Further evidence for altered myelin biosynthesis and glutamatergic dysfunction in schizophrenia

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Abstract
Recent studies have provided evidence for neuronal and oligodendrocyte-related abnormalities being associated with schizophrenia. However, the functional interplay and causal relationship between these two abnormalities is poorly understood. In this report, we provide data that identify myelin and fatty-acid biosynthesis dysfunction in schizophrenia based on post-mortem brain studies (prefrontal cortex) utilizing parallel metabolic and transcriptomics investigations. We detected a significant up-regulation of N-acetylaspartate (NAA) by HPLC analysis. Microarray and Q-PCR investigations revealed mRNA abnormalities for several enzymes involved in NAA metabolism. Additionally, glutamatergic neurotransmission components were also found to be affected. Our results suggest that, apart from the previously reported alterations in myelin-related protein synthesis, myelin synthesis itself may be directly affected in schizophrenia as indicated by changes in key enzymes involved in NAA metabolism. A decrease in NAA catabolism in oligodendrocytes would severely reduce acetate levels required to produce myelin lipids and may subsequently affect glutamatergic neurotransmission.

Introduction
The aetiology of schizophrenia remains elusive. There is strong evidence that a number of genetic factors contribute significantly to schizophrenia susceptibility, however, the interpretation of findings is frequently hampered by a lack of knowledge of the interactions between candidate molecules (Harrison and Weinberger, 2005). Similarly, diverse neurotransmitter systems have been implicated in the disease process based on the action of antipsychotic medication and the potential of illicit drugs to induce schizophrenia-like symptoms (Goff and Coyle, 2001). It is generally believed that multiple neuronal signalling pathways are affected simultaneously with glutamatergic and dopaminergic neurotransmission thought to be at the core of schizophrenia symptoms (Goff and Coyle, 2001; Harrison and Weinberger, 2005). At the same time, several expression profiling and imaging studies found abnormalities in lipid-, myelin- and oligodendrocyte-related pathways in schizophrenia and indeed bipolar disorder, implicating glial dysfunction in schizophrenia (Davis et al., 2003; Hakak et al., 2001; Mimmack et al., 2002; Pongrac et al., 2002; Tkachev et al., 2003). Previously, we have reported a down-regulation of the oligodendrocyte lineage transcription factor 2 (Olig 2) at the mRNA level (Tkachev et al., 2003). More recently, an association study has implicated variation in Olig 2 with genetic susceptibility to develop schizophrenia either solely or as part of a network of genes implicated in myelination and oligodendrocyte function (Georgieva et al., 2006).

In this report, we have combined microarray and quantitative PCR (Q-PCR) techniques with a small-scale metabolomics study on post-mortem brain tissue. Brain samples from the prefrontal cortex of 15 schizophrenia patients and 15 matched controls
were obtained from the Stanley Brain Collection (Stanley Medical Research Institute, Bethesda, USA). The combined application of gene expression studies and concurrent investigation of metabolic cellular processes allowed for the identification of dysfunctional metabolically linked pathways in schizophrenia. Here we present novel results supporting overlapping abnormalities in myelin- and glutamatergic function in schizophrenia (Figure 1).

**Materials and methods**

**Tissue collection**

Freshly frozen prefrontal cortex tissue (Brodmann area 9) was obtained from the Neuropathology Consortium of the Stanley Brain Collection. Tissue blocks with equivalent amounts of white and grey matter were used for total RNA extraction. Demographic and clinical characteristics of patient and control populations, as well as the methods of tissue harvest, preparation and storage have previously been described in detail (Torrey et al., 2000). The Stanley Brain Collection is well-matched for age, pH, sex, race, side of the brain and post-mortem interval (Table 1) (Torrey et al., 2000).

**HPLC sample preparation**

Frozen tissue samples were cut and homogenized in 10 vol. of ice-cold 0.1 M perchloric acid (HClO₄) by sonication. The resulting homogenates were centrifuged at 13000 g for 15 min at 4 °C. Supernatants were collected and the centrifugation process was repeated to remove all traces of cell debris. Separate aliquots were then prepared and used immediately for the quantification of N-acetylaspartate (NAA).

**NAA quantification by HPLC**

Ion-pairing HPLC with UV detection at 210 nm [Dionex AD20; Dionex, Sunnyvale, USA, range 0.05 AUFS (absorbance unit full scale)] was used for NAA quantification. Separation was achieved using a buffer containing 2.8 mM tetrabutylammonium hydroxide (Sigma-Aldrich, Gillingham, UK), 25 mM KH₂PO₄ and 2.5% methanol (pH 7.0). The flow rate was 0.4 ml/min (CMA 250). The column used was a SphereClone ODS2, size 150 × 3.20 mm, 3 μm (Phenomenex, Macclesfield, Cheshire, UK). A Gilson 231 autosampler was used for all injection procedures. All samples were quantified in triplicate and calibrated with a standard NAA solution (Sigma-Aldrich).

**RNA extraction and cDNA synthesis**

Total RNA was extracted from post-mortem prefrontal cortices (Brodmann area 9) of schizophrenia and control brains using the Tri-reagent RNA extraction protocol (Sigma) in conjunction with a mechanical homogenizer (Ribolyser, Thermohybaid,

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**Figure 1.** Pathway diagram. The pathway diagram schematically shows the relationship between measured metabolites and corresponding enzymes. Dashed downward arrows (↓) indicate down-regulation, whereas the dashed upward arrow (↑) represents up-regulated genes as detected in the schizophrenia prefrontal cortex compared to the control group.
New York, NY, USA). RNA quality was assessed using a high-resolution electrophoresis system (Agilent Technologies, Palo Alto, CA, USA). RNA samples (0.5 mg) were reverse-transcribed into cDNA using a site-directed mutant form of MMLV reverse transcriptase (PowerscriptTM; Clontech, Mountain View, CA, USA) by random hexamer priming (Mimmack et al., 2004).

Microarray analysis

Microarray expression analysis was carried out as previously described (Tkachev et al., 2003). In brief, total RNA (5 μg) was used to make double-stranded cDNA (Superscript Gibco-BRL, Gaithersburg, USA). In vitro transcribed transcripts were hybridized to the human genome U133A array (Affymetrix, Santa Clara, CA, USA). Arrays were scanned in the Affymetrix GeneChip scanner and images were processed using Microarray Analysis Suite 5 (Affymetrix). Based on the quality control procedures a final set of 11 schizophrenia and 11 control arrays (from the total 30 brains mentioned above) were used to generate the microarray result.

Q-PCR

Q-PCR was performed using an ABI Prism 7900HT sequence detection system (PE Applied Biosystems, Foster City, CA, USA) coupled to liquid-handling robotics (Matrix, Plate Mate Plus liquid handling robot). Q-PCR reactions (10 μl) were performed using the TaqMan Universal PCR Master mix and transcript-specific MGB probes (PE Applied Biosystems) according to the manufacturer’s instructions. The house-keeping gene (Beta-2-Microglobulin) was always quantified simultaneously with all candidate genes analysed (Mimmack et al., 2004). All samples were assayed in triplicate for the highest degree of accuracy and precision. A full set of 15 schizophrenia and 15 control samples was used in Q-PCR analysis.

Statistics

An unpaired (two-tailed) t test was used to identify significant changes in NAA levels. This test was also used to detect statistically significant changes in gene expression levels for each gene analysed by quantitative PCR and microarray experiments. Analyses of covariance (ANCOVA) was performed with HPLC, Q-PCR and microarray gene expression data as the dependent variable in each case in order to adjust for the effect of the following possible confounders: age, gender, duration of illness, fluphenazine equivalent drug exposure, family history, CNS medication use at time of death, alcohol and drug abuse. No single variable mentioned above appeared to significantly influence the reported gene and/or metabolite changes in the control vs. the disease group, so that the summaries presented in Table 2 effectively describe a series of unpaired t tests.

Results

Increased NAA levels and down-regulation of aspartoacylase mRNA

NAA is synthesized from aspartate and acetyl-CoA by aspartate N-acetyltransferase (ANAT) within neuronal mitochondria (Figure 1). We found a significant 1.30-fold up-regulation \( (p=0.017) \) of NAA in post-mortem schizophrenia tissue (prefrontal cortex; \( n=15 \)) compared to a matched control group (\( n=15 \)) by HPLC analysis. It is known that NAA is hydrolysed to acetyl groups and aspartate by the enzyme aspartoacylase (ASPA), which is predominantly expressed in oligodendrocytes in the brain (Figure 1) (Baslow et al., 1999).

In keeping with the observed changes in NAA levels, we found ASPA down-regulated in the schizophrenia prefrontal cortex, both in our microarray study.
Q-PCR analysis. Fold differences and Student’s two-tailed t test arrays were used in the microarray analysis. Fifteen schizophrenia and 15 matched control samples were included in the microarray study and by Q-PCR (−1.61-fold, p = 0.049; Table 2). Further investigation of NAAG neurotransmission involving its receptor mGluR3 revealed a significant mGluR3 down-regulation (p = 0.025), which was confirmed by Q-PCR (−1.60-fold, p = 0.029), providing additional evidence for NAAG/glutamate neurotransmission dysfunction in schizophrenia (Tsai et al., 1995).

**Table 2. Microarray and Q-PCR results of genes associated with NAA turnover in schizophrenia vs. matched control brain samples**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Microarray</th>
<th>Q-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartoacylase</td>
<td>ASPA</td>
<td>Fold Δ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p value</td>
</tr>
<tr>
<td>Glutamate carboxypeptidase 2</td>
<td>FOLH1</td>
<td>−2.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.067</td>
</tr>
<tr>
<td>Plasma glutamate carboxypeptidase</td>
<td>PGCP</td>
<td>−1.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.026</td>
</tr>
<tr>
<td>Glutamate receptor metabotropic 3</td>
<td>GRM3</td>
<td>−1.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.025</td>
</tr>
</tbody>
</table>

The table shows microarray and Q-PCR results for genes associated with the turnover of NAA. Eleven schizophrenia and 11 control samples were used in the microarray analysis. Fifteen schizophrenia and 15 matched control samples were included in the Q-PCR analysis. Fold differences and Student’s two-tailed t test p values were calculated. Significant p values (p < 0.05) and corresponding fold differences are shown in bold. Fold differences represent the ratios of disease/control (+) and control/disease (−) values.

(--1.78-fold, p = 0.097) and by Q-PCR (−1.61-fold, p = 0.049; Table 2). A reduction in ASPA activity could result in the observed increases in NAA levels. The key role of ASPA is to provide NAA-derived acetyl groups for incorporation into fatty acids and lipids (Mehta and Namboodiri, 1995). Additionally, acetyl groups produced by ASPA from NAA are directly incorporated into lipid components of myelin, consequently the enzyme shows a high level of activity in purified myelin (Chakraborty et al., 2001).

**FOLH1 and mGluR3 mRNA down-regulation**

NAA can also be produced from N-acetylaspartyl-glutamate (NAAG) by FOLH1 (folate hydrolase/glutamate carboxypeptidase 2). However, this pathway is a minor contributor to the overall NAA pool due to the low concentrations of NAAG within the human brain. The FOLH1 enzyme hydrolyses NAAG to NAA and glutamate (Figure 1). FOLH1, also known as NAALADase (N-acetylated α-linked acidic dipeptidase), was −2.05-fold down-regulated (p = 0.067) in our microarray study and by Q-PCR (−2.60-fold, p = 0.003) (Table 2). A reduction of FOLH1 enzymatic activity in the schizophrenia prefrontal cortex has also been found in an independent brain collection (Tsai et al., 1995). Microarray analysis also revealed a significant −1.53-fold down-regulation (p = 0.026) of PGCP (blood plasma glutamate carboxypeptidase) in the investigated schizophrenia brain collection (Table 2). PGCP is closely related to FOLH1 in terms of enzymatic activity, initially purified and cloned from human placenta, the enzyme is secreted and can also be found in blood plasma. When PGCP is expressed in transfected cells it was found to hydrolyse NAAG. However, PGCP down-regulation could not be validated by Q-PCR (Table 2). Further investigation of NAAG neurotransmission involving its receptor mGluR3 revealed a significant mGluR3 down-regulation (p = 0.025), which was confirmed by Q-PCR (−1.60-fold, p = 0.029), providing additional evidence for NAAG/glutamate neurotransmission dysfunction in schizophrenia (Tsai et al., 1995).

**Discussion**

Several studies have identified a down-regulation of myelination- and oligodendrocyte-related genes in schizophrenia (Hakak et al., 2001; Pongrac et al., 2002; Tkachev et al., 2003). The observed up-regulation of NAA in the schizophrenia post-mortem brain also supports the view of myelin/oligodendrocyte dysfunction in schizophrenia. NAA is the second most abundant metabolite in the brain after glutamate and is also considered to be the major source of acetyl groups within the central nervous system, which are directly incorporated into myelin and other fatty acids/lipids (Chakraborty et al., 2001; Mehta and Namboodiri, 1995). The enzymatic activity responsible for NAA synthesis (EC 2.3.1.17) has been ascribed to neuronal mitochondria and referred to as ANAT, however, the gene responsible has yet to be identified. The concurrent down-regulation of the principal NAA-hydrolysing enzyme ASPA (aspartoacylase/aminoacylase 2), primarily expressed in oligodendrocytes provides further evidence for oligodendroglial dysfunction in schizophrenia and may suggest a reduction in synthesis of myelin lipid components. Myelin consists of proteins (30%) and lipids (70%), therefore, slight alterations in the levels of myelin lipid synthesis influenced by ASPA.
down-regulation may be linked to the white-matter volume decrease seen in schizophrenia (Davis et al., 2003). NAA is effectively a natural precursor of brain lipids, synthesized within neuronal mitochondria and then axonally transported in an energy-dependent manner for utilization by the myelin-associated enzyme ASPA (Chakraborty et al., 2001). The developmental expression pattern of ASPA correlates with that of other structural myelin proteins, such as myelin basic protein (MBP) and proteolipid protein (PLP1), which have been shown to be differentially expressed in schizophrenia (Kimani et al., 2003; Pongrac et al., 2002; Shiota et al., 1989; Tkachev et al., 2003). Conversely, in-vivo magnetic resonance spectroscopic (MRS) studies have found a decrease in NAA levels in living schizophrenia patients (Bertolino et al., 1998). We believe that our results can not be directly compared to the in-vivo MRS findings due to the high degradability of NAA, which reaches up to 46% in the first 24 h post-mortem (Battistuta et al., 2001). In our study, the average post-mortem intervals were 23.7 and 33.7 h for control and schizophrenia groups respectively. Therefore, the increase in NAA seen in our study could be linked to decreases in oligodendrocyte-specific ASPA expression and may reflect a post-mortem effect. Of relevance here is the example of Canavan disease (CD), a rare early-onset spongiform leukodystrophy known to present with increased in-vivo NAA levels [up to 1000-fold in extracellular fluid (ECF)] (Baslow and Resnik, 1997). This autosomal recessive disorder is caused by mutations in the ASPA gene producing an inactive enzyme, which results in progressive myelin degradation and eventual loss of oligodendrocytes (Baslow and Resnik, 1997). Furthermore, NAA biosynthesis is closely linked to mitochondrial energy metabolism (Clark, 1998), which is of interest as a number of studies have shown a reduction in ATP utilization in the frontal lobe of schizophrenia patients (Volz et al., 2000). Mitochondrial dysfunction and oxidative stress in schizophrenia have also been the main finding of our large-scale functional genomics investigation involving parallel transcriptomics, metabolomics and proteomics investigations in schizophrenia post-mortem brains (Prabakaran et al., 2004). Oligodendrocytes are also considered to be most susceptible to oxidative stress damage due to reduced antioxidant defence mechanisms and a high metabolic rate. Thus, it is also possible that abnormalities in NAA homeostasis in schizophrenia may be secondary to the mitochondrial dysfunction.

In addition to the abnormalities detected in NAA metabolism discussed above, we found that the second NAA biosynthetic pathway, operating through NAAG hydrolysis, was also dysregulated in schizophrenia. N-acetylated-gamma-linked-acidic dipeptidase (NAALADase) activity is required for NAAG hydrolysis to NAA and glutamate (Figure 1). Glutamate carboxypeptidase 2 [also known as prostate-specific membrane antigen (PSMA)] is the enzyme with such biochemical activity (EC 3.4.17.21), although it is now designated as folate hydrolase (FOLH1). Decreases in NAALADase biochemical activity have already been identified in schizophrenia using an independent brain collection (Tsai et al., 1995). FOLH1 expression was down-regulated in our study (Table 2). Apart from the enzyme FOLH1, plasma glutamate carboxypeptidase (PGCP) has also been shown to possess NAALADase activity. PGCP is related to FOLH1, initially identified in blood plasma. Microarray results showed a significant down-regulation of PGCP in the schizophrenia group (Table 2), however, we were unable to cross-validate this result by Q-PCR (Table 2). The down-regulation of FOLH1 expression may be a result of the increased NAA and down-regulation of ASPA, as FOLH1 is not an oligodendrocyte-specific gene being predominantly expressed in astrocytes (Baslow, 2000). Astrocytes and oligodendrocytes are distinct glial cell populations that communicate between each other and with neurons. Our data provide conflicting insights regarding neuronal NAA metabolism in the schizophrenia brain, since in-vivo decreases of NAA have been reported (i.e. assuming initially unequal amounts of NAA between control and schizophrenia groups at the time of death). Instead, it is proposed that the down-regulation of ASPA may result in a local/glial increase in NAA concentrations (as NAA is catabolized more slowly), which is possible even if overall NAA is lower in schizophrenia brains compared to normal controls. This proposed local increase in NAA is in turn reducing the FOLH1-catalysed NAAG hydrolysis in astrocytes thus affecting NAAG-related neurotransmission (Figure 1). NAALADase activity converting NAAG into NAA and glutamate is particularly important, since NAAG is a partial agonist of N-methyl-D-aspartate receptors and a highly selective agonist for the metabotropic glutamate receptor 3 (mGluR3/GRM3), which has been associated with schizophrenia (Harrison and Weinberger, 2005). Notably, mGluR3 expression was also significantly reduced in the schizophrenia brain in both microarray and Q-PCR analyses (Table 2). These results positively support glutamatergic dysfunction in schizophrenia, however, possibly as a secondary effect to the observed glial metabolic alterations.
In summary, our results suggest a number of biochemical abnormalities associated with the biosynthesis of lipid components of myelin, which are also metabolically linked to NAAG/glutamatergic neurotransmission in the schizophrenia brain. However, it is not clear whether these changes represent primary or secondary factors in the disease process, nor are we able to rule out the influence of antipsychotic drug treatment effects.

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Statement of Interest
None.

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