GABA Transporters GAT-1 and GAT-3 in the Human Dorsolateral Prefrontal Cortex in Schizophrenia

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\textbf{Key Words}
GABA transporter 1 (GAT-1) and 3 (GAT-3), schizophrenia, \textsuperscript{3}H\textsuperscript{3}GABA, \textsuperscript{3}H\textsuperscript{3}b-alanine

\textbf{Abstract}
This study aimed to investigate the binding affinity of \textsuperscript{3}H\textsuperscript{3}GABA and \textsuperscript{3}H\textsuperscript{3}b-alanine to GABA transporters GAT-1 and GAT-3 in the human dorsolateral prefrontal cortex (Brodman’s area 9) in schizophrenia. Using post-mortem tissue from individuals diagnosed with schizophrenia (n = 6) and control subjects (n = 6), the density of GAT-1 was established by displacing \textsuperscript{3}H\textsuperscript{3}GABA with muscimol, and for GAT-3 \textsuperscript{3}H\textsuperscript{3}b-alanine was used. Data analysis showed a significant decrease of GAT-1 levels (45\%), and a significant increase of GAT-3 density (23\%) within the dorsolateral prefrontal cortex of individuals diagnosed with schizophrenia when compared to age- and sex-matched controls. The observed decrease of GAT-1 could be explained as a consequence of the GABA hypo-function or the result of volumetric shrinkage of the cerebral cortex previously reported in this disease. The observed elevation of GAT-3 levels could be due to a compensatory effect for any functional loss of GABA re-uptake by the decreased GAT-1 levels.

\textbf{Introduction}
GABA is the main inhibitory neurotransmitter within the human central nervous system. Previous studies have suggested that a decrease in GABAergic activity may contribute to some of the observed symptoms in schizophrenia [1]. Hypo-GABAergic neurotransmission is thought to be responsible for the observed up-regulation of GABA\textsubscript{A} receptor subunits [2, 3], in conjunction with a 40\% down-regulation of GAT-1 transporter expression in the dorsolateral prefrontal cortex in schizophrenia [4, 5].

GAT-1 has been localised to axon cartridges of inhibitory chandelier cells in post-mortal human cerebral cortex [5] as well as rat brain [6]. GAT-1 is thought to regulate the effect and duration of GABA in the synaptic cleft [7].

Previous binding studies have shown that GAT-1 has a very high binding affinity for GABA and nipecotic acid [8]. GAT-3, on the other hand, has been shown to have a higher binding affinity for b-alanine than for GABA [8] and was reported to be present predominantly in astrocytic processes [9]. However, a recent study also found GAT-3 immunoreactivity in the cytoplasm of pyramidal cells in rat cortex [10]. The function of GAT-3 is not fully understood, but Minelli et al. [9] suggested that it might function as a glial uptake system for GABA to ensure that...
GABA does not leak out of the synaptic cleft. Due to its predominately glial nature, GAT-3 would not transport GABA back to the presynaptic site, but would allow the metabolism of GABA into succinic acid by using the enzyme GABA-transaminase in the glial mitochondria [11].

The aim of the present study was to investigate possible changes in the GABA transporters, GAT-1 and GAT-3, in the dorsolateral prefrontal cortex (Brodman’s area 9), since previous studies have reported a decrease of GAT-1 immunoreactivity [12, 13] as well as a volumetric shrinkage of cerebral cortex, which is thought to be responsible for possible disturbances within the cortico-cortical and thalamo-cortical connections in schizophrenia [5, 14].

### Patients and Methods

#### Materials

[^H]GABA and [^3H]β-alanine were obtained from NEN (Ontario, Canada). Muscimol and nipeoic acid were purchased from Sigma, Castle Hill, Australia, and β-alanine from Fluka, Castle Hill, Australia. All other reagents were standard laboratory chemicals.

#### Brain Tissue Details

Human brain tissue was obtained from the NSW Tissue Resource Centre. Ethical approval (99/0115) was obtained from the University of Sydney Human Ethics committee. Brain tissue from 6 cases with a diagnosis of schizophrenia was matched by age and sex with 6 control cases to minimise effects of these potentially confounding variables. Table 1 shows a demographic summary of the two tissue cohorts.

All tissue was screened for neuropathological abnormalities and categorised by diagnosis. Any tissue exhibiting evidence of infectious diseases or any physiological or anatomical damage that could have been associated with removal of the brain or processing was excluded.

The study was conducted in a blind fashion with the clinical details only becoming available on completion of experiments and data analysis. From all subjects the prefrontal cortical area, defined as Brodmann’s area 9 [15], was blocked and sectioned coronally at 20-μm intervals on a cryostat. Adjacent sections from each brain were mounted onto glass slides, fixed in 4% paraformaldehyde and then stained with cresyl violet to distinguish between the different cortical layers.

#### Autoradiography Procedure

The mounted sections were first washed in Tris-buffer (50 mM TRIZMA BASE, 50 mM NaCl, pH 7.4) twice for 15 min to remove endogenous ligands, which may have otherwise competed with radio-labelled compounds for the site of interest. Following the washes, radioactive solution was applied to the sections (350 μl/section) before an incubation period of 40 min at 4°C. The sections were briefly dipped into ice-cold Tris buffer to remove excess radioactive ligand, then in milli-Q water (4°C) to eliminate any remaining salts from the buffer. The sections were dried for 2–3 h under a stream of air from a hair dryer at room temperature and then placed into an autoradiography cassette with 2 tritium standard micro-scales (Amersham Pharmacia Biotech, Castle Hill, Australia). Sections were exposed to either the Kodak Biomax MS-1 Hyperfilm (Amersham Pharmacia Biotech) or Hyperfilm [^3H] (Amersham Pharmacia Biotech) under dark room conditions. Exposure time to the film was 3–4 weeks at a storage temperature of −20°C.

#### GAT-1 Studies Using[^H]GABA

[^H]GABA binding to GAT-1 transporters was studied in the presence of muscimol (500 nM, to block binding to GABA<sub>B</sub> and GABA<sub>A</sub> receptors) and β-alanine (100 nM; to block binding to GAT-3 transporters) and in the absence of calcium ions (thus blocking interaction with GABA<sub>B</sub> receptors). Specific binding was defined as the difference between the[^H]GABA with muscimol (500 nM) in the absence and presence of 100 nM nipeoic acid.

Muscimol acts at ionotropic GABA receptors as a potent partial agonist (EC<sub>50</sub> 2 μM) at human recombinant GABA<sub>B</sub> receptors and as a somewhat less potent full agonist (EC<sub>50</sub> 27 μM) at GABA<sub>A</sub> receptors [16]. It acts on GABA transporters only at very high concentrations (K<sub>1</sub> 1 mM) [17].

To ensure that the concentration of muscimol would not interfere with GAT-1-specific binding sites, a blocking experiment using rat brain tissue was conducted where[^H]muscimol was displaced by the GAT-1 substrate nipeoic acid and the GAT-3 substrate β-alanine. It was shown that the concentration of muscimol employed (< 500 nM) did not interfere with GAT-1- and GAT-3-specific binding sites, which is consistent with previous studies [17, 18].

#### GAT-3 Studies Using[^3H]β-Alanine

The necessary concentration of[^3H]β-alanine for subsequent experiments involving human brain tissue was established by carrying out a[^3H]β-alanine dose-response pilot study in rat brain tissue. Six different concentrations were tested. The concentration gradient for[^3H]β-alanine was 5, 20, 50, 100 and 200 nM; 500 nM β-alanine (Fluka, Castle Hill, Australia) was used to ascertain the level of nonspecific binding.

#### Cortical Layer Distribution

Cresyl violet-stained sections from each case were used to distinguish between the different cortical layers [19] which were sup-

<table>
<thead>
<tr>
<th>Sex</th>
<th>Control group (n = 6)</th>
<th>Schizophrenia group (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>43.7 ± 5.8</td>
<td>44.3 ± 11.1</td>
</tr>
<tr>
<td>Post mortem delay, h</td>
<td>18.3 ± 5.7</td>
<td>22.6 ± 8.47</td>
</tr>
<tr>
<td>pH</td>
<td>6.15 ± 0.5</td>
<td>6.39 ± 0.24</td>
</tr>
<tr>
<td>Brain weight, g</td>
<td>1.455 ± 108</td>
<td>1.428 ± 161</td>
</tr>
<tr>
<td>DSM-IV-R diagnosis</td>
<td>clinically normal</td>
<td>chronic paranoid schizophrenia</td>
</tr>
</tbody>
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Fig. 1. [H]GABA binding to GAT-1 in the presence of muscimol (500 nM) and β-alanine (100 nM) in the prefrontal cortex of controls (UC = upper cortical layers, LC = lower cortical layers) and patients with schizophrenia (UP = upper cortical layers, LP = lower cortical layers). The GAT-1 density was significantly decreased (p < 0.05) by 43.5% in the upper and 47.8% in the lower cortical layers.

Fig. 2. GAT-3-binding density with [H]β-alanine in the prefrontal cortex of controls and patients with schizophrenia. A significant increase (p < 0.05) of 23% was found for all cortical layers in the schizophrenia tissue compared to controls.

Statistical Analysis
Paired Student's t test was performed using SPSS (PC Version 3.0a, SPSS Inc, Chicago, Ill, USA). Due to the small sample size, an additional power analysis of the estimated t values was carried out using PASS 2002 (Power Analysis and Sample Size Software, NCSS Statistical Software, Kaysville, Utah, USA). The criterion for significance was p < 0.05.

Results

GAT-1 Distribution in the Dorsolateral Prefrontal Cortex
Nissl-stained brain sections permitted the identification and quantification of upper (layers I–III) and lower (layers IV–VI) cortical layers, as shown in figure 1. Analysis of binding data indicated that the final density was due to the presence of a single population of GAT-1-binding sites for upper and lower cortical layers.

The B_max for both the upper (10.45 ± 2.25 nCi/mg tissue) and lower (8.49 ± 1.9 nCi/mg tissue) cortical layers was significantly decreased (p < 0.05) in schizophrenia tissue when compared to control tissue (15.69 ± 2.71 and 11.68 ± 7.28 nCi/mg tissue equivalent for upper and lower cortical layers, respectively). In the schizophrenia tissue, GAT-1 density was, on average, decreased by 43.5% in the upper and 47.8% in the lower cortical layers compared to matched controls.

The dissociation constant K_i was similar for control (~0.33 ± 0.04 for upper and ~0.27 ± 0.03 for lower cortical layers) and schizophrenia tissue (~0.38 ± 0.1 for upper and ~0.35 ± 0.12 for lower cortical layers). This showed that any decrease in GAT-1 density was due to a loss of transporter sites (B_max) rather than a change in affinity of binding (K_i).

GAT-3 Distribution in the Dorsolateral Prefrontal Cortex
When measuring GAT-3 density, no significant difference was found for density distribution throughout all 6
cortical layers. [3H]β-Alanine saturation as a function of $B_{max}$ for mean control (9.29 ± 0.3084 nCi/mg tissue equivalent) and schizophrenia (12.03 ± 0.8447 nCi/mg tissue equivalent) tissue is shown in figure 2.

The mean GAT-3 density of schizophrenia tissue was significantly increased by 23% ($p < 0.05$) when compared to the control tissue. Power analysis showed that the power of this $t$ test was 0.998, which indicates that the sample size was sufficient to demonstrate the size of the effect observed.

The dissociation constants ($K_D$) were not significantly different for control and schizophrenia tissue ($p > 0.05$), which indicates that binding occurred at a single transporter site.

**Discussion**

*The Role of GAT-1 in the Dorsolateral Prefrontal Cortex in Schizophrenia*

Lewis [5] and Pierri et al. [13] have previously reported an average decrease of 40% of GAT-1 immunoreactivity in Brodmann’s area (BA) 9/46 in schizophrenia. Our autoradiographic studies support these immunohistochemical data in that [3H]GABA binding to GAT-1 is significantly decreased in the dorsolateral prefrontal cortex in schizophrenia in comparison to controls. Specifically, we found an average decrease in GAT-1 binding of 43.5% for the upper cortical layers of BA 9 and 47.8% for the lower cortical layers in schizophrenia tissue. Using autoradiography it was possible to illustrate that the decrease of GAT-1 binding sites is due to an anatomical loss rather than a defect in the binding sites of the transporter [20].

The observed volumetric reduction within the dorsolateral prefrontal cortex in schizophrenia [15, 19] is thought to occur via a reduced volume of neuronal processes and synaptic contacts such as axon cartridges [5]. This reduction of axon cartridges has been associated with the decrease of GAT-1 density in this cortical region, as shown in earlier studies using immunohistochemistry [4, 12, 21] as well as in this study by radioligand-binding autoradiography.

The decrease of GAT-1 levels seems to occur mainly in layers II-IV [13], which is the same area where an elevation of GABA_A receptors has been reported in schizophrenia [2, 22]. Therefore the decrease of GAT-1 seems to be strongly associated with an increase of GABA_A receptors. The decrease of synaptic GABA levels may occur in two ways; firstly, through decreased re-uptake of GABA into the presynaptic terminal due to a decrease of available GAT-1 transporter sites, and secondly, by a compensatory increase in GAT-3, which in turn would induce the metabolism of GABA in glia to succinic acid. The observed increase in GABA_A receptor expression [22] on postsynaptic pyramidal neurons might compensate for the decreased synaptic GABA concentration, and therefore may underlie any decrease in excitatory output or increase in inhibitory output to other cortical and subcortical areas [5].

*Elevation of GAT-3 Density in the Dorsolateral Prefrontal Cortex in Schizophrenia*

A major finding of this study was the significant increase in GAT-3 density (23%) within the dorsolateral prefrontal cortex in schizophrenia compared to matched controls. This finding may be explained by a possible glial proliferation or an increase in GAT-3 expression within astrocytes [23]. Since previous studies have shown that GAT-3 is not only found on astrocytic processes but also on pyramidal cells [10], an elevation of GAT-3 levels may take place to compensate for any functional loss of GABA re-uptake by the decreased GAT-1 levels in schizophrenia.

All 6 cases with a diagnosis of schizophrenia had a documented history of neuroleptic medication use. This fact raised the issue that the use of antipsychotic drugs may be associated with gliosis, even though it is generally agreed that a classic form of gliosis is not a pathological feature of the schizophrenic cortex [24]. Thus, since astrocytic processes are thought to play an essential role in the regulation of neuronal and metabolic activity of the cortex, the induction of glial proliferation by antipsychotics may counteract some of the observed transmitter imbalances observed in schizophrenia and other psychotic illnesses. This may aid in restoring some normal functional capacity to cortical circuits disrupted by the disease [25]. Whether the observed increase in GAT-3 density is due to glial proliferation as a result of neuroleptic medication remains to be investigated [25].

The current study has demonstrated significant schizophrenia-specific changes of GAT-1 (average 6-layer decrease of 45%) and GAT-3 (average 6-layer increase of 23%) densities within the dorsolateral prefrontal cortex. These findings may either be connected for the reasons hypothesised or may be due to distinct mechanisms necessitating further investigation. Additional studies are required to determine whether these changes are compensatory to other disease-specific mechanisms.
Acknowledgements

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