years vs 17.4 years), higher daily doses of anti-psychotics (773.8 mg/day vs 1143.9 mg/day) and lower educational status (13 years vs 11.7 years) (Table 1). We also observed a 1.5-fold higher number of hospitalizations in group 4 compared with group 1 subjects. In this study,[37] we could not exclude the possibility that high plasma pentosidine levels were a consequence of high doses and long exposure to antipsychotic medication. Therefore, further studies focused on drug-naïve patients will be required to address these issues.

Table 1. Clinical features of schizophrenics with and without carbonyl stress (modified and adapted from Miyashita *et al.*[37])

	Group 1 ($n = 67$) Normal pentosidine and normal pyridoxal	Group 4 ($n = 26$) High pentosidine and low pyridoxal	Fold change vs group 1
Biochemical variables			
Pentosidine†	41.5 ± 11.6	123 ± 85.6	2.97***
Pyridoxal‡	9.5 ± 5.7	3.4 ± 1	0.36***
GLO1 enzymatic activity	7.06 ± 0.77	6.32 ± 0.97	0.9**
Clinical variables			
Inpatients/outpatients	16/51	21/5	3.38***
Education duration (years)	13 ± 2.6	11.7 ± 2.6	0.9*
Hospitalization duration (years)	4.2 ± 9.2	17.4 ± 16.9	4.17**
Anti-psychotics (mg/day; CP equivalent)	773.8 ± 652.4	1143.9 ± 743.6	1.48*

*$P < 0.05$ (vs group 1); **$P < 0.001$ (vs group 1); ***$P < 0.0001$ (vs group 1).
†Cut-off point for high plasma pentosidine levels, 62.9 ng/mL (the mean + 2SD of healthy controls). ‡Low pyridoxal levels, <6 ng/mL (male) and <4 ng/mL (female).
CP, chlorpromazine; GLO, glyoxalase I.

THERAPEUTIC POTENTIAL OF PYRIDOXAMINE TO PREVENT CARBONYL STRESS

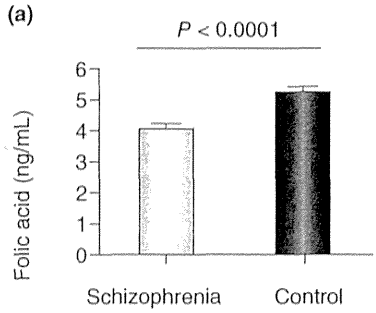
As mentioned above, vitamin B_6 consists of three components, namely, pyridoxamine, pyridoxine and pyridoxal. Of the three, only pyridoxamine is able to prevent AGE accumulation by amine-chemistry.[54] To further validate our hypothesis, we are planning a phase II clinical trial for carbonyl stress-induced schizophrenia, using pyridoxamine. Using evidence-based medicine, we are attempting to adapt translational research into medical care for carbonyl stress-induced schizophrenia.

In addition to vitamin B_6 metabolism, Glo1 metabolites are linked to other pathways, such as those involving glutathione, methionine, homocysteine, folate and amino acids, as described in AGP *e*Figure 1.[35] Glo1 metabolism is also associated with glycolysis, the pentose phosphate pathway and lipid/protein metabolism.[55] In our preliminary data, schizophrenics show lower folic acid concentrations and higher levels of homocysteine, relative to healthy control subjects (folic acid, $\chi^2 = 42.21$, $P < 0.0001$, OR = 8.84, 95%CI = 4.16–18.81; homocysteine, $\chi^2 = 24.76$, $P < 0.0001$, OR = 7.13, 95%CI = 3.12–16.28) (Fig. 5). Patients with higher homocysteine and lower folate levels showed high pentosidine accumulation and pyridoxal depletion. These results imply an imbalance of one-carbon metabolism[56] in schizophrenics with altered Glo1 metabolism. These metabolic systems are functionally integrated and act homeostatically. Therefore, for us to understand the full diversity of biological systems that exists in both the diseased and healthy states, it is important to evaluate combined data from *in vitro* and *in vivo* sources.[57]

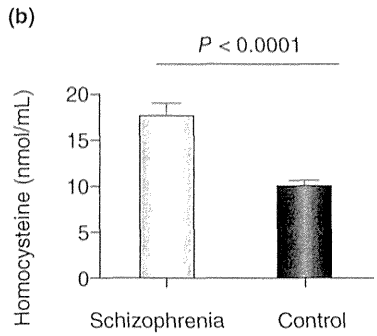
CONCLUSIONS

Our study is the first investigation into *GLO1* alterations and enzymatic activity in schizophrenics, and to highlight its link to idiopathic carbonyl stress, defined as pentosidine accumulation and pyridoxal depletion. Although more studies are needed to understand the molecular mechanisms precipitated by carbonyl stress in the central nervous system, carbonyl stress undoubtedly represents a new target for medication without neurotransmitter-based concepts in the treatment of schizophrenia. In particular, schizophrenic patients with low pyridoxal and high pentosidine may well benefit clinically from pyridoxamine treatment. A better understanding of the molecular mechanisms that promote the pathophysiology of carbonyl stress-related schizophrenia could drive improvements in difficult-to-treat negative symptoms and cognitive dysfunction, thereby improving the quality of life for patients.

Future omics studies combining comprehensive molecular and clinical information will lead to new



Folic acid	Low group	Normal group
Schizophrenia	116 cases (38.9%)	182 cases (61.1%)
Healthy control subjects	8 cases (6.7%)	111 cases (93.3%)
$X^2 = 42.21$; d.f. = 1; $P < 0.0001$; Odds ratio = 8.843; 95%CI = 4.159 - 18.81		



Homocysteine	High group	Normal group
Schizophrenia	41 cases (51.3%)	39 cases (48.8%)
Healthy control subjects	9 cases (12.9%)	61 cases (87.1%)
$X^2 = 24.76$; d.f. = 1; $P < 0.0001$; Odds ratio = 7.125; 95%CI = 3.119 - 16.28		

Figure 5. Glyoxalase metabolites are linked to other pathways. Schizophrenics showed (a) lower folic acid concentrations and (b) higher levels of homocysteine. CI, confidence interval.

discoveries, allowing for more accurate diagnostic subdivisions for schizophrenia and subsequent treatment regimens.

ACKNOWLEDGMENTS

This study was supported by a grant from the Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation by the Japan Society for the Promotion of Science fellowship: The collaborative study between Japan and United Kingdom on molecular mechanisms of glycation and oxidative stress in psychiatric disorders. We especially thank Professor Paul J. Thornalley and Dr Naila Rabbani. We thank members of the host research teams for technical assistance: Drs Mingzhan Xue, Jinit Masania, Fozia Shaheen and Attia Anwar in Clinical Sciences Research Laboratories, Warwick Medical School, University of Warwick, University Hospital. We also thank Hiroko Yuzawa at the Institute of Medical Sciences, Tokai University for the measurement of plasma pentosidine levels. We are grateful for the expert technical assistance of Izumi Nohara, Mayumi Arai and Nanako Obata. Additionally, we thank Ikuyo Kito and Sachie Kogoku at Tokyo Metropolitan Institute of Medical Science for assistance with the preparation of this manuscript.

REFERENCES

1. Giusti-Rodríguez P, Sullivan PF. The genomics of schizophrenia: Update and implications. *J. Clin. Invest.* 2013; 123: 4557–4563.
2. Kim Y, Zerwas S, Trace SE, Sullivan PF. Schizophrenia genetics: Where next? *Schizophr. Bull.* 2011; 37: 456–463.
3. Ng F, Berk M, Dean O, Bush AI. Oxidative stress in psychiatric disorders: Evidence base and therapeutic implications. *Int. J. Neuropsychopharmacol.* 2008; 11: 851–876.
4. O'Donnell P, Do KQ, Arango C. Oxidative/nitrosative stress in psychiatric disorders: Are we there yet? *Schizophr. Bull.* 2014. doi: 10.1093/schbul/sbu048
5. Fournier M, Ferrari C, Baumann PS *et al.* Impaired metabolic reactivity to oxidative stress in early psychosis patients. *Schizophr. Bull.* 2014. doi: 10.1093/schbul/sbu053
6. Hovatta I, Juhila J, Donner J. Oxidative stress in anxiety and comorbid disorders. *Neurosci. Res.* 2010; 68: 261–275.
7. Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium. Genome-wide association study identifies five new schizophrenia loci. *Nat. Genet.* 2011; 43: 969–976.

39. Koike S, Bundo M, Iwamoto K *et al.* A snapshot of plasma metabolites in first-episode schizophrenia: A capillary electrophoresis time-of-flight mass spectrometry study. *Transl. Psychiatry* 2014; 4: e379.
40. Ge X, Xu XY, Feng CH, Wang Y, Li YL, Feng B. Relationships among serum C-reactive protein, receptor for advanced glycation products, metabolic dysfunction, and cognitive impairments. *BMC Neurol.* 2013; 13: 110.
41. Kaddurah-Daouk R. Metabolic profiling of patients with schizophrenia. *PLoS Med.* 2006; 3: e363.
42. Rabbani N, Thornalley PJ. Quantitation of markers of protein damage by glycation, oxidation, and nitration in peritoneal dialysis. *Perit. Dial. Int.* 2009; 29: S51–S56.
43. Rabbani N, Thornalley PJ. Glyoxalase Centennial Conference: Introduction, history of research on the glyoxalase system and future prospects. *Biochem. Soc. Trans.* 2014; 42: 413–418.
44. Rabbani N, Thornalley PJ. Glycation research in amino acids: A place to call home. *Amino Acids* 2012; 42: 1087–1096.
45. Rabbani N, Chittari MV, Bodmer CW, Zehnder D, Ceriello A, Thornalley PJ. Increased glycation and oxidative damage to apolipoprotein B100 of LDL cholesterol in patients with type 2 diabetes and effect of metformin. *Diabetes* 2010; 59: 1038–1045.
46. Rabbani N, Shaheen F, Anwar A, Masania J, Thornalley PJ. Assay of methylglyoxal-derived protein and nucleotide AGEs. *Biochem. Soc. Trans.* 2014; 42: 511–517.
47. Thornalley PJ. The glyoxalase system in health and disease. *Mol. Aspects Med.* 1993; 14: 287–371.
48. Rabbani N, Thornalley PJ. Methylglyoxal, glyoxalase 1 and the dicarbonyl proteome. *Amino Acids* 2012; 42: 1133–1142.
49. Thornalley PJ, Waris S, Fleming T *et al.* Imidazopurinones are markers of physiological genomic damage linked to DNA instability and glyoxalase 1-associated tumour multidrug resistance. *Nucleic Acids Res.* 2010; 38: 5432–5442.
50. Rabbani N, Xue M, Thornalley PJ. Activity, regulation, copy number and function in the glyoxalase system. *Biochem. Soc. Trans.* 2014; 42: 419–424.
51. Rabbani N, Thornalley PJ. Dicarbonyl proteome and genome damage in metabolic and vascular disease. *Biochem. Soc. Trans.* 2014; 42: 425–432.
52. Miyashita M, Arai M, Yuzawa H *et al.* Replication of enhanced carbonyl stress in a subpopulation of schizophrenia. *Psychiatry Clin. Neurosci.* 2014; 68: 83–84.
53. Arai M, Koike S, Maekawa T *et al.* Carbonyl stress. *Jpn J Psychiatr Treat.* 2011; 26: 1589–1596 (in Japanese).
54. Voziyan PA, Hudson BG. Pyridoxamine as a multifunctional pharmaceutical: Targeting pathogenic glycation and oxidative damage. *Cell. Mol. Life Sci.* 2005; 62: 1671–1681.
55. Bélanger M, Yang J, Petit JM, Laroche T, Magistretti PJ, Allaman I. Role of the glyoxalase system in astrocyte-mediated neuroprotection. *J. Neurosci.* 2011; 31: 18338–18352.
56. Krebs MO, Bellon A, Mainguy G, Jay TM, Frieling H. One-carbon metabolism and schizophrenia: Current challenges and future directions. *Trends Mol. Med.* 2009; 15: 562–570.
57. Tanaka H. Omics-based medicine and systems pathology. A new perspective for personalized and predictive medicine. *Methods Inf. Med.* 2010; 49: 173–185.

8. Sullivan PF, Daly MJ, O'Donovan M. Genetic architectures of psychiatric disorders: The emerging picture and its implications. *Nat. Rev. Genet.* 2012; 13: 537–551.
9. Kim AH, Parker EK, Williamson V, McMichael GO, Fanous AH, Vladimirov VI. Experimental validation of candidate schizophrenia gene ZNF804A as target for hsa-miR-137. *Schizophr. Res.* 2012; 141: 60–64.
10. Timms AE, Dorschner MO, Wechsler J *et al.* Support for the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia from exome sequencing in multiplex families. *JAMA Psychiatry* 2013; 70: 582–590.
11. Huang JT, Leweke FM, Tsang TM *et al.* CSF metabolic and proteomic profiles in patients prodromal for psychosis. *PLoS ONE* 2007; 2: e756.
12. Yao JK, Dougherty GG Jr, Reddy RD *et al.* Homeostatic imbalance of purine catabolism in first-episode neuroleptic-naïve patients with schizophrenia. *PLoS ONE* 2010; 5: e9508.
13. Yang J, Chen T, Sun L *et al.* Potential metabolite markers of schizophrenia. *Mol. Psychiatry* 2013; 18: 67–78.
14. Orešič M, Tang J, Seppänen-Laakso T *et al.* Metabolome in schizophrenia and other psychotic disorders: A general population-based study. *Genome Med.* 2011; 3: 19.
15. Domenici E, Willé DR, Tozzi F *et al.* Plasma protein biomarkers for depression and schizophrenia by multi analyte profiling of case-control collections. *PLoS ONE* 2010; 5: e9166.
16. Kikuchi S, Shinpo K, Takeuchi M *et al.* Glycation–a sweet tempter for neuronal death. *Brain Res. Brain Res. Rev.* 2003; 41: 306–323.
17. Thornalley PJ. The glyoxalase system: New developments towards functional characterization of a metabolic pathway fundamental to biological life. *Biochem. J.* 1990; 269: 1–11.
18. Jaisson S, Gillery P. Evaluation of nonenzymatic posttranslational modification-derived products as biomarkers of molecular aging of proteins. *Clin. Chem.* 2010; 56: 1401–1412.
19. Thornalley PJ. Glyoxalase I–structure, function and a critical role in the enzymatic defence against glycation. *Biochem. Soc. Trans.* 2003; 31: 1343–1348.
20. Miyata T, de Strihou C, Kurokawa K, Baynes JW. Alterations in nonenzymatic biochemistry in uremia: Origin and significance of 'carbonyl stress' in long-term uremic complications. *Kidney Int.* 1999; 55: 389–399.
21. Ott C, Jacobs K, Haucke E, Navarrete Santos A, Grune T, Simm A. Role of advanced glycation end products in cellular signaling. *Redox Biol.* 2014; 2: 411–429.
22. Kirk RL, Theophilus J, Whitehouse S, Court J, Zimmet P. Genetic susceptibility to diabetes mellitus: The distribution of properdin factor B (Bf) and glyoxalase (GLO) phenotypes. *Diabetes* 1979; 28: 949–951.
23. Perkins BA, Rabbani N, Weston A *et al.* Serum levels of advanced glycation endproducts and other markers of protein damage in early diabetic nephropathy in type 1 diabetes. *PLoS ONE* 2012; 7: e35655.
24. Miyata T, de Strihou C, Imasawa T *et al.* Glyoxalase I deficiency is associated with an unusual level of advanced glycation end products in a hemodialysis patient. *Kidney Int.* 2001; 60: 2351–2359.
25. Kouidrat Y, Amad A, Desailloud R *et al.* Increased advanced glycation end-products (AGEs) assessed by skin autofluorescence in schizophrenia. *J. Psychiatr. Res.* 2013; 47: 1044–1048.
26. Katsuta N, Ohnuma T, Maeshima H *et al.* Significance of measurements of peripheral carbonyl stress markers in a cross-sectional and longitudinal study in patients with acute-stage schizophrenia. *Schizophr. Bull.* 2014. doi: 10.1093/schbul/sbt234
27. Toyosima M, Maekawa M, Toyota T *et al.* Schizophrenia with the 22q11.2 deletion and additional genetic defects: Case history. *Br. J. Psychiatry* 2011; 199: 245–246.
28. Distler MG, Palmer AA. Role of glyoxalase 1 (Glo1) and methylglyoxal (MG) in behavior: Recent advances and mechanistic insights. *Front. Genet.* 2012; 250: 1–10.
29. Hambsch B. Altered glyoxalase 1 expression in psychiatric disorders: Cause or consequence? *Semin. Cell Dev. Biol.* 2011; 22: 302–308.
30. Fujimoto M, Uchida S, Watanuki T *et al.* Reduced expression of glyoxalase-1 mRNA in mood disorder patients. *Neurosci. Lett.* 2008; 438: 196–199.
31. Junaid MA, Kowal D, Barua M, Pullarkat PS, Sklower Brooks S, Pullarkat RK. Proteomic studies identified a single nucleotide polymorphism in glyoxalase I as autism susceptibility factor. *Am. J. Med. Genet. A* 2004; 131: 11–17.
32. Politi P, Minoretti P, Falcone C, Martinelli V, Emanuele E. Association analysis of the functional Ala111Glu polymorphism of the glyoxalase I gene in panic disorder. *Neurosci. Lett.* 2006; 396: 163–166.
33. Ledig M, Doffoel M, Ziessel M *et al.* Frequencies of glyoxalase I phenotypes as biological markers in chronic alcoholism. *Alcohol* 1986; 3: 11–14.
34. Kuhla B, Boeck K, Schmidt A *et al.* Age- and stage-dependent glyoxalase I expression and its activity in normal and Alzheimer's disease brains. *Neurobiol. Aging* 2007; 28: 29–41.
35. Arai M, Yuzawa H, Nohara I *et al.* Enhanced carbonyl stress in a subpopulation of schizophrenia. *Arch. Gen. Psychiatry* 2010; 67: 589–597.
36. Arai M, Koike S, Oshima N *et al.* Idiopathic carbonyl stress in a drug-naive case of at-risk mental state. *Psychiatry Clin. Neurosci.* 2011; 65: 606–607.
37. Miyashita M, Arai M, Kobori A *et al.* Clinical features of schizophrenia with enhanced carbonyl stress. *Schizophr. Bull.* 2013. doi: 10.1093/schbul/sbt129
38. McCarthy MI, Abecasis GR, Cardon LR *et al.* Genome-wide association studies for complex traits: Consensus, uncertainty and challenges. *Nat. Rev. Genet.* 2008; 9: 356–369.

Kawakami et al. Acta Neuropathologica Communications 2014, 2:40
http://www.actaneurocomms.org/content/2/1/40

ACTA NEUROPATHOLOGICA COMMUNICATIONS

RESEARCH Open Access

Tau accumulation in the nucleus accumbens in tangle-predominant dementia

Ito Kawakami[1,2], Masato Hasegawa[3], Tetsuaki Arai[1,4], Kenji Ikeda[1,5], Kenichi Oshima[6], Kazuhiro Niizato[6], Naoya Aoki[1,2], Katsuse Omi[2], Shinji Higashi[1], Masato Hosokawa[1], Yoshio Hirayasu[2] and Haruhiko Akiyama[1*]

Abstract

Background: Tangle-predominant dementia (TPD) is characterized neuropathologically by numerous neurofibrillary tangles in the limbic areas with no or occasional senile plaques throughout the brain. TPD is an under-recognized disease, while it is a common cause of dementia in those over 80 years of age. In the present study, we describe hyperphosphorylated tau (tau) accumulation in the nucleus accumbens (Acb) in patients with TPD.

Results: We investigated immunohistochemically the brain tissues from 7 patients with TPD, 22 with Alzheimer disease (AD) and 11 non-demented aged subjects. In the Acb of all 7 TPD patients, a considerable number of tau positive neurons were found together with many neuropil threads. The tau deposits in the Acb were labeled with all the anti-tau antibodies used in the present study. They included conformational change-specific, phosphorylation-specific and phosphorylation-independent antibodies. The Acb consists of the predominant medium-sized neurons with a small number of large neurons. Both the cell types were affected by tau pathology in TPD. Tau accumulation in the majority of such neurons appeared to be pretangle-like, diffuse deposits with only occasional paired helical filament formation. Tau positive neurons were also found in the Acb in some AD and non-demented aged subjects but much fewer in the majority of cases. The immunoblot analyses of fresh frozen samples of the Acb and parahippocampal cortex from 3 TPD and 3 AD patients revealed that the insoluble tau in the Acb was a mixture of the 3- and 4-repeat isoforms.

Conclusions: To our knowledge, this is the first report on the occurrence of tau accumulation in the Acb in TPD. The Acb receives direct and massive projections from the hippocampal CA1 and subiculum where neurofibrillary tangles are known to occur more frequently in TPD than in AD. The prevalence of abnormal tau accumulation in the Acb in TPD may support the idea that abnormal tau aggregation propagates via neural circuits. In all but one TPD cases used in this study, delusion was a consistent clinical feature. Whether the Acb tau accumulation is related to the psychiatric symptoms in TPD may be an issue for further investigation.

Keywords: Neurofibrillary tangle, Alzheimer disease, Propagation, Delusion

Introduction

Tangle-predominant dementia (TPD), which is also referred to as neurofibrillary tangle predominant dementia, limbic neurofibrillary tangle dementia or senile dementia of the neurofibrillary tangle type, is a poorly understood and under-recognized tauopathy. TPD has been reported to comprise 0.7 to 5.8% of elderly patients with dementia [1-3]. TPD is characterized neuropathologically by numerous neurofibrillary tangles (NFT) in the limbic areas with no or occasional senile plaques throughout the brain. The clinical features of TPD include the late-adult onset, which is over 80 years in the majority of cases, and slow progression of dementia as compared with Alzheimer's disease (AD). In patients with TPD, there is a propensity for the memory disturbance to be conspicuous with relative preservation of other cognitive functions. However, it is hard to distinguish TPD from AD on a clinical basis and, thus, diagnosis of TPD in most cases is only made postmortem.

The etiology of TPD is unknown. NFT in TPD consist of both 3-repeat (3R) and 4-repeat (4R) isoforms of hyperphosphorylated tau (tau), and the neuronal cell types

* Correspondence: akiyama-hr@igakuken.or.jp
[1]Dementia Research Project, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan
Full list of author information is available at the end of the article

bearing NFT in TPD are similar to those in AD. TPD seems to be a disorder that is related to AD, if it is not an atypical form of AD. TPD, as a subtype of tauopathy, is also included in the group described as neuropathologically-defined frontotemporal lobar degeneration [4,5]. In fact, cortical lesions in TPD are localized to the mediobasal temporal cortex. Thus, the situation of TPD in the groups of dementing neurodegenerative diseases remains unclear from both clinical and neuropathological points of view.

A neuropathological characteristic of TPD is the heavy accumulation of NFT in the hippocampal regions, with few or occasional NFT in neocortical areas beyond the collateral sulcus. Compared with AD patients in which a similar number of NFT occurs in the hippocampal regions, neuronal cell loss, tissue rarefaction and gliosis are less prominent in TPD, even in NFT rich areas. Changes in the neocortex are modest, with well-preserved laminar structures and unremarkable neuronal cell loss. The cortical expansion of NFT in TPD is considered to follow in principle the hierarchical pathway described in AD by Braak and Braak [6] but to be limited to stage IV. In the hippocampal regions, the density of NFT is higher than in AD [7] and ghost tangles are very frequent [3]. Tau pathology in the subcortical structures in TPD has not been well studied. The occurrence of NFT in the amygdala, the nucleus basalis of Meynert, the substantia nigra and the locus coeruleus, regions where NFT frequently occur in AD cases, have been reported in TPD [3,8].

The nucleus accumbens (Acb) is located in the region where the caudate head and the rostral putamen meet near the septum pellucidum (Figure 1). The Acb and the olfactory tubercle form the ventral striatum in the forebrain. The Acb is a key component of the limbic striatal loop in which the Acb receives fibers from the prefrontal cortex, amygdala, hippocampus and ventral tegmental area (VTA) and projects to the ventral pallidum [9-12]. The ventral pallidum sends axonal projections to the dorsomedial thalamic nucleus, which then projects to the prefrontal cortex to close the loop [13,14]. The dopaminergic input from the VTA modulate the activity of this loop [15]. The Acb is considered to be involved in cognition, emotion and emotional behaviors such as pleasure, fear, aggression, addiction and reward [16,17]. The limbic striatal loop is, therefore, one of the major targets of studies on the pharmacological actions of anti-psychotic drugs [18,19].

In the present study, we found the frequent and consistent tau accumulation in the Acb in TPD. Tau positive neurons were also found in the Acb in some AD and on-demented aged subjects but much fewer in the majority of such cases. We speculate that the lesions in the Acb play a role in some psychiatric symptoms such as delusion, which is often conspicuous in TPD.

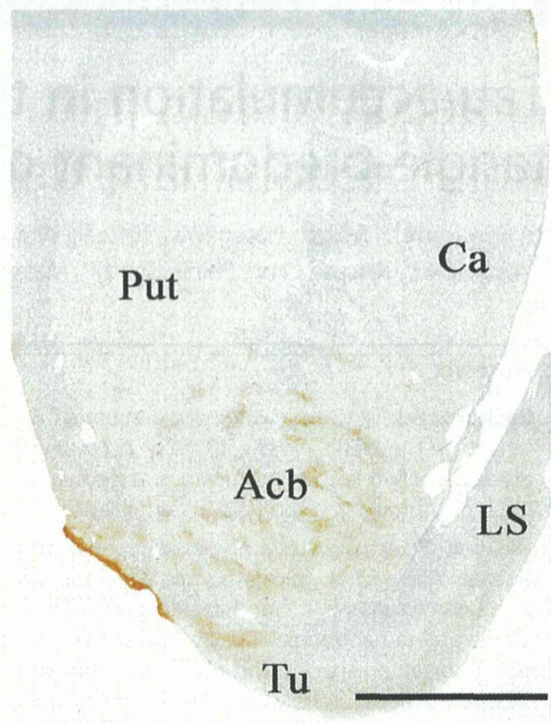


Figure 1 A semi-macro photograph of the basal ganglia from a TPD case stained with AT8. Faint immunoreaction is seen in the nucleus accumbens (Acb), lateral septal nucleus (LS) and the olfactory tubercle (Tu) even at this low power magnification. Ca: caudate nucleus; Put: putamen. Scale bar = 1 cm.

Materials and methods

We used brain tissues, archived in our laboratory, from 7 patients with TPD, 22 with AD and 11 subjects without dementia or other neurological disease. The demography, Braak and Braak's NFT stages and brain weight in each patient group are summarized in Table 1. Diagnoses were initially made on a clinical basis and were confirmed in every case by neuropathological examination. Clinical and neuropathological diagnoses of TPD followed the descriptions in previous articles [3,7,20]. Diagnoses of AD were made if the CERAD plaque score was 'C' [21] and the Braak and Braak's NFT stage was IV or higher [6]. In TPD and AD cases with the NFT stage III or IV, Lewy body pathology was confirmed to be absent or mild/stage 1 [22] in the hippocampus, parahippocampal gyrus and temporal neocortex to exclude the possibility of Dementia with Lewy bodies. In all cases, the patient or, in any case where the patient had died, his/her next of kin gave the written consent for autopsy and postmortem analyses for research purposes. This study was approved by the ethics committee in the Tokyo Metropolitan Institute of Medical Science and was performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki and its later amendments.

Table 1 Summary of patient groups used in this study

	TPD		AD		Non-demented
Braaks' NFT stages	III or IV	IV	V	VI	I-III
Number of cases	7	10	8	4	11
Gender (male/female)	1/6	6/4	4/4	2/2	7/4
Age at death*	88.4 ± 7.2	81.5 ± 8.5	86 ± 3.5	81.5 ± 8.8	81 ± 7.0
Disease duration (y)*	4.7 ± 2.9	6.1 ± 6.7	6.2 ± 3.5	6.6 ± 6.0	n/a
Brain weight (g)*	1,137 ± 135.3	1,134 ± 174.8	1,119 ± 77.2	1,008 ± 156.5	1,146 ± 77.1

TPD, Tangle-predominant dementia; *AD*, Alzheimer's disease; *NFT*, neurofibrillary tangles.
*Data are shown as mean ± S.D.

For routine neuropathological examinations, formalin-fixed, paraffin-embedded brain blocks were cut into 10 μm thick sections and stained with hematoxylin and eosin (HE), Klüver-Barrera, modified Gallyas-Braak and methenamine silver staining. Tissue sections of the mediobasal temporal cortex containing the hippocampus, entorhinal cortex and temporal neocortex were stained for tau and amyloid β protein (Aβ) by immunohistochemistry. Sections of the rostral striatum with the Acb and the septal nuclei were stained for tau. In TPD cases, additional tau immunohistochemistry was performed for the nucleus basalis of Meynert, amygdala and substantia nigra. The hippocampus, parahippocampal gyrus and adjacent temporal neocortex were also stained for phosphorylated α-synuclein and phosphorylated TDP-43 in TPD cases.

For more detailed immunohistochemical analyses, small blocks of brain the tissues were dissected at autopsy and fixed in 4% paraformaldehyde (PFA) for 2 days. The cryocut sections of 30 μm thickness were used for the high sensitive, free-floating immunhistochemical staining [23]. The antibodies used in this study are listed in Additional file 1: Table S1. The primary antibody labeling was visualized with 3,3′-diaminobenzidine as a chromogen, in combination with the Envision Plus® kit (Dako Japan, Tokyo). For enhanced thioflavin-S staining, tissue sections were pretreated with $KMnO_4$ for 20 min and, subsequently, with sodium borohydride for 4 min [24]. Sections were then stained with 0.05% thioflavin-S in 50% ethanol in the dark for 8 min, followed by differentiation in two changes of 80% ethanol for 10 sec each time and three washes in large volumes of distilled water. Following incubation in a high salt solution containing 411 mM NaCl, 8.1 mM KCl, 30 mM Na_2HPO_4 and 5.2 mM KH_2PO_4, pH 7.2 at 4°C for 30 min, sections were briefly rinsed with distilled water and observed by fluorescence microscopy.

For immunoelectron microscopy, both post-embedded and pre-embedded procedures were used. For the former, the 4% PFA-fixed small tissues were embedded in LR White Resin® (London Resin, U.K.) without further fixation. The ultra-thin sections were stained with AT8, which was followed by incubation with anti-mouse IgG conjugated with 10 or 20 nm gold colloidal particles (BBinternational, U.K.). For the pre-embedding procedure, the 4% PFA-fixed free-floating sections were stained with AT8 in combination with Alexa Fluor 488 FluoroNanogold anti-mouse IgG (Nanoprobes, U.S.A.). Following examination by fluorescence microscopy to localize the positive labeling, the sections were postfixed with 2% glutaraldehyde and then treated with HQ Silver Enhancement Kit (Nanoprobes, U.S.A.). After the treatment with 1% osmium tetroxide, which was followed by 2% uranyl acetate, the sections were embedded in epoxy resin (Querol 812, Nissin EM, Japan). Ultrathin sections were cut and observed by a transmission electron microscope (JEM-1400, JEOL, Japan).

For immunoblot analyses, fresh frozen samples of the Acb and the parahippocampal cortex were obtained from 3 TPD cases (cases 3, 4 and 6) and 3 AD cases. The Braak and Braak's NFT stages of the AD cases were 4, 5 and 6, respectively. Brain tissue was homogenized in 2 vol of TS buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.5), with a mixture of protease inhibitors and centrifuged at 200,000 g for 20 minutes at 4°C. The supernatant was taken as the soluble fraction and the pellet was used to further extract the sarkosyl-insoluble fraction as described previously [25]. Dephosphorylation of the sarkosyl-insoluble fractions was performed by incubation of the samples with Escherichia coli alkaline phosphatase (type III, Sigma) as described previously [25]. HT7, a pan-tau monoclonal antibody (Additional file 1: Table S1), was used for immunoblotting. Primary antibody labeling on the membranes was visualized with 3,3′-diaminobenzidine as a chromogen, in combination with a Vectastain ABC kit® (Vector Lab., USA).

For semiquantitative analyses of immunohistochemically stained tissue sections, the density of AT8 positive tau accumulation was graded to be 0 for absent, 1 for low, 2 for intermediate and 3 for high, based on microscopic observations at ×200 magnification. The Acb, septal nuclei, caudate nucleus, hippocampal CA1, entorhinal cortex and temporal neocortex were assessed in TPD, AD and non-demented aged subjects. The Mann–Whitney *U* test was used for statistical analyses using Graph Pad Prism 4 software (Graph Pad Software, U.S.A.).

Results

TPD cases used in the present study

The demographic, pathologic, and clinical information of the TPD cases used in the present study is summarized in Tables 1 and 2. In general, both the clinical and neuropathological features are similar to those described in previous reports [1-3,7,26,27]. The average age at death is higher than that in AD. Moderate dementia was noted in 5 of the 7 cases but the other two were diagnosed as having mild cognitive impairment. Delusion was evident in 6 cases. Brain atrophy was mild, if present, and senile plaques were either absent or rare. Lacunar infarcts were seen in the globus pallidus in 2 cases. In all cases, heavy tau accumulation was seen in the limbic regions in the forms of NFT, diffuse cytoplasmic accumulations and neuropil threads. Tau accumulation was heavier in the subiculum and the CA1 region than in the entorhinal and transentorhinal cortices. Tau was also deposited in the amygdala, the septal nuclei and the basal nucleus of Meynert, and, less frequently, in the caudate nucleus and substantia nigra. A small amount of tau was found in the temporal neocortex but only in 3 cases. Such limbic-predominant distribution of tau pathology is consistent with previous reports [1,2,26,28]. A small number of argyrophilic grains were present in 2 cases.

Tau accumulation in the Acb in TPD

In addition to the previously reported tau distribution, we found a considerable number of tau positive neurons in the Acb in all TPD cases used in this study (Figures 1 and 2). Similarly to the hippocampus, numerous neuropil threads were associated with tau positive neurons (Figure 2A). The tau positive neurons and neuropil threads were labeled with all the anti-tau antibodies used in the present study (Figures 2A-D). They included conformational change-specific, phosphorylation-specific and phosphorylation-independent antibodies (Additional file 1: Table S1). The staining pattern varied, which partly depends on the affinity of the antibody and the localization of the antigen epitope recognized by each antibody. Preservation of the epitope in tissue sections is affected by aggregation, degradation and post-mortem processing such as fixation. The majority of tau positive neurons in the Acb showed pretangle-like, diffuse or granular accumulation of tau in the cytoplasm (Figures 2B). Flame-like NFT, the common form in the hippocampus in TPD, were also present but not frequent (Figure 2B, arrow). The vast majority of tau positive neurons were medium sized but, occasionally, large neurons were also stained positively for tau (Figure 2D, arrow). Tau positive neurons and threads were not distributed evenly in the Acb. Rather, areas with sparse- and dense-tau pathology were intermingled (Figure 2E).

Table 2 Demography and basic clinical and neuropathological features of TPD cases

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Age at death	89	102	90	85	89	78	86
Sex	F	F	F	F	F	M	F
Dementia	+	+	+	+	MCI	MCI	+
Psychiatric symptoms							
Delusion	+	+	+	+	+	+	-
Anxiety	+	-	-	-	-	-	-
Depression	-	-	-	+	-	-	-
Brain weight (g)	940	970	1170	1300	1230	1220	1130
Atrophy	mi(Fr)	mi(Fr/T)	-	-	-	-	-
Plaque stage (1)	0	0	A*	0	0	0	0
NFT stage (1)	III	III	III	III	IV	III	III
Argyrophilic grain stage (2)	0	0	0	0	II	II	0
Hippocampal sclerosis	-	-	+	-	-	-	-
Vascular lesions	+	-	-	+	-	-	-
α-synuclein (hip/T**)	+§	-	-	-	-	-	-
TDP-43 (hip/T**)	-	-	-	-	-	-	-
Acb tau score	3	2	2	3	3	2	3

F, Female; *M*, Male; *MCI*, Mild cognitive impairment; *mi*, Mild; *Fr*, Frontal; *T*, Temporal; *Acb*, The nucleus accumbens. *A small number of diffuse Aβ deposits were seen in the temporal cortex. The Acb tau score was determined according to the method described in the text. (1) The senile plaque and NFT staging were based on the description by Braak and Braak [6]. (2) The argyrophilic grain staging was based on the description by Saito et al. [29]. **Immunohistochemistry for α-synuclein and TDP-43 was performed in tissue sections of the hippocampus, parahippocampal gyrus and adjacent temporal neocortex. § α-Synuclein pathology in this case was mild, corresponding to stage 1 by the 3rd report of the DLB consortium [22].

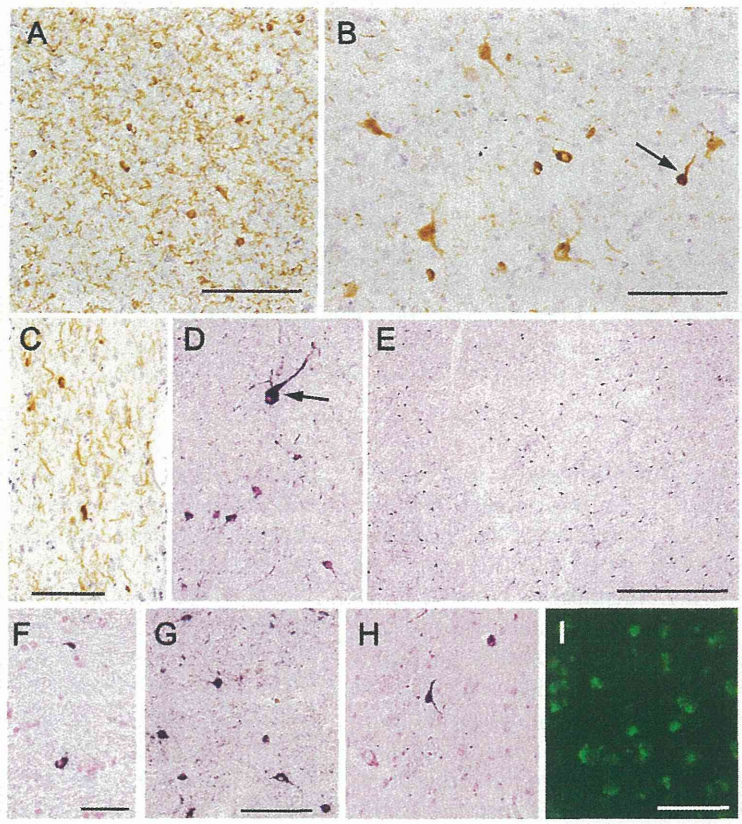


Figure 2 Tau accumulation in the Acb in TPD. A through **G** are immunohistochemistry with phosphorylation or conformational change specific tau antibodies. **A**, **B**, **C** and **I**: 4% paraformaldehyde-fixed, frozen-cut, 30 μm thick Sections. **D** through **H** are formalin-fixed, 10 μm thick, paraffin Sections. **A**: immunohistochemistry with AP422. Tau positive neurons are associated with many neuropil threads. Scale bars = 200 μm. **B**: immunohistochemistry with PHF-1. The majority of tau positive neurons show pretangle-like, diffuse or granular cytoplasmic labeling. Among them, apparent NFT are also seen but less frequently (arrow). Scale bar = 100 μm. **C**: immunohistochemistry with MC1, a conformational change specific antibody. Scale bar = 100 μm. **D** through **G** are immunohistochemistry with AT8. **D**: the vast majority of tau positive neurons are of medium-size but, occasionally, large neurons are also stained positively for tau (arrow). At the same magnification as **C. E**: tau positive neurons are not evenly distributed in the Acb. Scale bar = 500 μm. **F**: a glial coiled body. Scale bar = 25 μm. **G** and **H**: the nearby sections from the same case with AT8 immunohistochemistry **(G)** and Gallyas-Braak staining **(H)**. **I**: thioflavin S staining reveals granular cytoplasmic labeling of neurons. Scale bar = 100 μm.

Occasional glial coiled bodies were seen in the majoriy, if not all, of the cases (Figure 2F). Occurrence of glial coiled bodies in other brain regions in TPD has been reported previously [3]. Gallyas-Braak staining labeled only a small number of NFT in the Acb, while tau immunohistochemistry of nearby sections from the same patient revealed many positive cells (Figure 2G and 2H). Enhanced thioflavin-S staining labeled many neurons (Figure 2I).

The density of tau positive neurons and neuropil threads varied somewhat among the TPD cases. In TPD, no clear association was seen between the degree of Acb tau pathology and the Braak and Braak's NFT stage or the presence or absence of Aβ deposits, argyrophilic grains [29] and vascular lesions (Table 2). Despite the consistent tau accumulation in the Acb in TPD, we were not able to find severe neuronal loss or gliosis by HE staining.

Immunoelectron microscopy of the Acb in TPD with a tau antibody, AT8, revealed positive labeling of granular structures in the neurons (Figure 3A). Small and sparse bundles of short filamentous structures were occasionally seen to be stained positively for AT8 in the neuronal cytoplasm and neuropil (Figure 3B). Some of them showed morphology consistent with paired helical filaments (PHF). Thus, the ultrastructure of tau accumulation in the Acb was different from that in the hippocampal CA1 region, where dense and long bundles of PHF were frequent and intensely labeled for AT8 (Figure 3C).

Tau pathology in the Acb in AD and non-demented aged subjects

We then investigated the Acb in AD and non-demented, aged subjects. Tau positive neurons were found in some,