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学 位 論 文

The investigation of ALDH4A1 expression in the
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- genetic neuropathology

(統合失調症死後脳における ALDH4A1 に
ついてのジェネティックニューロパソロジー)

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論文内容要旨 (和文)

The investigation of ALDH4A1 expression in the postmortem brains from patients with schizophrenia-genetic neuropathology

(統合失調症死後脳における ALDH4A1 についてのジェネティックニューロパソロジー)

統合失調症は人口の 1%弱が罹患する比較的頻度の高い精神疾患であるが、未だ分子メカニズムは解明されていない。統合失調症の発症には遺伝的な関与が疑われており遺伝学的研究が盛んに行われてきたが、統合失調症発症そのものを表現型とした場合、研究間で一致した見解が得られてこなかったため、脳画像所見や神経生理学的所見などを中間表現型として遺伝子との関連を検討する研究が現在主流となっている。我々は、統合失調症死後脳における分子発現を中間表現型として、統合失調症発症に関わる遺伝子多型との関連を解析し、統合失調症発症のメカニズムを解明するための研究を行ってきた。我々は新規の技術による前頭前皮質のプロテオーム解析 (2DICAL 法) によって統合失調症において Aldehyde dehydrogenase 4 family member A1 (ALDH4A1) が著明に上昇していることを見出した。ALDH4A1 はプロリンが代謝されてグルタミン酸に至る経路の中の酵素の一つであり、統合失調症の末梢単核球における遺伝子発現の増加や統合失調症死後脳脳梁におけるタンパク発現量の増加が報告されている。本研究ではより定量性の高いタンパク質測定法である ELISA 法を用い、統合失調症の病態において重要であると考えられている前頭前野、上側頭回について、統合失調症、双極性障害、健常対照例、計 64 例の死後脳の ALDH4A1 の発現量を測定した。また同じサンプルセットの一部における ALDH4A1 の発現量とプロリン代謝経路に関わる分子の遺伝子多型 (SNPs) との関連を解析した。その結果、前頭前野と上側頭回で統合失調症群及び双極性障害において ALDH4A1 が有意に上昇していたことが分かった。また前頭前野の ALDH4A1 発現量は、プロリン代謝に関わる分子であるプロリダーゼの遺伝子の SNPs (rs33823、rs153508) と P5C シンセターゼの遺伝子の SNPs (rs10882639) とそれぞれ有意に相関することも分かった。

統合失調症の主要な病態仮説の 1 つに、NMDA 受容体の機能低下が統合失調症の陽性症状および陰性症状を引き起こすというグルタミン酸仮説があるが、なぜ NMDA 受容体機能低下が引き起こされるかは解明されていない。統合失調症患者においてコラーゲン分解しプロリンを合成するプロリダーゼ活性が亢進していること、統合失調症患者の血漿中でプロリンが上昇していることが報告されており、今回見出した統合失調症患者死後脳における ALDH4A1 発現上昇の所見を合わせて考えると、統合失調症患者においてプロリンの合成、分解が亢進した結果グルタミン酸産生過剰を招き、二次的に NMDA 受容体の機能低下につながる可能性が示唆された。この知見はドパミン仮説に基づく既存の抗精神病薬とは異なる新規の観点からの創薬につながる可能性がある。

Abstract

The molecular mechanisms underlying schizophrenia remain largely unclear. Until now, we have consistently investigated the functional correlations between schizophrenia risk alleles and the molecular expression profiles of postmortem brain samples from patients with schizophrenia. Recently, we identified multiple proteins significantly altered in the postmortem prefrontal cortex (PFC) of patients with schizophrenia using a novel liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Among these proteins, aldehyde dehydrogenase 4 family member A1 (ALDH4A1) was especially elevated in patients with schizophrenia. However, few studies have focused on the association between ALDH4A1 expression and schizophrenia. Therefore, in this study, we investigated levels of ALDH4A1 protein expression in the prefrontal cortex (PFC) and superior temporal gyrus (STG) in postmortem brains from 24 patients with schizophrenia, 8 patients with bipolar disorder, and 32 controls using enzyme-linked immunosorbent assay (ELISA). Moreover, we explored the associations between ALDH4A1 expression and genetic variants in enzymes associated with proline metabolism, including ALDH4A1 [schizophrenia (n=22), bipolar disorder (n=6), controls (n=11)]. We found that ALDH4A1 levels were significantly elevated in both the PFC and STG in patients with schizophrenia and bipolar disorder. Furthermore, ALDH4A1 expression levels in the

PFC were significantly associated with the following three single-nucleotide polymorphisms (SNPs): rs10882639, rs33823, rs153508. These findings indicate that altered expression of ALDH4A1 may reflect the potential molecular mechanisms underlying the pathogenesis of schizophrenia and bipolar disorder, and may aid in the development of novel drug therapies.

Introduction

The lifetime prevalence of schizophrenia is 0.30–0.66%, occurring at a rate of 10.2–22.0 per 100,000 each year [1]. In addition to positive symptoms such as auditory hallucinations, patients with schizophrenia experience negative symptoms including a lack of interest in social interaction, lack of motivation, anhedonia, and thought disorders [2]. Cognitive impairment, such as decline of executive function, selective attention, and working memory, may also be present [3]. Although many individuals experience these symptoms, the molecular mechanisms underlying schizophrenia remain largely unclear.

Various neuroimaging, neurophysiological, neuropathological, and genetic methods are used to investigate the brains of patients with schizophrenia. Among them, postmortem investigations have become increasingly important, as they have the potential to elucidate molecular phenotypes and region-specific alterations that may be related to the pathophysiology of schizophrenia. Furthermore, given the recent evidence regarding somatic mutations in the brain, such studies may further our understanding of how genetic structures including rare mutations affect the molecular signatures of brain functions.

Due to the strongly associated genetic factors, numerous studies have utilized genetic methods to investigate the mechanisms underlying schizophrenia. However, the results of these genetic association studies are inconsistent. Researchers have relied on

neurobiological characteristics to determine endophenotypes (intermediate phenotypes). Recent studies have investigated the functional correlations between schizophrenia risk alleles and intermediate phenotypes using neuroimaging, as well as cognitive and neurophysiological indicators. Kleinman et al. proposed a novel strategy known as "genetic neuropathology" for identifying the ultimate intermediate phenotype based on the expression of mRNA or protein in the postmortem brain [4]. They hypothesized that insights into mechanisms underlying psychiatric diseases and identification of novel therapeutic targets can be obtained by assessing the association between genetic variation and the relevant molecular phenotypes in the postmortem brain [4]. Indeed, recent postmortem brain studies [5] [6] [7] [8], including those by us [9] [10], have adopted this strategy, providing unique insight into the genetic and molecular mechanisms underlying schizophrenia.

Previously, we identified multiple proteins significantly altered in the postmortem prefrontal cortex (PFC) of patients with schizophrenia using a novel liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Among these proteins, aldehyde dehydrogenase 4 family member A1 (ALDH4A1) was especially elevated in the brains of patients with schizophrenia [11]. ALDH4A1 is an enzyme that dehydrogenizes glutamic- γ -semi-aldehyde—a metabolite in the conversion of proline to

glutamate. Two previous reports have focused on the association between ALDH4A1 and schizophrenia. A previous microarray study demonstrated that expression of the *ALDH4A1* gene is increased in the peripheral blood cells of patients with schizophrenia [12]. In addition, a postmortem nano-LC-MS/MS study reported increased ALDH4A1 expression in the corpus callosum of patients with schizophrenia [13].

While one previous study has examined the expression of ALDH4A1 in the postmortem corpus callosum of patients with schizophrenia, no studies have focused on other brain regions. Previous researchers have focused heavily on the PFC and superior temporal gyrus (STG) in postmortem studies of schizophrenia [14], as negative symptoms and cognitive deficiencies are thought to reflect frontal lobe dysfunction among affected patients [15]. Indeed, functional neuroimaging studies have revealed that patients with schizophrenia exhibit decreased blood flow in the frontal regions, relative to general cerebral perfusion. One report noted that functional neuroimaging findings and neuropsychological performance reflect prefrontal dysfunction in patients with schizophrenia [16]. Heschl's gyrus (HG), which is located in the STG, plays an important role in auditory perception [17]. A functional MRI study by Dierks et al. revealed that HG activation is associated with auditory hallucinations in patients with schizophrenia [18]. The STG is the major generator of mismatch negativity (MMN), which occurs via the

comparison between sound deviations and the neural trace of preceding sounds stored in auditory sensory memory [19]. Because NMDA antagonists attenuate the magnitude of MMN, N-methyl-D-aspartate (NMDA) receptor transmission is presumed to generate MMN [20]. Indeed, some studies have reported dysfunction of glutamate systems in the STG among patients with schizophrenia. For example, a postmortem study by Le Corre et al. reported increased mRNA expression of the NMDA receptor NR1 subunit splice variant in the STG of patients with schizophrenia [21]. In addition, ligand binding studies have reported increased NMDA receptor density in the STG of patients with schizophrenia [22]. Kasai et al. reported that schizophrenia is associated with attenuated MMN, as well as progressive decreases in gray matter volume in HG [23] [24] [25]. Taken together, these findings suggest that the STG is involved in auditory hallucinations in patients with schizophrenia. As previously mentioned, the PFC and STG are thought to be strongly related to the pathology of schizophrenia. Thus, we investigated the expression of ALDH4A1 in the PFC and STG of patients with schizophrenia.

To further clarify the pathology of schizophrenia, we investigated the functional correlations between schizophrenia risk alleles and molecular expression profiles in the postmortem brains of patients with schizophrenia, which we hypothesized would reflect intermediate phenotypes. We increased the number of samples relative to that used in our

previous study and measured postmortem levels of ALDH4A1 expression in the PFC and STG of 24 patients with schizophrenia, 8 patients with bipolar disorder, and 32 healthy controls using enzyme-linked immunosorbent assay (ELISA). We then analyzed the relationship between ALDH4A1 expression levels and gene polymorphisms (SNPs) of proline metabolism-containing molecules (*PRODH*, *ALDH4A1*, *ALDH18A1*, *PYCR1*, *OAT*, *PEPD*).

Materials and methods

Human brain samples

Postmortem brain samples from patients with schizophrenia, bipolar disorder, and controls were obtained from the Fukushima Brain Bank and the Brain Research Institute at the University of Niigata, as previously described [9] [26] [27]. The present study was approved by the Ethics Committee of Fukushima Medical University and Niigata University, and was conducted in accordance with the Declaration of Helsinki. Detailed demographic characteristics for the 24 patients with schizophrenia, eight patients with bipolar disorder, and 32 controls is provided in Table 1. The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) was used to diagnose both schizophrenia and bipolar disorder. For each patient with schizophrenia, the daily dosage of antipsychotics within 3 months before death is indicated as the chlorpromazine equivalent dose (mg/day) (Table 1).

Protein expression analysis via ELISA

Sections (approximately 100 mg) of gray matter were selected from Brodmann area 10 (BA10) (i.e., PFC) and BA22 (i.e., STG) in the frozen brains. The tissues were suspended in lysis buffer (N-PER Neuronal Protein Extraction Reagent, ThermoFisher, Japan)

supplemented with protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, Japan) and phosphatase inhibitors (PhosSTOP, Roche, Japan), following which they underwent sonication for 50 seconds (30% duty cycle) at an output level of 5 (Ultrasonic processor W385, Astrason Heat Systems-Ultrasonics, NY, USA). The samples were centrifuged ($40,000 \times g$ for 30 min), and the total protein concentrations of the supernatants were measured via a bicinchoninic acid assay (Protein Assay BCA Kit, Fuji Film, Tokyo, Japan) using bovine serum albumin (BSA) as a control. Levels of ALDH4A1 expression were determined using an ELISA kit (OKEH02446, Aviva Systems Biology, USA), in accordance with the manufacturer's protocol. Levels of ALDH4A1 expression were normalized against the total protein concentration.

SNP genotyping

SNP data were determined in previous studies [10]. In brief, genomic DNA was extracted from the frozen cerebellum or occipital cortex. Genotyping was performed using HumanCoreExome -24 v1.0 Beadchip (Illumina, Tokyo, Japan) on an iScan system (Illumina). In the present study, we examined 30 SNPs associated with proline metabolism (Table 2). For analyses of the associations between SNPs and protein expression, we excluded SNPs with call rates <99% and minor allele frequencies <5%.

Statistical analysis

Analyses of covariance (ANCOVA) were used to compare levels of protein (ALDH4A1) expression among the groups (schizophrenia, bipolar disorder, and control). Diagnosis and genotype were set as independent variables, while brain pH and postmortem interval (PMI) were set as covariates. There were two missing values for PMI and three for brain pH. Each missing value was represented using the average value. Fisher's least significant difference (LSD) post hoc comparisons were performed to evaluate group differences. In addition, Spearman's rank correlation coefficients were used to assess the association between protein expression and the following: age at death, PMI, brain pH, and estimated total dosage of neuroleptic drugs. T-tests were used to assess the association between sex and levels of ALDH4A1 expression. A p value <0.05 was considered significant in these tests. For SNP analysis, the false discovery rate (FDR) was used to control for multiple comparisons [28]. We reported a corrected p value with an FDR at $q = 0.05$ in the SNP study. Statistica (version 10.0, Statsoft Inc., Tulsa, OH) was used for ANCOVA, and Sigma Plot software (version 11, Systat Software, Inc., CA) was used for all tests that did not involve FDR and ANCOVA values. The association study between gene polymorphisms and schizophrenia was conducted using SNPStats (<http://bioinfo.iconcologia.net/SNPstats>).

Results

We examined levels of ALDH4A1 expression in the PFC and STG of patients with schizophrenia, bipolar disorder, and controls via ELISA. Our ANCOVA analysis revealed that levels of ALDH4A1 expression in the PFC were significantly different between schizophrenia, bipolar disorder and controls [F (2,57) =3.59, $p < 0.05$]. ALDH4A1 levels in PFC were higher in patients with schizophrenia ($p < 0.05$) and bipolar disorder ($p < 0.05$) than in controls (Figure 1a). Similarly, ANCOVA analysis revealed that levels of ALDH4A1 expression in the STG were significantly different between schizophrenia, bipolar disorder and controls [F (2,58) =10.36, $p < 0.05$]. ALDH4A1 levels in STG were higher in patients with schizophrenia ($p < 0.05$) and bipolar disorder ($p < 0.05$) than in controls (Figure 1b).

We then examined the associations between ALDH4A1 expression and sex, age at death, PMI, and brain pH using Pearson correlation coefficients (Figure 2). In patients with schizophrenia, we also examined associations with the daily dosage of antipsychotics in the 3 months prior to death. No significant effects of sex, age at death, or antipsychotic dose were observed on ALDH4A1 expression in any brain region examined. However, we observed a significant association between levels of ALDH4A1 expression in the PFC and PMI ($p < 0.05$, $r = 0.459$)/brain pH ($p < 0.005$, $r = 0.265$). We also

observed a significant association between levels of ALDH4A1 expression in the STG and brain pH ($p < 0.005$, $r = 0.291$). Therefore, brain pH and PMI were set as covariates, as described in Materials and Methods section.

We examined the effects of SNPs in molecules associated with proline metabolism on levels of ALDH4A1 expression in patients with schizophrenia ($n = 22$), bipolar disorder ($n = 6$), and controls ($n = 11$). In our analysis of 111 SNPs of proline metabolism-associated molecules (*PRODH*, *ALDH4A1*, *ALDH18A1*, *PYCR1*, *OAT*, *PEPD*), 30 SNPs were retrieved for analysis using the criteria described in the Materials and Methods section. We then associations between these 30 SNPs and levels of ALDH4A1 expression in the PFC and STG. In the PFC, we observed significant associations between ALDH4A1 expression and three SNPs for *ALDH18A1* (rs10882639) and *PEPD* (rs33823, rs153508) (Figure 3). The rs10882639 SNP was associated with an allele dose-dependent effect: Individuals homozygous for the A allele and heterozygous AG individuals at rs10882639 exhibited significantly higher levels of ALDH4A1 expression than homozygous G individuals. The rs33823 SNP was also associated with allele dose-dependent effects: Individuals homozygous for the G allele and heterozygous AG individuals at rs33823 exhibited significantly higher levels of ALDH4A1 expression than homozygous A individuals. Furthermore, we also observed a

significant effect of SNP rs153508 on the expression of ALDH4A1: Individuals homozygous for the G allele and heterozygous for AG at rs153508 exhibited significantly higher levels of ALDH4A1 expression than homozygous A individuals. In the STG, we observed no associations between ALDH4A1 expression and SNPs.

Discussion

The present study is the first postmortem investigation to examine levels of ALDH4A1 expression in the PFC and STG in patients with schizophrenia. We further examined the relationship between levels of ALDH4A1 expression and genetic polymorphisms involved in proline metabolism. Our results indicated that ALDH4A1 levels were significantly increased in the PFC and STG of patients with schizophrenia or bipolar disorder.

ALDH4A1 is among the enzymes involved in the metabolism of proline to glutamate, which is the most downstream component of the pathway (Figure 4). ALDH4A1 is expressed in all regions in the brain and is somewhat strongly expressed in the hippocampus and caudate nucleus (THE HUMAN PROTEIN ATLAS <https://www.proteinatlas.org/>). It is ubiquitously present in various cells and is an enzyme located downstream of the pathway that metabolizes proline to glutamate in mitochondria (Figure 4). The glutamate produced is converted to α -ketoglutarate and exits from the mitochondria to the cytoplasm via an α -ketoglutarate-malate carrier (OGC) [29]. Aspartate aminotransferases (AST), is an enzyme that converts α -ketoglutarate into glutamate, is present in the cytoplasm. Thus, higher expression of ALDH4A1 in schizophrenia may result in increased glutamate and α -ketoglutarate levels in mitochondria, inducing cytoplasmic glutamate and glutamine upregulation in the

astrocyte and presynaptic glutamate increase, thereby leading to increased glutamate release into the synaptic cleft.

The relationship between schizophrenia and glutamate has attracted attention. The glutamate hypothesis, one of the major pathological hypotheses of schizophrenia, was proposed because overuse of phencyclidine (PCP), an antagonist of the NMDA receptor, causes hypofunction of the NMDA receptor, resulting in positive and negative symptoms [30]. It is known that in anti-NMDA receptor encephalitis, which exhibits symptoms similar to those of schizophrenia, an autoantibody of the NMDA receptor causes internalization of the NMDA receptor and thus a decrease of NMDA receptor function [31]. Neurotoxicity by excessive release of glutamate is thought to occur because of disinhibition of glutamate nerve terminals caused by NMDA receptor hypofunction [32]. However, the mechanisms underlying NMDA receptor hypofunction remain to be elucidated. It was reported that NMDA receptors are internalized when activated by glutamate [33] and that the internalization of NMDARs not only reduced the number of cell surface NMDARs but also inhibited the activity of the non-internalized surface NMDARs [34]. Due to the increase in glutamate it is possible that downregulation of NMDA receptors by internalization or phosphorylation of NMDA receptors may occur. Glutamate can be synthesized via four pathways. The first involves synthesis from α -

ketoglutarate by the action of an aminotransferase, the second from glutamate dehydrogenase via the addition of free ammonia to α -ketoglutarate, the third via the synthesis of glutamine to glutaminase, and the fourth via proline metabolism. Glutamate that is used as a transmitter is generally synthesized from glutamine at the presynaptic terminal and is stored in synaptic vesicles. Furthermore, it is known to be involved in the pathology of schizophrenia, and several studies have reported the relationship between schizophrenia and glutamate. Glutamate release induced by non-competitive NMDA receptor agonists has been reported to increase prefrontal gray matter in model animals, resulting in schizophreniform-like behaviors [35]. Postmortem brain studies have shown that glutamate levels were lower in the schizophrenia than in the control groups [36], and that glutamate was higher in the schizophrenia group in the prefrontal region [37]. Furthermore, no significant difference between glutamate levels in the caudate nucleus, nucleus accumbens, frontal cortex, amygdala, thalamus of was observed between the schizophrenia and control groups [38]. Thus, no consensus on schizophrenia and glutamate levels has been obtained in postmortem brain studies. Furthermore, a metabolome study revealed an elevated serum glutamate levels in schizophrenia patients[39]; however, glutamate levels in the cerebrospinal fluid were observed to be lower in schizophrenic patients [40] in one study and in a few others, no difference in

these levels were observed compared with those in the control group [41] [42] [36]. Hence, these results are inconsistent. A magnetic resonance spectroscopy (MRS) study revealed that patients with acute schizophrenia had high levels of glutamine and patients with chronic schizophrenia had low levels of glutamate and glutamine [43] [44]. In addition, glutamine levels were high in the cingulate gyrus and thalamus in first episode schizophrenia patients, but glutamate and glutamine levels were low in the cingulate gyrus in patients with schizophrenia in the chronic stage after treatment [45-47]. In studies comparing results before and after drug treatment, glutamine levels were higher than normal in the anterior cingulate gyrus and thalamus of untreated schizophrenic patients, and were significantly reduced in the thalamus after 30 months of antipsychotic treatment [48]. Moreover, higher levels of glutamate were observed in the striatum of antipsychotic-naïve patients with first-episodic psychosis than in controls. After antipsychotic treatment for 4 weeks, glutamate levels were not significantly different in the striatum [49]. These results suggest that hyperactivity of glutamate mediated neurotransmission occurs in the acute phase in schizophrenia, but these levels normalize following treatment. Conversely, glutamate neurotransmission decreases in the chronic phase.

The relationship between schizophrenia and glutamate receptors have been examined in previous studies. Glutamate receptors include ionotropic receptors (NMDA

receptor, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, kainate receptor) and metabotropic receptors and their association with schizophrenia has been studied. NMDA receptors have several subunits [GluN1 (NR1), GluN2A-2D (NR2A-2D), GluN3A-3B (NR3A-3B)]. Postmortem brain studies have reported decreased NR1 mRNA expression and elevated NR2B mRNA expression in the hippocampus [50] and increased NR3A mRNA expression in the dorsolateral prefrontal cortex (DLPFC) of schizophrenia patients [51]; however, another study reported that expression of NR1 and NR3A proteins in the DLPFC was not significantly different between the schizophrenia and control group [52]. Thus, consistent results were not obtained. Neuroimaging studies reported decreased ligand binding of NMDA receptors in the hippocampus and frontal lobe in untreated schizophrenia patients [53]. The AMPA receptor has four subunits, namely GluA1-A4 (GluR1-GluR4). Postmortem brain studies have investigated the binding of selective ligands and changes in the expression of each subunit in patients with schizophrenia, and in the medial temporal lobe including the hippocampus, ligand binding and a decrease in the expression of GluA1 and GluA2 in schizophrenia patients was observed; [50, 54] however, these results lack reproducibility. Changes in ligand binding in the prefrontal cortex were unclear [54], but a decrease in GluA2 mRNA expression was reported [55] [56]. The kainate receptor is an ion channel

formed by five subunits GluK1-GluK5. Collaborative research using our postmortem brain showed decreased GluK4 expression in the prefrontal cortex of schizophrenia patients. Among the 8 types of metabotropic glutamate receptors, mGlu1-mGlu8, mGlu2 and mGlu3 agonists improve positive and negative symptoms of schizophrenia [57]. Studies on the postmortem brains have shown the unchanged expression pattern and amount of mGlu 2/3 protein [58] and decreased mGlu2 and unchanged mGlu3 mRNA levels [59] in the prefrontal cortex of schizophrenia patients. Increased mGlu1 mRNA and protein expression has been reported in the prefrontal cortex of schizophrenia [60] [61], but the changes in the prefrontal cortex and hippocampus of mGlu5 mRNA have not been elucidated [62]. Experiments in animals, have shown that the glutamate release in the frontal lobe increases in rats administered with phencyclidine (PCP), and mGlu 2/3 agonists that suppress this phenomenon improve abnormal behaviors [63]. Developmental studies using mGlu 2/3 agonists for treating acute schizophrenia had reached the advanced stages, but the therapeutic effects were somewhat lower than olanzapine [57], and have hence been discontinued.

Previous studies have demonstrated that some neuropsychiatric disorders appear to be related to hyperprolinemia [64] [65]. Several studies have suggested that glutamate excitotoxicity may underlie neurological dysfunction in patients with hyperprolinemia

[66] [67] [68]. Previous reports have indicated that proline decreases Na^+ , K^+ , ATPase, and acetylcholinesterase activity and induces oxidative stress in the rat brain [69] [70], and that plasma proline levels are increased in patients with schizophrenia [39] [71]. Additional studies have reported that hyperprolinemic patients with schizophrenia are significantly older at the first psychiatric hospitalization, suggestive of later onset. However, hospital stays are approximately 46% longer in patients with hyperprolinemia than in those without, suggesting that proline may be associated with the clinical picture of schizophrenia [72]. Previous studies reporting that the onset of schizophrenia in patients with hyperprolinemia is significantly delayed are of great interest. Considering that proline is an agonist of the NMDA receptor GluN1 glycine site [73] and that it enhances NMDA receptor function [74], the onset of schizophrenia may have been delayed in these patients. Deficiencies in proline oxidase, an enzyme that converts proline to D-1-pyrroline-5-carboxylate (P5C), can also cause hyperprolinemia [75]. The *PRODH* gene encoding proline oxidase is located on chromosome 22 (22q 11.2). Carriers with chr 22 q 11. 2 deficiency exhibit a morbidity rate of 20-30% for schizophrenia, and this deletion is among the most well-known risk factors for schizophrenia [76] [77] [78]. These findings indicate that hyperprolinemia may be related to clinical symptoms of 22q11 deletion syndrome, including the onset of schizophrenia. However, if proline

oxidase is deficient or decreased in a patient with the 22q11.2 deletion syndrome, the efficiency of the metabolic cascade from proline to glutamine is decreases, so that even if hyperprolinemia occurs, glutamate excess cannot occur. Factors other than glutamate excess seem to be involved in schizophrenia onset in 22q11.2 deletion syndrome patients. For example, the deleted region of 22q11.2 deletion syndrome includes schizophrenia related genes, such as *COMT*, which codes an important enzyme in monoamine catabolism; *ZDHHC8*, which codes a palmitoyl transferase enzyme; and *DGCR8*, a key enzyme in the microRNA synthetic pathway [79]. One previous study reported that serum prolidase activity was significantly higher in the schizophrenia group than in the control group [80], in accordance with the finding that proline production is upregulated in patients with schizophrenia [81]. Other studies have demonstrated that prolidase activity in the production of proline from collagen is elevated in patients with schizophrenia, along with plasma proline concentrations.

Conversely, that an increase in ALDH4A1 occurred secondarily in response to the energy demand by neurons (e.g., overactivity of STG when auditory hallucinations are active) generated in schizophrenic conditions cannot be ruled out. However, in the PFC, cerebral blood flow reduction is observed in schizophrenia, and brain hypoactivity is assumed and energy demand is expected to decline; however, ALDH4A1 increased

in the PFC. Thus, the increase in ALDH4A1 is not only due to the increase in energy demand.

Based on the accumulated evidence, we hypothesized that glutamate production is enhanced via increases in the expression of enzymes involved in degradation such as prolidase and ALDH4A1, and that the pathway from proline to glutamate is accelerated in patients with schizophrenia. Furthermore, glutamate production may be excessive, which may in turn decrease NMDA receptor function. Thus, inhibiting the function of ALDH4A1 and preventing excessive glutamate production may aid in the treatment of schizophrenia. Disulfiram and cyanamide, which are currently used in the treatment of alcoholism, also exert an inhibitory effect on ALDH4A1. However, inhibition of ALDH4A1 may cause hyperprolinemia and other adverse effects. Some studies have reported that disulfiram treatment for alcoholism does not alter Positive and Negative Syndrome Scale (PANSS) scores in patients with schizophrenia [82]. Nonetheless, further studies are required in order to determine the potential application of ALDH4A1 inhibition in the treatment of schizophrenia.

Our findings also indicated that ALDH4A1 expression was also elevated in the PFC and STG of patients with bipolar disorder. Previous mass spectrometry studies have analyzed the postmortem brains of patients diagnosed with mental illness (schizophrenia,

bipolar disorder, depression) in relation to those of healthy controls. Such studies have examined changes in amino acid levels and glucose metabolism, revealing that psychiatric disorders are associated with increases in proline levels [83]. An additional study reported that serum prolidase activity was significantly higher in patients with bipolar disorder than in healthy controls [84]. A meta-analysis of glutamate concentration measurement via ¹H-MRS further reported that brain glutamate concentrations are increased in patients with bipolar disorder [85]. These findings indicate that schizophrenia and bipolar disorder may share the tendency toward increased proline metabolism and glutamic acid production. Although the results of our study support this notion, our sample included only a small number of patients with bipolar disorder. Thus, our findings should be interpreted with caution.

In the present study, we observed that ALDH4A1 expression levels were significantly associated with the following three SNPs: rs10882639, rs33823, and rs153508. The rs10882639 SNP is a genetic polymorphism of Δ -1-pyrroline-5-carboxylane (P5C) synthetase, while rs33823 and rs153508 are genetic polymorphisms of prolidase. P5C synthetase is an enzyme that functions inversely with ALDH4A1, decomposing glutamic acid into glutamic- γ -semi-aldehyde. Prolidase is an enzyme that generates proline from collagen. We could not confirm that each gene of the 3 SNPs

identified in this study had a cis-acting effect on mRNA expression using Brain Cloud (<http://braincloud.jhmi.edu/>). All three SNPs reflecting ALDH4A1 expression in this study were intronic (iSNPs), located in the intergenic region (IGR) of the DNA sequence. Although the IGR is a subset of noncoding DNA, recent studies have indicated that iSNPs play key roles in regulating mRNA transcription rate, splicing, and maturation [86, 87]. Changes in the nucleotide sequence within the intron can affect splicing, as the sequence is recognized by various splicing-related factors within introns of heterologous nuclear RNA (hnRNA; alternative term for pre-mRNA). Most schizophrenia-associated SNPs are thought to be iSNPs, and several studies have reported the role of iSNPs in psychiatric disorders [88]. Such findings suggest that the three SNPs identified in the present study play undetermined roles in schizophrenia.

It may be possible to predict levels of ALDH4A1 expression in patients with schizophrenia by examining SNPs of P5C synthetase and prolidase in peripheral tissues (i.e., blood, saliva). Furthermore, patients with schizophrenia exhibiting increased ALDH4A1 expression may benefit from ALDH4A1 inhibition. As only one ALDH4A1 SNP associated with one genotype was observed in the present study, further studies are required to determine whether other SNPs can be observed in patients with schizophrenia. Future studies should also examine expression levels and gene polymorphisms for proline

and other molecules involved in proline metabolism.

The present study possesses several limitations of note. First, confounding effects of disease-related factors including medication may have affected the expression of ALDH4A1. Although we did not observe any effects of antipsychotic drugs on the levels of ALDH4A1 expression in this study, additional animal studies are necessary to investigate the effects of chronic antipsychotic administration on the expression of these proteins in the PFC and STG. Second, our study population was relatively small, particularly for a genetic study. Therefore, our findings must be verified via postmortem examination in a larger brain cohort. Third, the age of each sample was slightly advanced, and there was considerable variation in PMI. Therefore, we corrected the results via statistical methods. Fourth, ALDH4A1 is also expressed in regions, such as the thalamus, nucleus accumbens, and striatum, in which dopamine system abnormality is assumed, other than the PFC and STG assessed in this study, and may be involved in schizophrenia pathology; however ALDH4A1 expression in these regions were not measured in this study. Further studies conducted will include these analyses. Fifth, the protein (P5C synthetase, Prolidase) encoded by the three SNPs (rs 10882639, rs 33823, and rs 153508) involved in ALDH4A1 expression were not measured in this study. However, our future study measures the expression of these proteins and investigates the significance of the

trans-acting effect on ALDH4A1 expression. We also measure other proteins involved in proline metabolism.

In summary, the findings of the present study demonstrate that ALDH4A1 expression is elevated relative to control levels in the postmortem brains of patients with schizophrenia and bipolar disorder. Furthermore, such increases were accompanied by genetic polymorphisms in molecules associated with proline metabolism. Such results provide new insight into the potential pathogenic mechanisms underlying schizophrenia and bipolar disorder.

Figure legends

Figure 1. Expression of ALDH4A1 in the diagnostic groups. Mean expression of ALDH4A1 in the PFC (a) and STG (b) in patients with schizophrenia, patients with bipolar disorder, and control participants. ELISA was used to measure protein expression. Error bars indicate the SE of the mean. P values presented over a bar indicate that the difference between these groups was significant based on a post hoc Fisher's LSD test. PFC, prefrontal cortex; STG, superior temporal gyrus; ELISA, enzyme-linked immunosorbent assay; SE, standard error; LSD, least significant difference, Sz, schizophrenia; BP: bipolar disorder; Cont, controls.

Figure 2. Association between levels of ALDH4A1 expression and each confounding factor.

PFC, prefrontal cortex; STG, superior temporal gyrus; PMI, postmortem interval; CPeq, chlorpromazine equivalent.

Figure 3. The effects of SNPs in molecules associated with proline metabolism on levels of ALDH4A1 expression. There was a significant association between ALDH4A1 expression in the PFC and rs10882639 (a), rs33823 (b), and rs153508 (c).

Error bars indicate the SE of the mean. Asterisks presented over a bar indicate that the

difference between these groups was significant based on a post hoc Fisher's LSD test.

SNP, single-nucleotide polymorphism; PFC, prefrontal cortex; SE, standard error; LSD, least significant difference.

Figure 4. The proline metabolism system. The metabolism of proline involves two other amino acids (glutamate and ornithine) and six enzymes [64].

P5C, Δ -1-pyrroline-5-carboxylane; TCA, tricarboxylic acid; OGC, α -ketoglutarate-malate carrier; N, System N transporter (astrocytic from); A, System A transporter (neuronal from); AST, aspartate aminotransferases.

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Figure 1

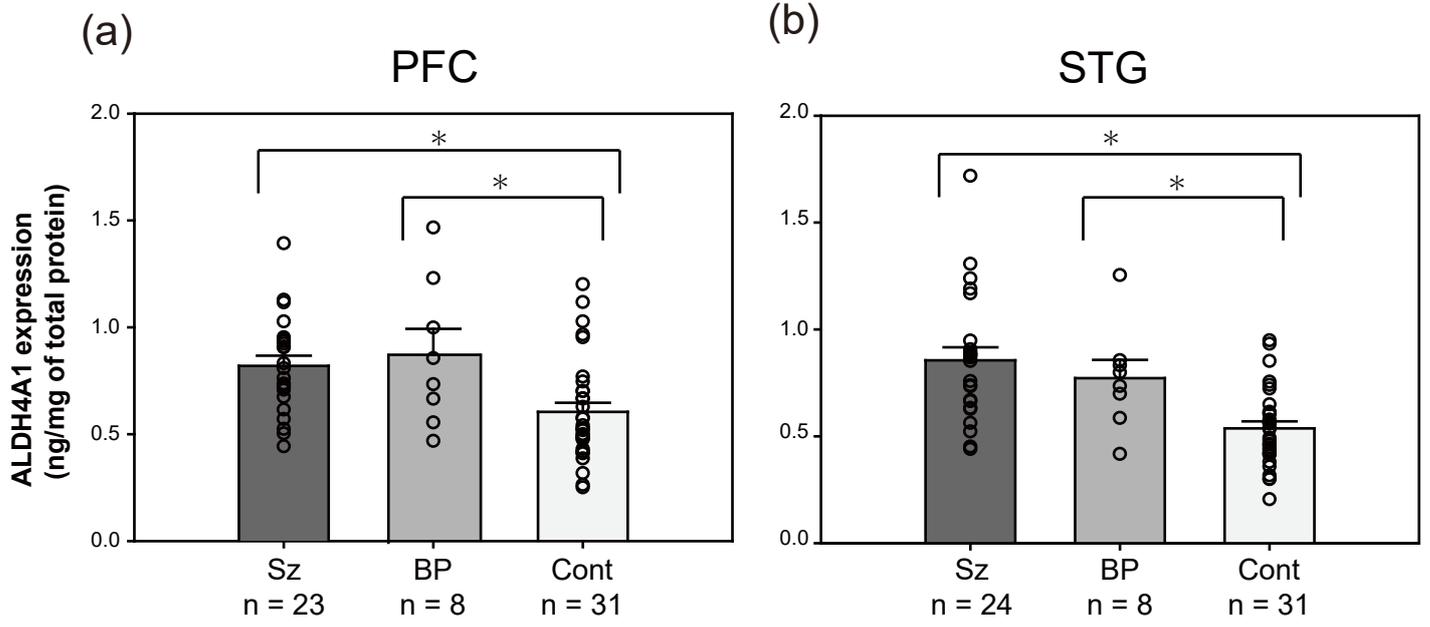


Figure 2

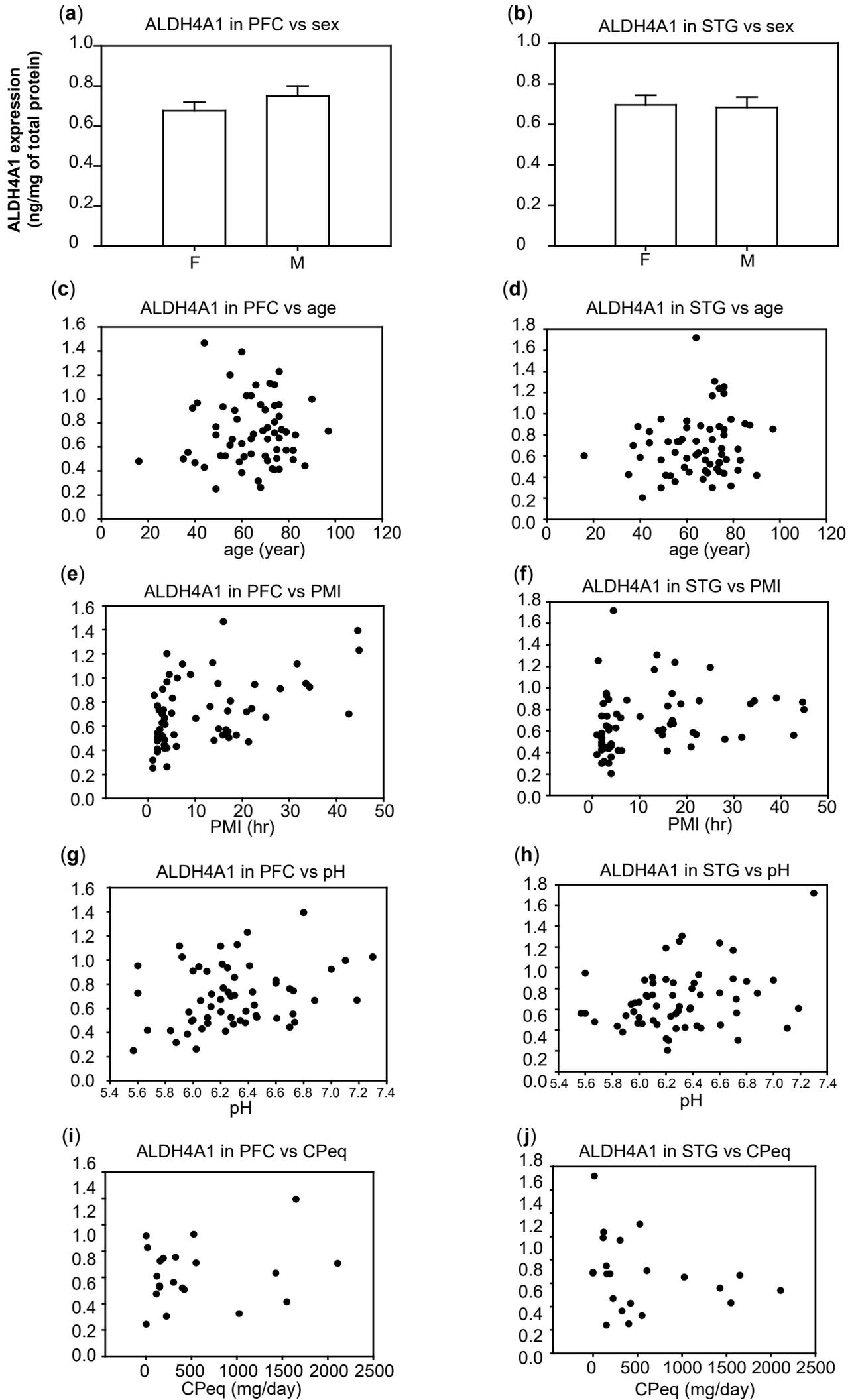


Figure 3

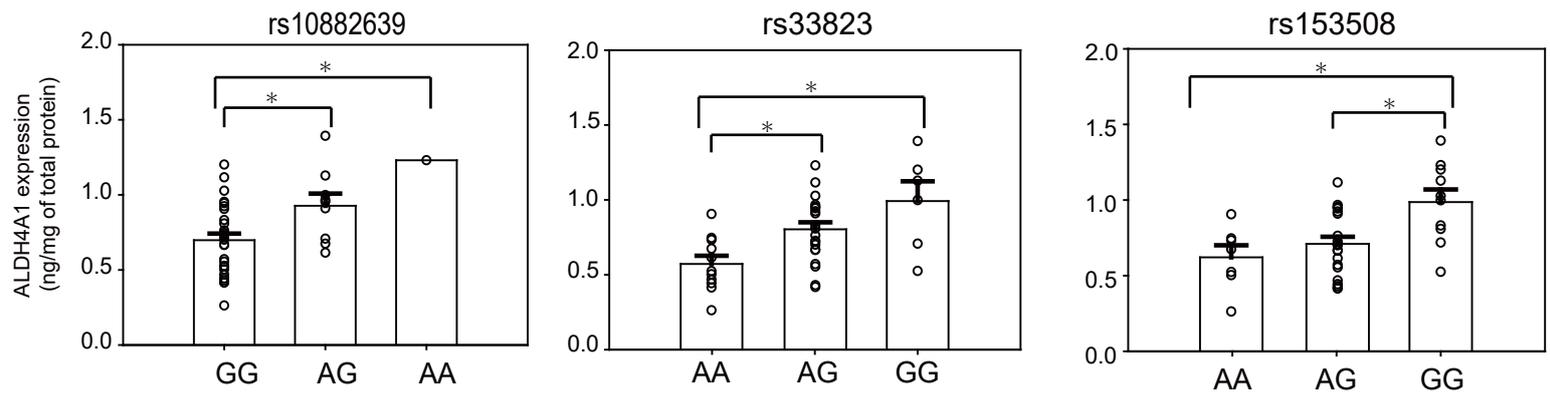
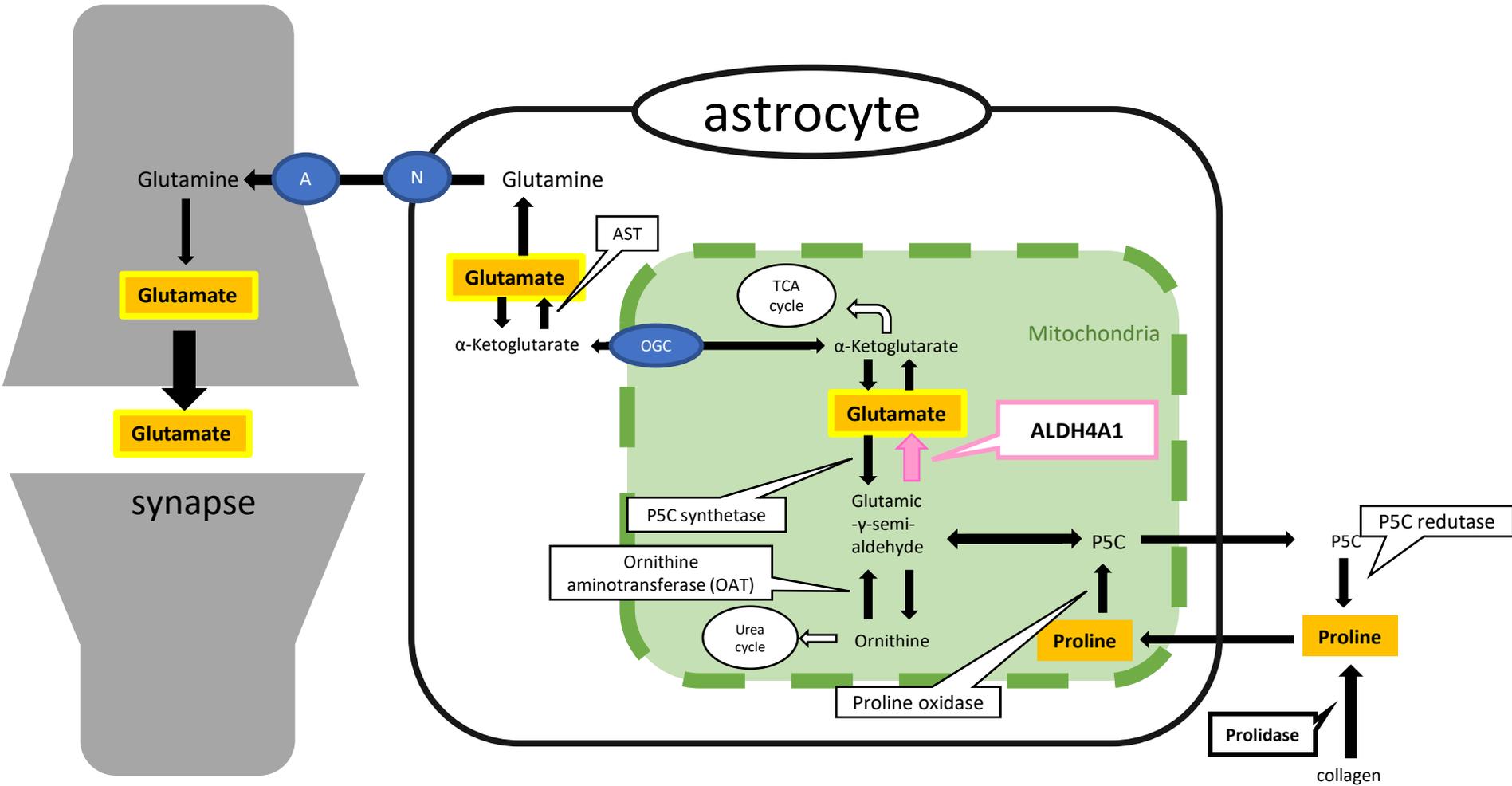


Figure 4



Tables

Table 1. Characteristics of post mortem brain samples

	Schizophrenia	Bipolar Disorder	Control	Sz vs Cont p value
Number of sample	24	8	32	
Sex	M15, F9	M4, F4	M18 ,F14	p=0.638 ※1
Mean age at death (year)	68.4±11.0	64.5±23.4	62.1±15.0	p=0.086 ※2
Mean PMI (h)	16.5±11.8	14.9±14.1	8.1±10.6	p=0.009 ※2
Mean brain pH	6.3±0.4	6.4±	6.2±0.4	p=0.591 ※2
Mean CP eq (mg/day)	545.9±608.2	-	-	-

※1 Pearson's chi-square test

※2 T-test

SZ; Schizophrenia, Cont; control, PMI; postmortem interval, CP eq: The daily dosage of antipsychotics within 3 months before death is indicated as the chlorpromazine equivalent dose.

Table 2. Candidate SNPs tested for association with ALDH4A1 expression.

Genotyping of 30 SNPs for molecules associated with proline metabolism (*PRODH*, *ALDH18A1*, *OAT*, *PEPD*) was performed using a HumanCoreExome -24 v1.0 Beadchip on an iScan system (Illumina, Tokyo, Japan).

SNP	Gene	Protein	Chr	MapInfo	Alleles	intron/exon	Mutation(s)	FDR p value
exm1584429	PRODH	Proline oxidase	22	18910355	[T/G]	exon	T275N	0.090
exm1584453	PRODH	Proline oxidase	22	18912678	[A/G]	exon	W185R	0.493
rs2238732	PRODH	Proline oxidase	22	18915347	[T/C]	intron	Silent	0.126
rs4819756	PRODH	Proline oxidase	22	18912678	[A/G]	exon	W185R	0.493
exm844571	ALDH18A1	P5C synthetase	10	97386497	[T/G]	exon	S372Y	0.828
exm844584	ALDH18A1	P5C synthetase	10	97388162	[A/G]	exon	T299I	0.828
rs10509688	ALDH18A1	P5C synthetase	10	97385242	[A/G]	intron	Silent	0.493
rs10748634	ALDH18A1	P5C synthetase	10	97385662	[T/G]	intron	Silent	0.948
rs10882639	ALDH18A1	P5C synthetase	10	97368990	[A/G]	intron	Silent	0.03 *
rs2275273	ALDH18A1	P5C synthetase	10	97397227	[T/C]	intron	Silent	0.561
rs2296692	ALDH18A1	P5C synthetase	10	97373966	[A/C]	intron	Silent	0.827
rs10794058	OAT	OAT	10	126100523	[A/G]	intron	Silent	0.493
exm1453731	PEPD	Prolidase	19	33878837	[A/G]	exon	L435F	0.493
exm1453740	PEPD	Prolidase	19	33878977	[T/C]	exon	R388H	0.526
exm2272783	PEPD	Prolidase	19	33906123	[T/C]	intron	Silent	0.526
exm-rs731839	PEPD	Prolidase	19	33899065	[A/G]	intron	Silent	0.493
exm-rs8182584	PEPD	Prolidase	19	33909710	[T/G]	intron	Silent	0.625
rs10420551	PEPD	Prolidase	19	33973913	[A/G]	intron	Silent	0.98
rs10853926	PEPD	Prolidase	19	33883248	[T/C]	intron	Silent	0.526
rs153508	PEPD	Prolidase	19	34009392	[T/C]	intron	Silent	0.03 *
rs17531264	PEPD	Prolidase	19	33897223	[A/G]	intron	Silent	0.526
rs33823	PEPD	Prolidase	19	34000725	[T/C]	intron	Silent	0.03 *
rs33825	PEPD	Prolidase	19	33989245	[A/G]	intron	Silent	0.493
rs3786897	PEPD	Prolidase	19	33893008	[A/G]	intron	Silent	0.493
rs4362488	PEPD	Prolidase	19	33914944	[A/G]	intron	Silent	0.526

rs7248389	PEPD	Prolidase	19	33932315	[T/C]	intron	Silent	0.526
rs7258937	PEPD	Prolidase	19	33938800	[T/C]	intron	Silent	0.145
rs754986	PEPD	Prolidase	19	33902809	[A/G]	intron	Silent	0.526
rs889140	PEPD	Prolidase	19	33889000	[A/G]	intron	Silent	0.583
rs9676705	PEPD	Prolidase	19	33906764	[A/G]	intron	Silent	0.526

ALDH, aldehyde dehydrogenase; OAT, ornithine aminotransferase; P5C, Δ -1-pyrroline-

5-carboxylate; PEPD, peptidase; PRODH, proline dehydrogenase.

* significant with FDR p value < 0.05